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Culture models for the study of amino acid transport and metabolism

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1. Glutamine uptake and release by primary astrocytes

Glutamine (Gln) transport in mammalian cells is mediated by a variety of amino acid transporters (more details in the text below). Kinetic studies and substrate specific inhibitory experiments in *in vitro* cultured cells have demonstrated that Gln transport in both in astrocytes and neurons mainly involves three sodium-dependent systems: A, ASC and N, and one sodium-independent system: L (1). Transporters belonging to these systems primarily mediate inward transport, but they can also function in outwardly Gln transport.

Several questions relevant to regulation of Gln transport in the CNS cells may be conveniently addressed by *in vitro* uptake and release assays. Studies in the presence of sodium-free or sodium-containing medium, as well as competitors specific for particular transporter families can dissect out the involvement of each system in Gln transport under the tested conditions. In this unit, protocols are presented for performing the uptake and release studies with radiolabeled Gln in primary astrocyte culture. These protocols can be easily modified for the investigation of Gln transport in other cultures and cell lines. A representative example of L-[G-³H]-Gln uptake by primary astrocytes in control conditions is shown in Fig. 1.

2. Materials

2.1. Buffers and Reagents

1. Primary astrocytes culture grown to at least 75% confluence in 24-well culture plates
2. L-[G-³H] Gln (*e.g.*, specific activity: 49.0 Ci/mmol; Amersham Biosciences, Piscataway, NJ)
3. L-glutamine (Gln; *e.g.*, Sigma, St. Louis, MO)

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4. Incubation media (see recipe)
5. L-histidine (His; *e.g.*, Sigma, St. Louis, MO)
6. L-threonine (Thr; *e.g.*, Sigma, St. Louis, MO)
7. 2-methylaminoisobutyric acid (MeAIB; *e.g.*, Sigma, St. Louis, MO)
8. L-leucine (Leu; *e.g.*, Sigma, St. Louis, MO)
9. 6-Diazo-5-oxo-L-norleucine (DON; *e.g.*, Sigma, St. Louis, MO)
10. 1 M NaOH
11. Scintillation fluid (*e.g.*, Fisher Scientific, Pittsburgh, PA)
12. DC Protein Assay Kit (Bio-Rad, Hercules, CA)

2.2. Equipment

1. Incubator suitable for mammalian tissue cultures (37° C, 5% CO₂)
2. Scintillation counter (*e.g.*, 6500, Beckman Coulter, Fullerton, CA)
3. Scintillation vials and caps (*e.g.*, Fisher Scientific, Pittsburgh, PA)
4. Equipments for measuring protein content: microplate reader set to 750 nm of excitation wavelength (*e.g.*, FlexStation and SoftMax Pro Program, Molecular Devices, Sunnyvale, CA)

Note: When working with radioactivity, appropriate precautions must be taken to avoid contamination of personnel conducting the study and surroundings. The experiments must be performed in a designated area following the guidelines of the local radiation safety committee and the user must be authorized to use such materials.

3. Gln uptake assay procedure

Each experimental treatment should be replicated in at least 4 wells in a minimum of 3 independently isolated cultures. Power analysis should be used a priori to determine the number of necessary replicates which will vary based on the variability of the results and the set alpha value.

1. Culture the cells in 24-well plates until they are >75% confluent.
2. Prepare incubation media (IM):
3. Adjust pH to 7.4

3.1. Kinetic analysis:

1. Prepare stock of non-radioactive (“cold”) 20 mM Gln in IM (+ or - NaCl)
2. Prepare radioactive mixture: cold Gln + 1 µCi of radiolabeled (“hot”) L-[G-³H] Gln (1 tube per 4 wells):
3. Remove culture medium

4. Wash the cells three times using warm (37° C) IM (2 ml / well for each wash)
5. Preincubate culture with IM (250 µl / well) for 30 min at 37° C
6. Remove IM and add radioactive mixture (250 µl / well)
7. Incubate for 5 min at 37° C
8. Remove radioactive mixture into a radioactive waste container
9. Terminate the reaction - wash the cells three times using ice-cold IM (2 ml / well for each wash)
10. Pour the washes into the radioactive waste container
11. Add 450 µl of 1 M NaOH
12. Incubate for 30 min. at 37° C
13. Transfer 400 µl of lysate to the scintillation vials and add 4 ml of scintillation fluid
14. Prepare control for “hot” Gln: put 0.25 µl of L-[G-³H] Gln into the scintillation vial and add 4 ml of scintillation fluid
15. Measure ³H activity using liquid scintillation counter
16. Take 5 µl of lysate for protein concentration assay
17. Measure protein content in the samples following the manufacturer’s protocol
18. Calculate results using following equation:

$$\frac{(1.125 * v_p / v_{iso}) * (C_{Gln} / 4)}{t * 90 * m_p} \quad [nmol/min/mg]$$

- v_p – activity in 400 µl of lysate [dpm]
- v_{iso} – activity in 0.25 µl of isotope [dpm]
- C_{Gln} – concentration of cold Gln [µM]
- t – time of incubation of cells in radioactive mixture [min.]
- m_p – protein content in 5 µl of lysate [mg]

3.2. Competition analysis:

1. Prepare stock of cold 20 mM Gln in IM (+ NaCl)
2. Prepare stocks of 20 mM amino acids (His, Thr, MeAIB, Leu) in IM (+ NaCl)
3. Prepare radioactive mixture: cold Gln + 1 µCi of hot L-[G-³H] Gln + combination of amino acid according to each System substrate specificity (1 tube per 4 wells):
4. Remove culture medium
5. Wash the cells three times using warm (37° C) IM (2 ml / well for each wash)

6. Preincubate culture with IM (250 μ l / well) for 30 min at 37° C
7. Remove IM and add radioactive mixture (250 μ l / well)
8. Incubate for 5 min at 37° C
9. Remove the radioactive mixture into the radioactive waste container
10. Terminate the reaction - wash the cells three times using ice-cold IM (2 ml / well for each wash)
11. Pour the washes into the radioactive waste container
12. Add 450 μ l of 1 M NaOH
13. Incubate for 30 min. at 37° C
14. Transfer 400 μ l of lysate to the scintillation vial and add 4 ml of scintillation fluid
15. Prepare control for “hot” Gln: put 0.25 μ l of L-[G-³H] Gln into the scintillation vial and add 4 ml of scintillation fluid
16. Measure ³H activity using liquid scintillation counter
17. Take 5 μ l of lysate for protein concentration assay
18. Measure protein content in the samples following manufacturer’s protocol
19. Calculate the uptake using the equation described above (point 13 of kinetic analysis protocol)

4. Gln efflux assay procedure

IM used in efflux assays contains DON – an inhibitor of Gln-requiring enzymes, to prevent L-[G-³H] Gln breakdown in the cells.

1. Prepare IM (+ or - NaCl) with 50 μ M DON
2. Prepare stock of cold 20 mM Gln in IM (+ NaCl)
3. Prepare stocks of 20 mM amino acids (His, Thr, MeAIB, Leu) in IM (+ NaCl)
4. Prepare radioactive mixture: 0.5 μ l of cold Gln + 4 μ l of L-[G-³H] Gln + 995.5 μ l of IM (+ NaCl) (1 tube per 4 wells)
5. Prepare efflux medium (1 tube per 4 wells):
6. Remove culture medium
7. Wash the cells three times using warm (37° C) IM (2 ml / well for each wash)
8. Preincubate culture with IM (250 μ l / well) for 30 min at 37° C
9. Pre-load the cells with radiolabeled Gln: remove IM and add radioactive mixture (250 μ l / well)
10. Incubate for 30 min at 37° C

11. Remove the radioactive mixture into the radioactive waste container
12. Wash the cells three times using warm (37° C) IM (2 ml / well for each wash)
13. Pour the washes into the radioactive waste container
14. Add warm efflux medium (250 µl / well)
15. Incubate for 10 min. at 37° C
16. Aspirate the medium and save it for scintillation counting
17. Add 450 µl of 1 M NaOH
18. Incubate for 30 min. at 37° C
19. Transfer 230 µl of efflux medium (point 16) to the scintillation vial and add 4 ml of scintillation fluid
20. Transfer 400 µl of NaOH lysate to the scintillation vial and add 4 ml of scintillation fluid
21. Measure ³H activity using liquid scintillation counter
22. Calculate amount of Gln released from the cells in 10 min. normalized to amount of preloaded Gln (results should not be expressed in nmol of released Gln / min / mg of protein, because the concentration of intracellular Gln is not known) using the following equation:

$$[(1.087 * V_e)/(1.087 * V_e + 1.125 * V_i)] * 100\%$$

- V_e – activity measured in 230 µl of efflux medium [dpm]
- V_i – activity measured in 400 µl of lysate [dpm]

5. Background of glutamine transport in CNS

Active Gln transport across the plasma membrane is essential for the supply of this amino acid for cellular metabolism. In the CNS, Gln plays a key role in neuron – glia interactions predominantly via astrocyte-mediated control of the turnover of neuronally-derived Glu and γ -aminobutyric acid (GABA), the principal excitatory and inhibitory neurotransmitters, respectively (2, 3). In addition to its specific role in neurotransmission, Gln also supports cellular energy requirements (5). A portion of Glu derived from Gln can be oxidized to an intermediate of tricarboxylic acid cycle (TCA) - alpha-ketoglutaric acid (α KG) (Fig. 2) (6).

Upon its synaptic release during neurotransmission, Glu is taken up largely by astrocytes (7, 8). In the astrocytes Glu is converted into Gln via a highly active and glia-specific enzyme, glutamine synthetase (GS) and subsequently released back into the extracellular space. Once taken up by juxtaposed neurons, Gln serves as a precursor for neurotransmitter synthesis, Glu or GABA in reactions catalyzed by phosphate-activated glutaminase (PAG) in glutamatergic neurons or by PAG and glutamic acid decarboxylase (GAD) in GABAergic neurons, respectively. Gln passage across the astrocytic and neuronal plasma membranes is

mediated by specific transporting proteins and is a key factor in the so called glutamine-glutamate-GABA cycle (Fig. 2) (9, 10).

Transporters are integral membrane proteins characterized by their ability to mediate movement of small molecules across the membranes by active transport or facilitated diffusion (11). Traditionally, mammalian amino acid transporters have been assigned to different transport systems, depending on their kinetic and regulatory properties, substrate specificity, pH sensitivity and ion dependence (12). Gln transport in mammalian tissues is mediated mainly by the sodium-dependent systems: A, ASC and N, and the sodium-independent system L (13). Table 6 summarizes some of the properties that distinguish these four transport systems. Recently, several proteins belonging to these systems have been characterized at the molecular level. A list of Gln transporters is presented in Table 7.

6. Functional characteristics of the systems transporting Gln in the CNS

6.1. Sodium dependent systems

6.1.1 System A (SNAT1 and SNAT2)—System A is widely expressed in mammalian cells and catalyzes Na⁺-dependent transport of neutral short-chain amino acids with preference for alanine, glutamine and serine. It recognizes model substrate 2-(methylamino)isobutyric acid (MeAIB) and exhibits marked inhibition at low extracellular pH. System A transporters couple amino acid transport with the Na⁺ electrochemical potential gradient with 1:1 stoichiometry (14). System A is able to mediate symport of amino acid and Na⁺ ion without countertransporting an additional compound, while the majority of other transporters works as the antiporters. Activity of system A can be stimulated by several factors, including amino acid starvation, hormones and growth factors (15 – 17).

System A transporter – SNAT1 (previously referred to as ATA1, GlnT, SA2, SAT1) is composed of 481 amino acids. SNAT1 transports all zwitterionic, aliphatic amino acids and displays high affinity for glutamine, alanine, asparagines and cysteine. In the CNS, SNAT1 is highly expressed in glutamatergic and GABAergic neurons *in situ* and in cultures (18, 14). This transporter was also found in dopaminergic neurons of the substantia nigra and in cholinergic motoneurons. SNAT1 is not expressed in astrocytes but was found in other non-neuronal components, namely luminal membranes of the ependyma (19).

Another transporter belonging to the system A - SNAT2 (previously known as ATA2, SA1, or SAT2) is a protein consisting of 506 amino acids. This transporter operates by a mechanism similar to SNAT1, but differs from the latter in its substrate specificity as it shows high affinity for proline. The expression of SNAT2 is more widespread than SNAT1: this transporter has been found in all tested tissues. In the CNS, SNAT2 transporter is enriched in glutamatergic neurons and in spinal motoneurons (20). High levels of SNAT2 mRNA have also been found in glia and in endothelial cells comprising the blood-brain barrier (21).

6.1.2. System ASC (ASCT1, ASCT2)—System ASC was originally named for three of the preferred substrates, alanine, serine, cysteine (1). This system is known to function as

an exchanger capable of mediating both influx and efflux of these amino acids. Distinguishing characteristics of system ASC from the other Na⁺-dependent systems include insensitivity to pH changes. The first isolated isoform of system ASC from human brain - transporter ASCT1 is ubiquitously expressed, but does not recognize Gln (22, 23). The second isoform, ASCT2, was cloned from rat astrocyte cultures. This transporter is composed of 539 amino acids (4). ASCT2 accepts Gln with high affinity and is responsible for a highly efficient Gln uptake by cell lines and tumors (24 – 26). In astrocytes, this transporter is mainly responsible for Gln efflux by obligatory exchange with extracellular amino acids (27).

6.1.3. System N (SNAT3, SNAT5)—System N shows narrow substrate specificity to amino acids containing nitrogen in their side chain, such as Gln, histidine and asparagine. Other properties of system N include high sensitivity to pH and substitution for Li⁺ in place of Na⁺. *In situ* hybridization in brain sections and immunohistochemistry in primary cell cultures identified a glial localization of system N isoform - SNAT3 (previously referred to SN1). In human SNAT3 is composed of 504 amino acids (28). This transporter is responsible for the inward and outward transport of Gln, the direction depending on the Gln and pH gradients (27, 28). Marked immunoreactivity of SNAT3 was observed in glia adjacent to glutamatergic and GABAergic synapses and cell bodies, suggesting this transporter is a major mediator of Gln efflux from astrocytes and supplying Gln for neurotransmitter synthesis in neurons (29).

Another system N isoform- SNAT5 (known also as a SN2) was found in a variety of tissues, including the brain. SNAT5 protein is composed of 471 amino acids and is 63% identical to SNAT3. Similarly to SNAT3, SNAT5 mediates Na⁺/amino acid cotransport and countertransport of H⁺. These transporters differ in their substrate profile: SNAT3 can recognize classic system N substrates, while SNAT5 favors serine (30, 31).

6.2. Sodium independent systems

6.2.1. System L (LAT1 and LAT2)—System L catalyzes Na⁺-independent transport of neutral amino acids with high affinity for leucine and is inhibited by synthetic model substrate 2-aminobicyclo [2,2,1]heptane-2-carboxylic acid (BCH). This system has been shown to be involved in efflux as well as in influx of amino acids (1). In the brain, system L is the major transport system of the blood-brain barrier (32). Low-affinity and high-capacity uptake of glutamine via this system was observed in both astrocytes and neurons. LAT1 and LAT2, two molecular isoforms belonging to the system L exist as heterodimers with the 4F2 heavy chain (33). LAT1 is a protein of 506 amino acids and has 12 transmembrane spanning domains. LAT2, which exhibits similar topology to LAT1, is composed of 535 amino acids. The mRNAs coding for both LAT1 and LAT2 are detectable in cultured astrocytes as well as cultured neurons (34). These transporters differ in substrate specificity; LAT1 has a narrow substrate profile, whereas LAT2 is able to transport many neutral amino acids. Studies have shown that LAT2 recognizes Gln with higher affinity than LAT1 (32).

Summary

Studies in recent years have provided evidence that carrier-mediated glutamine transport between astrocytes and neurons is a key factor in the glutamate-glutamine-GABA cycle. The molecular bases of Gln passage in CNS have been investigated extensively over the last few years. Gln transport in CNS involves the following systems: (a) sodium-dependent: system N; system ASC; system A and (b) sodium-independent: system L. Here we are presenting protocols for performing the uptake and release studies of radiolabeled Gln by different systems in primary astrocyte culture. Moreover, in this unit, the basic properties of glutamine-glutamate-GABA cycle are discussed, including aspects of Gln transport and metabolism

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Fig 1.

A representative example of L-[G-³H]-Gln uptake in primary astrocytes in control conditions. Experiments were performed in the presence of 32-fold excess of the following amino acids: MeAIB + Thr + Leu (selection for system N); MeAIB + His + Leu (selection for system ASC); Thr + His + Leu (selection for system A); MeAIB + Thr + His (selection for systems L). Results are mean $\pm$ S.D. of three independent experiments. Abbreviations: His – histidine, Leu-leucine, MeAIB-2-(methylamino)isobutyric acid Thr – threonine

Fig 2.

Schematic representation showing the involvement of amino acid transporter systems in Gln – Glu – GABA cycle between astrocytes and neurons. Glutamate released from presynaptic terminals is transported to the astrocytes, where it is converted to Gln by the glutamine synthetase (GS). In turn, Gln is released into the extracellular space and taken up by neurons, where Glu is regenerated via phosphate-dependent glutaminase. Glu may then be subsequently converted into GABA. In both astrocytes and neurons some portion of Glu is utilized for the synthesis of alpha-ketoglutaric acid, which enters the tricarboxylic acid cycle (TCA). For additional details please refer to the text above. Abbreviations: GAD - glutamic acid decarboxylase, GDH - glutamate dehydrogenase, GS - glutamine synthetase, αKG - Alpha-ketoglutaric acid, PAG - phosphate activated glutaminase, NAA – neutral amino acids, TCA- tricarboxylic acid.

Table 1.

Preparation of incubation medium for Gln uptake by both sodium-dependent and -independent carriers.

		+NaCl (pH 7.4)							
IM volume		50 ml	100 ml	200 ml	300 ml	400 ml	500 ml	600 ml	1 l
		[mg]							
150 mM	NaCl	438	877	1753	2629	3507	4383	5259	8766
3 mM	KCl	11.2	22.4	44.8	67.2	89.6	112	134	224
2 mM	CaCl ₂	11.1	22.2	44.4	66.6	88.8	111	133	222
0.8 mM	MgCl ₂	3.8	7.6	15.2	22.8	30.5	38.1	45.7	163
5 mM	glucose	45	90	180	270	360	450	540	900
10 mM	HEPES	119	238	477	715	953	1191	1430	2383

Table 2.

Preparation of incubation medium for Gln uptake by sodium-independent carriers

		-NaCl (pH 7.4)							
IM volume		50 ml	100 ml	200 ml	300 ml	400 ml	500 ml	600 ml	1 l
		[mg]							
150 mM	choline chloride	1047	2094	4188	6282	8376	10470	12564	20940
3 mM	KCl	11.2	22.4	44.8	67.2	89.6	112	134	224
2 mM	CaCl ₂	11.1	22.2	44.4	66.6	88.8	111	133	222
0.8 mM	MgCl ₂	3.8	7.6	15.2	22.8	30.5	38.1	45.7	76.2
5 mM	glucose	45	90	180	270	360	450	540	900
10 mM	HEPES	119	238	477	715	953	1191	1430	2383

Table 3.

Preparation of radioactive mixture for kinetic analysis

Gln [mM]	0.158	0.5	1	2	4	8	10
				[μl]			
cold Gln	7.9	25	50	100	200	400	500
L-[G- ³ H] Gln	1	1	1	1	1	1	1
IM (+ or - NaCl)	991.85	974.75	949.75	899.75	799.75	599.75	499.75

Table 4.

Preparation of radioactive mixture for competition analysis.

		investigated system			
final concentration		A	N	ASC	L
		[μl]			
cold Gln	0.158 mM	7.9	7.9	7.9	7.9
L-[G- ³ H] Gln		1	1	1	1
His	5 mM	250	-	250	250
Thr	5 mM	250	250	-	250
MeAIB	5 mM	-	250	250	250
Leu	5 mM	250	250	250	-
IM (+ NaCl)		241.85	241.85	241.85	241.85

Table 5.

Preparation of efflux medium.

	final concentration	investigated systems					
		all	Na-independent	A	N	ASC	L
		[μl]					
His	5 mM	-	-	250	-	250	250
Thr	5 mM	-	-	250	250	-	250
MeAIB	5 mM	-	-	-	250	250	250
Leu	5 mM	-	-	250	250	250	-
IM (+ NaCl)		1000	-	250	250	250	250
IM (- NaCl)		-	1000	-	-	-	-

Table 6.

Functional characteristic of the systems involved in Gln transport in mammalian CNS. Abbreviation: BCH- 2-aminobicyclo [2,2,1]heptane-2-carboxylic acid, MeAIB-2-(methylamino)isobutyric acid.

Features	Systems			
	A	ASC	N	L
Neutral amino acids	Short chain	Short chain	Containing side-chain nitrogen	Bulky
Synthetic model substrates	MeAIB			BCH
Inhibition by low pH	+	–	+	–
Na ⁺ -dependence	+	+	+	–

Table 7.

Glutamine transporters in the CNS. Abbreviations: Ala – alanine, Asn – asparagine, Cys – cysteine, Gln – glutamine, Gly – glycine, His – histidine, Leu – leucine, Ser – serine, Thr – threonine.

Transporter	Mechanism	Substrate specificity	Localization in brain
System ASC			
ASCT2	antiport	Ala, Ser, Cys, Thr, Gln, Asn	astrocytes
System N			
SNAT3	cotransport with Na ⁺ / antiport with H ⁺	Gln, Asn, His	astrocytes
SNAT5	cotransport with Na ⁺ / antiport with H ⁺	Gly, Asn, Ala, Ser, Gln, Met, His	astrocytes
System A			
SNAT1	cotransport with Na ⁺	Gln, Asn, His	neurons
SNAT2	cotransport with Na ⁺	Gln, Asn, His	astrocytes, neurons
System L			
LAT1	antiport	Leu, Ala, Ser, Gln	astrocytes, neurons
LAT2	antiport	Leu, Ala, Ser, Gln	astrocytes, neurons