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Phylogenetic Studies on the Synaptic Vesicle Glutamate Transport System

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The ATP-dependent uptake of glutamate into synaptic vesicles isolated from mammalian brains is well characterized. Glutamate uptake requires an electrochemical proton gradient, is specific for glutamate over other amino acids, and is stimulated by chloride. To determine whether these characteristics are fundamental to the vesicular uptake system, vesicles were isolated from the brain and central nervous ganglia of several vertebrate and invertebrate species, which included goldfish, frogs, turtles, pigeons, rats, *Drosophila,* and crayfish, and these vesicles were assayed for glutamate uptake activity. ATP-dependent glutamate was found in all of the vertebrate species tested, but was not detected in *Drosophila* or crayfish vesicles. The nature of the vesicular uptake of glutamate was similar among all the vertebrates: the specificity for glutamate remained high, transport was energized by a vacuolar (V)-type ATPase, 2–4 mM chloride stimulated uptake three- to sixfold, and K_m for glutamate was between 0.5 and 2 mM. While these major characteristics of the uptake system remained conserved among the vertebrates tested, minor differences were seen in glutamate specificity, the steady-state level of glutamate obtained in the vesicles, and V_{max} of the glutamate uptake systems. These results indicate that the synaptic vesicle glutamate uptake system is present throughout the vertebrate class, and that while minor changes in the transport system have occurred, its major functional characteristics, such as stimulation by chloride and strict substrate specificity, have been conserved for over 350–400 million years.

It is well established that neurotransmitters such as catecholamines, glutamate, GABA, and glycine are readily accumulated in an ATP-dependent manner into synaptic vesicles within the CNS (Philippu and Beyer, 1973; Toll et al., 1977; Naito and Ueda, 1983, 1985; Fykse and Fonnum, 1988; Hell et al., 1988; Kish and Ueda, 1989; Floor et al., 1990). However, the role of synaptic vesicles in release of these neurotransmitters in the CNS has not been firmly established. Studies by Nicholls and Sihra (1986) strongly suggest that the exocytotic pool of glutamate originates from a noncytosolic site within the nerve terminal, and recent evidence by Kish and Ueda (1991) points to synaptic vesicles as the source of the glutamate that is released in a calcium-dependent manner from nerve terminals.

Because the vesicular pool of glutamate appears to be involved in glutamatergic synaptic transmission, it will be important to understand the role of glutamate transport in this event. The transport of glutamate into synaptic vesicles has been well characterized, and both active uptake (Naito and Ueda, 1985) and facilitative efflux (Carlson and Ueda, 1990) of glutamate have been shown to occur readily in isolated mammalian brain synaptic vesicles. The uptake of glutamate into mammalian synaptic vesicles is driven by an electrochemical membrane potential that is generated by a vacuolar (V)-type (ATPase nomenclature is that of Petersen and Carafoli, 1987) proton-pump ATPase (Naito and Ueda, 1985; Maycox et al., 1988; Shioi et al., 1989; Cidon and Sihra, 1989). The ATP-dependent glutamate uptake system is stimulated by chloride and is specific for glutamate over other amino acids, neurotransmitters, or glutamate receptor agonists/antagonists; it also has an unusually high K_m (about 1–2 mM) for uptake (Naito and Ueda, 1985; Kish and Ueda, 1989). These characteristics are in contrast to those of the brain plasma membrane glutamate transport system, which is sodium dependent, accepts aspartate as well as glutamate, and has a K_m for uptake in the 3–20 μM range (Logan and Snyder, 1972; Kanner and Sharon, 1978; Christensen and Makowske, 1983).

The role of chloride stimulation, stringent substrate selectivity, and low substrate affinity is not well understood. To determine whether these characteristics are fundamental to the function of glutamatergic synaptic vesicles in all species or are unique to mammalian brain vesicles, we have examined the transport of glutamate into synaptic vesicles isolated from evolutionarily diverse organisms. We have found that, though small differences are present among the vertebrate species examined, the major characteristics of vesicular glutamate transport remain conserved throughout vertebrate evolution.

Materials and Methods

Materials. Frogs were purchased from Sullivan Co., Nashville, TN, or Kons Scientific, Germantown, WI. Turtles were from Dr. D. Dawson, Department of Physiology, The University of Michigan, and goldfish were from Dr. R. Davis, Neuroscience Laboratory, The University of Michigan. Pigeons were purchased from Palmetto Pigeon Farm, Sumter, SC. Crayfish were purchased from a local fish market, and *Drosophila* heads, thoraxes, and abdomens were a generous gift of Dr. K. Ikeda, Beckman Research Institute, City of Hope, CA.

All glutamate analogs and agonists, ATPase inhibitors, and assay chemicals were purchased from Sigma, with the exception of bafilomycin, which was a generous gift of Dr. Erik Floor (Department of Physiology and Cell Biology, University of Kansas), which was supplied

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by Dr. K. Altendorf (Universität Osnabrück, Germany). L-[2,3-^{3}H]-glutamate (50 Ci/mmol) was purchased from Amersham.

Preparation of crude synaptic vesicles. Crude synaptic vesicles were prepared as specified in Kish and Ueda (1989). Briefly, *Drosophila* heads, thoraxes, and abdomens; crayfish central nervous ganglia; and whole brains from goldfish, frogs, turtles, pigeons, and rats were removed and homogenized in solution A (0.32 M sucrose, 0.5 mM calcium acetate, 1 mM magnesium acetate, and 1 mM $NaHCO_3$). The homogenate was centrifuged at 12,100 × g_{max} for 20 min (10,000 rpm; Sorvall SS-34 rotor). The pellets were lysed for 45 min in 6 mM Tris-maleate (pH, 8.1) and centrifuged at 43,500 × g_{max} for 15 min (19,000 rpm; Sorvall SS-34 rotor). The supernatant was centrifuged at 200,000 × g_{max} for 60 min (43,000 rpm; Beckman Ti45 rotor), and the crude synaptic vesicle pellets were resuspended in solution B (0.32 M sucrose, 1 mM dithiothreitol, and 1 mM $NaHCO_3$). Synaptic vesicles were stored in liquid nitrogen for up to 6 months, with no loss of glutamate uptake activity.

Assay for glutamate transport activity. Vesicular glutamate uptake was measured as described previously (Kish and Ueda, 1989). The standard uptake assay mixture (100 μl final volume) contained 10–50 μg crude vesicle protein, 0.25 M sucrose, 10 mM HEPES (pH, 7.4), 4 mM $MgSO_4$, 4 mM KCl, 2 mM potassium aspartate (HEPES/sucrose media), 0 or 2 mM Tris-ATP, and 0.5 mM potassium ^{3}H-glutamate (4 μCi). Crude vesicles (10 μl) and HEPES/sucrose media (70 μl) were prewarmed to 30°C, and the uptake reaction was initiated by the rapid addition of ^{3}H-glutamate with or without ATP (20 μl). Unless stated otherwise, the reaction mixture was incubated for 10 min, and uptake was quenched by the addition of 2.0 ml of ice-cold 0.15 M KCl, followed immediately by filtration through Millipore HAWP filters (25 mm, 0.45 μm). The assay tubes were washed twice with KCl solution, and the filters were washed three more times with KCl. The radioactivity retained on the filter was counted in Cytoscint ES scintillation cocktail (ICN, Costa Mesa, CA) using a Beckman LS 9000 liquid scintillation spectrophotometer. All values are corrected for the nonspecific ^{3}H-glutamate that bound to filters. Synaptic vesicle protein was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

Kinetic parameters were derived using a FORTRAN program described by Cleland (1979). The program applies the Gauss–Newton nonlinear least-square method to fit the data to

$$\log v = \log(V_{max} \cdot [S]/(K_m + [S])),$$

where v is the velocity of ATP-dependent glutamate uptake, [S] is the concentration of glutamate in mM, and V_{max} and K_m are the Michaelis–Menten constants for the maximal initial velocity and half-maximal concentration of glutamate, respectively.

Results

Time course studies of glutamate uptake in crude synaptic vesicles from five classes of vertebrates

Synaptic vesicles were isolated from the brains of rats, pigeons, turtles, frogs, and goldfish, which represent the five classes of vertebrates: mammals, avians, reptiles, amphibians, and fish. Glutamate uptake into these vesicles was measured in the presence and absence of 2 mM ATP (Fig. 1). All five of these species exhibit ATP-dependent glutamate uptake into synaptic vesicles in a manner that is consistent with that first described for bovine synaptic vesicles (Naito and Ueda, 1983). The time required to reach steady state varies among the species from 10 to 30 min, and the levels obtained at steady state differ substantially, but all five species display a 10–20-fold stimulation of uptake by ATP.

Effect of chloride on glutamate uptake activity

At concentrations of 1–10 mM, chloride stimulates glutamate uptake into bovine (Naito and Ueda, 1985), mouse (Fischer-Bovenkerk et al., 1988), and rat (Kish and Ueda, 1989; Hell et al., 1990) synaptic vesicles three- to fivefold, while concentrations above 10 mM show little stimulation or even inhibition. We have examined the effect of chloride on glutamate uptake in synaptic vesicles from other vertebrate species (Fig. 2). Low-millimolar concentrations of chloride stimulated uptake in all five species tested. When compared to a control experiment using potassium isethionate, a nonpermeant anion (data not shown), 2–4 mM chloride caused from a 3.3-fold stimulation in rat and goldfish vesicles to almost a sixfold stimulation in turtle and pigeon vesicles. Also, concentrations of chloride above 10 mM display a diminished ability to stimulate uptake, similar to that shown previously for rat, mouse, and bovine vesicles (Naito and Ueda, 1985; Fischer-Bovenkerk et al., 1988; Kish and Ueda, 1989).

Effect of glutamate analogs, agonists, and related amino acids

The glutamate translocator present in bovine and rat synaptic vesicles has been shown to be quite specific for glutamate (Naito and Ueda, 1985; Kish and Ueda, 1989). It does not transport L- or D-aspartate and displays stereoselectivity for L-glutamate over D-glutamate. It also has no affinity for other neurotransmitter amino acids such as glycine or GABA or for glutamate receptor agonists.

The effects of these reagents were tested on the various synaptic vesicle uptake systems, and similar results were seen (Table 1). When tested at 5 mM (on the uptake of 0.5 mM glutamate in all species and on 0.05 mM glutamate in turtle and frog), L-glutamate was more effective than D-glutamate in reducing the uptake of ^{3}H-L-glutamate, in all the species tested. Of the other amino acids and glutamate analogs tested, only α-methylglutamate and γ-methylene glutamate were potent inhibitors, both of which have been previously shown to inhibit glutamate uptake in mammalian vesicles (Naito and Ueda, 1985; Kish and Ueda, 1989). D- and L-aspartate, which have only one less methylene group in their carbon backbone, show little affinity for the vesicular glutamate transport system. This is interesting because both D- and L-aspartate are transported by the plasma membrane glutamate transporter (Christensen and Makowske, 1983). Also, glutamine, α-ketoglutarate, and the neurotransmitter amino acids glycine and GABA had little effect on the uptake of glutamate in any of these species.

The glutamate analogs NMDA, quisqualate, and kainate strongly interact with various glutamate receptors. In previous studies, these agents showed no inhibition of glutamate uptake in mammalian vesicles (Naito and Ueda, 1985; Kish and Ueda, 1989). When these agents were tested in the various species, they had no effect in rat, turtle, frog, and goldfish and had only a very slight effect in pigeon vesicles. The results from these analog studies suggest that the specificity of the glutamate translocator remains largely unchanged during the evolution from fish to mammals.

To further demonstrate that the patterns described above represent the true inhibitory potential of these neurotransmitter amino acids and glutamate analogs, the same agents were tested in frog and turtle vesicles (and can be compared to rat and bovine vesicles in Kish and Ueda, 1989, their Table III) using the same inhibitor concentration and tenfold less glutamate (Table 1). The results show a very similar pattern to those seen with 0.5 mM glutamate: L-glutamate is preferred over D-glutamate, and only α-methylglutamate and γ-methylene glutamate display significant inhibition of glutamate uptake.

Effect of membrane-potential–dissipating agents, ATPase inhibitors, and other agents

The vesicular uptake of glutamate (Naito and Ueda, 1985; Maycox et al., 1988; Shioi et al., 1989), glycine (Kish et al., 1989),

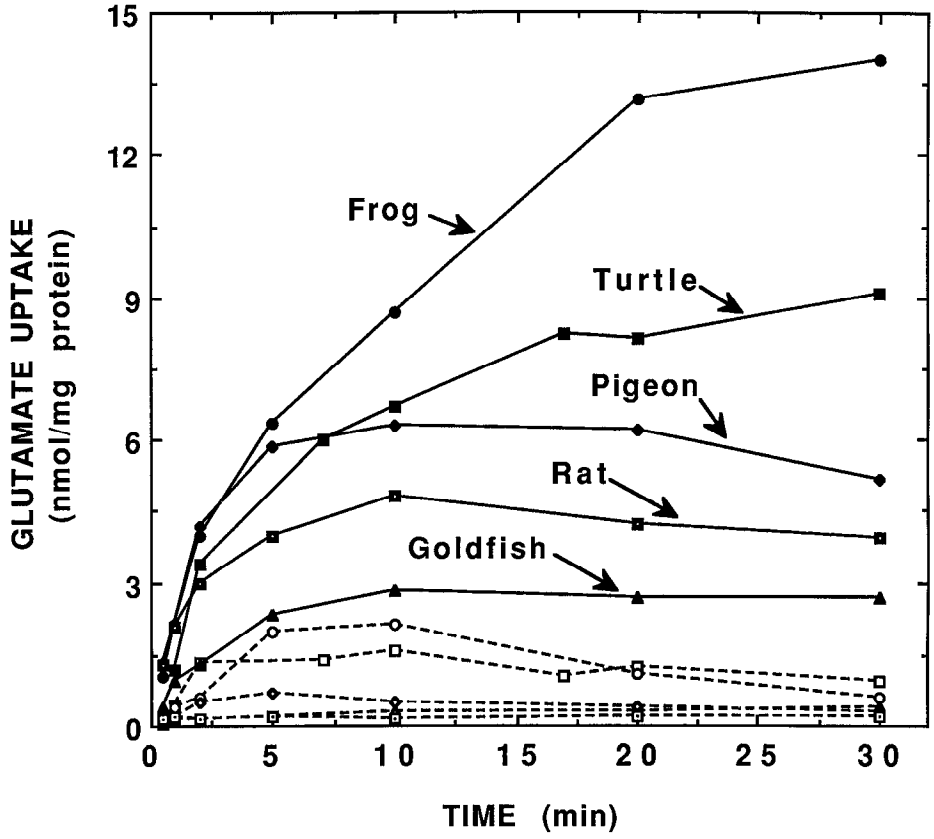

Figure 1. Time courses of ^{3}H-glutamate uptake into crude synaptic vesicles from goldfish, frog, turtle, pigeon, and rat brains. Crude synaptic vesicles from goldfish (*triangles*), frog (circles), turtle (*simple squares*), pigeon (*diamonds*), and rat (*squares* with *center dots*) brains were incubated for various times with 0.5 mM ^{3}H-glutamate in the absence (*open symbols, broken lines*) or presence (*solid symbols, solid lines*) of 2 mM ATP. The uptake reaction was stopped at the indicated times, and the amount of ^{3}H-glutamate taken up into the vesicles was determined in triplicate as described in Materials and Methods. The variation as measured by the SD was less than 5% of the uptake values for all data points.

GABA (Fykse and Fonnum, 1988; Hell et al., 1988; Kish et al., 1989), ACh (Yamagata and Parsons, 1989), and dopamine (Floor et al., 1990) requires the maintenance of a membrane potential where the inside of the vesicle is kept more positive than the outside. Studies using bovine or rat brain or spinal cord vesicles have shown that this membrane potential is supplied by a V-type ATPase (Kish and Ueda, 1989; Shioi et al., 1989; Floor et al., 1990). To determine if the V-type ATPase functions in this capacity throughout vertebrate species, we looked at the effect of various ATPase inhibitors on glutamate uptake in these five species (Table 1).

Oligomycin, a mitochondrial H^+-ATPase inhibitor (F-type ATPase), and ouabain and orthovanadate, inhibitors of Na^+/K^+- and K^+/H^+-ATPases (P-type ATPase) had no significant effect on the uptake of glutamate in any of the species tested. Carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP), a proton ionophore, *N*-ethylmaleimide (NEM), a sulfhydryl reagent, and bafilomycin, a specific inhibitor of V-type H^+-ATPases (Bowman et al., 1988), have all been shown to inhibit proton pumping in mammalian chromaffin granules (Cidon and Nelson, 1986), lysosomes (Moriyama and Nelson, 1989a), the Golgi apparatus (Moriyama and Nelson, 1989b), and synaptic vesicles (Floor et al., 1990). When these agents that inhibit V-type ATPases were used against the uptake of glutamate in brain vesicles from rat, pigeon, turtle, frog, and goldfish, ATP-dependent transport was virtually eliminated. These results suggest that the energization system for glutamate uptake is conserved throughout the entire vertebrate class.

Two other agents were tested to see if their actions differed in vesicles from different vertebrates. 4-Bromocriptine is an ergot tripeptide that has been shown to inhibit glutamate uptake competitively in rat synaptic vesicles (Carlson et al., 1989a). This agent also inhibited glutamate uptake into turtle, frog, pigeon, and goldfish vesicles. 3-Phosphoglycerate (3-PG), a glycolytic intermediate, has been found specifically to stimulate glutamate uptake (2-phosphoglycerate and other glycolytic intermediates do not stimulate glutamate uptake) into rat synaptic vesicles by as much as 100% (M. Feng and T. Ueda, unpublished observations). When 3-PG was tested, it was found to stimulate glutamate uptake on all five species, from about 20% up to almost 100%.

Michaelis–Menten kinetics of glutamate uptake

To determine whether the affinity of the glutamate translocator for glutamate has changed during evolution from fish to mammals, the concentration of glutamate was varied, and the initial rate of uptake was measured (Table 2). The transport of glutamate into both intact bovine synaptic vesicles and reconstituted rat vesicles has been shown to follow a classical saturable kinetic profile (Naito and Ueda, 1985; Carlson et al., 1989b) displaying a single K_m of about 1.6–2.0 mM. The values obtained from these species ranged from about 0.7 mM for pigeon vesicles to about 1.7 mM for turtle vesicles. The V_{max} for the initial uptake of glutamate uptake varied quite extensively among the species tested. Goldfish vesicles showed the lowest steady-state level of uptake but had the highest V_{max} (over 7 nmol/mg protein/min). In contrast, turtle vesicles displayed the second-highest steady-state level of uptake (Fig. 1), but the V_{max} was the lowest (1.66 nmol/mg protein/min).

Glutamate uptake in synaptic vesicles from invertebrates

A synaptic vesicle fraction was also prepared from two invertebrates: the major neural ganglion of the crayfish and the heads, abdomens, and thoraxes of *Drosophila*. No ATP-dependent glu-

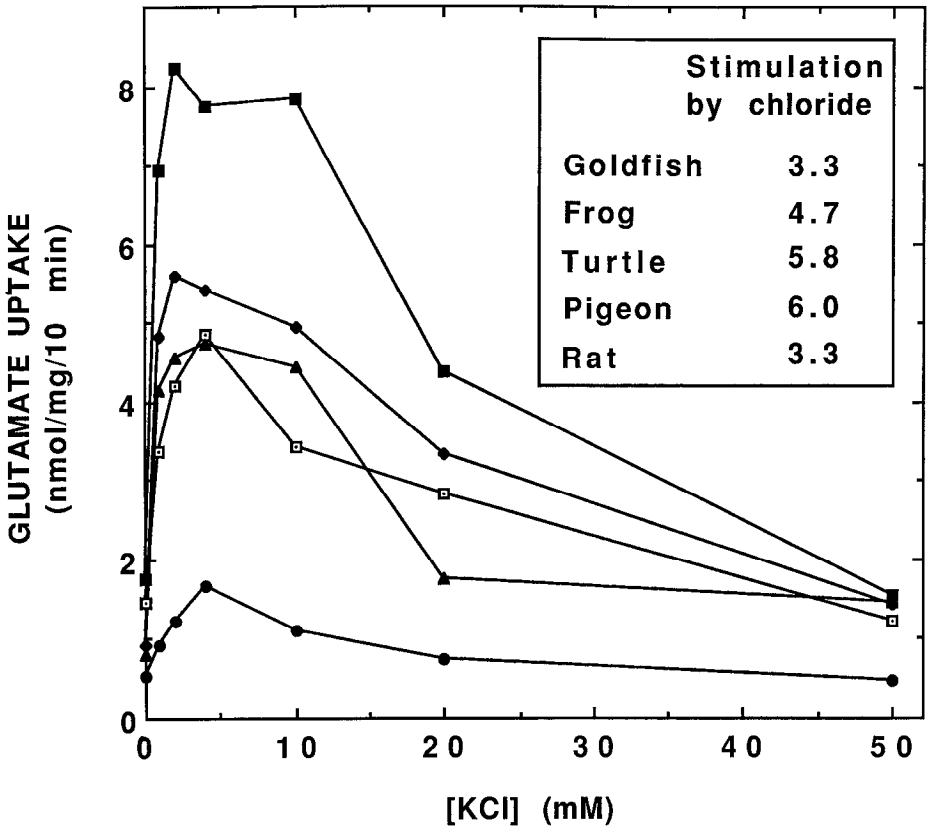

Figure 2. Effects of KCl on ATP-dependent ^{3}H-glutamate uptake into crude synaptic vesicles from various vertebrate brains. Crude synaptic vesicles from goldfish (●), frog (■), turtle (◆), pigeon (▲), and rat (□) brains were incubated with varying concentrations of KCl, and the uptake of 0.5 mM ^{3}H-glutamate was measured for 10 min in the presence or absence of 2 mM ATP. The *numbers* in the *inset* represent the average of triplicate determinations of the ATP-dependent glutamate uptake activity into the vesicles. The variation as measured by the SD was less than 5% of the uptake values for all data points. Vesicles incubated under identical conditions using potassium isethionate instead of KCl showed no difference in uptake activity from those vesicles incubated without KCl (data not shown). The values presented for the stimulation by chloride represent the maximal-fold stimulation of glutamate uptake by KCl.

tamate uptake could be measured in these vesicle fractions, at either low (5 μM) or high (500 μM) concentrations of glutamate.

Discussion

In this study, we have examined the major functional characteristics of glutamate uptake into brain synaptic vesicles and have found that these characteristics remain virtually unchanged among the major classes of vertebrates.

The uptake of several neurotransmitters such as glutamate, GABA, and glycine into mammalian brain vesicles (Naito and Ueda, 1985; Fykse and Fonnum, 1988; Hell et al., 1988; Kish et al., 1989), catecholamines into both mammalian brain vesicles and chromaffin granules (Toll et al., 1977), and ACh into *Torpedo* electric organ vesicles (Anderson et al., 1982) has been previously shown to be ATP dependent. We found this also to be the case with glutamate uptake into vesicles from avian (pigeon), reptilian (turtle), amphibian (bullfrog), and fish (goldfish) brain. The final steady-state levels of glutamate obtained in the vesicles from the various vertebrates varied greatly (Fig. 1). Variation in steady-state glutamate levels may be a function of the following vesicle characteristics: the vesicle size, the vesicular H^+-pump ATPase activity, or the vesicle population density in the vesicle fraction. The initial rate of uptake as expressed by the V_{max} of uptake also varied to a large extent among the species tested (Table 2). These differences in V_{max} may reflect variations in the catalytic turnover rate of the glutamate transport proteins, the number of transport proteins present per vesicle, or again, the vesicle population density in the vesicle fraction. It is unknown at this time which of these vesicle characteristics is responsible for the differences seen in either the steady-state level of glutamate or the V_{max} of glutamate uptake.

The uptake of glutamate into mammalian vesicles has been unequivocally demonstrated to be stimulated by low concentrations of chloride; it is typically stimulated three- to fivefold by 2–4 mM concentrations of chloride. This is in contrast to the uptake of glycine into synaptic vesicles (Kish et al., 1989), which is stimulated only slightly by 5 to 10 mM chloride, or that of GABA, which has been shown to be unaffected by millimolar concentrations of chloride (Fykse et al., 1989; Kish et al., 1989). This study shows that, in all of the species where glutamate uptake was observed, it was stimulated by 2–4 mM chloride. While the function of this stimulation is unclear, it is apparent from this study that the stimulatory effect of chloride is conserved among vertebrates, and this conservation suggests that the intracellular levels of chloride at the nerve terminal may play an important role in regulating the storage of glutamate within the synaptic vesicle.

The synaptic vesicle glutamate transport system is remarkably selective for the amino acid glutamate. Molecules that differ by as little as a single methylene group (such as aspartate or α-aminoadipate) are not recognized by the glutamate transporter (Naito and Ueda, 1985; Kish and Ueda, 1989). This selectivity of the transporter also appears to be strongly conserved throughout vertebrate evolution (Table 1). In all of the species tested, neither the neurotransmitter amino acids glycine and GABA nor aspartate isomers significantly inhibited glutamate uptake. Only α-methyl glutamate and γ-methylene glutamate inhibited vesicular glutamate uptake, and this inhibition was consistent among all of the species examined. The glutamate receptor agonists (NMDA, kainate, and quisqualate) did not inhibit glutamate uptake activity in most of the vertebrates tested. Two patterns were noticed in the inhibition patterns presented in Table 1. First, the glutamate uptake into rat vesicles showed

Table 1. Effects of glutamate analogs and agonists, ATPase inhibitors, and other compounds on glutamate uptake into crude synaptic vesicles from various species

	Percent of control ATP-dependent uptake						
Test agents[a]	Goldfish	Frog	(Frog)[b]	Turtle	(Turtle)[b]	Pigeon	Rat
Amino acids and analogs							
Control[c]	(1.74 ± 0.09)	(10.0 ± 0.2)	(1.2 ± 0.1)	(6.2 ± 0.1)	(0.7 ± 0.04)	(4.8 ± 0.2)	(4.2 ± 0.3)
L-Glutamate	19 ± 3	13 ± 3	(4 ± 1)	8 ± 5	(2 ± 4)	30 ± 2	25 ± 2
D-Glutamate	41 ± 2	39 ± 2	(29 ± 3)	31 ± 5	(15 ± 3)	55 ± 3	66 ± 4
L-Aspartate	95 ± 5	114 ± 6	(96 ± 3)	88 ± 4	(90 ± 3)	71 ± 3	101 ± 4
D-Aspartate	101 ± 5	107 ± 4	(97 ± 2)	98 ± 4	(88 ± 5)	92 ± 5	102 ± 4
α-Methyl-DL-glutamate	28 ± 4	23 ± 2	(14 ± 1)	22 ± 5	(19 ± 4)	35 ± 3	42 ± 6
γ-Methylene-DL-glutamate	41 ± 4	31 ± 3	(22 ± 4)	26 ± 1	(16 ± 2)	44 ± 3	50 ± 4
L-Glutamine	87 ± 6	100 ± 3	(90 ± 5)	97 ± 4	(87 ± 5)	78 ± 4	97 ± 2
α-Ketoglutarate	110 ± 5	101 ± 5	(99 ± 2)	92 ± 4	(85 ± 6)	97 ± 2	102 ± 4
γ-Aminobutyrate (10 mM)	114 ± 2	ND	(ND)	85 ± 6	(ND)	91 ± 2	106 ± 3
Glycine (10 mM)	114 + 4	ND	(ND)	84 ± 4	(ND)	99 ± 1	101 ± 0
Glutamate receptor agonists							
NMDA	100 ± 1	113 ± 5	(101 ± 4)	96 ± 3	(95 ± 5)	81 ± 3	101 ± 2
Kainate	110 ± 2	95 ± 3	(98 ± 3)	90 ± 3	(91 ± 6)	81 ± 4	104 ± 2
Quisqualate	92 ± 5	100 ± 1	(95 ± 5)	92 ± 4	(91 ± 3)	86 ± 3	124 ± 6
ATPase inhibitors/membrane-potential dissipators							
FCCP (10 μM)[d]	0 ± 1	0 ± 2	(0 ± 1)	0 ± 0	(0 ± 1)	0 ± 2	0 ± 3
Oligomycin (2.5 μg/ml)[d]	107 ± 5	ND	(101 ± 3)	114 ± 3	(98 ± 4)	110 ± 3	99 ± 1
Ouabain (1 mM)	99 ± 2	ND	(98 ± 2)	106 ± 4	(100 ± 2)	103 ± 4	100 ± 2
Vanadate (0.5 mM)	109 ± 4	ND	(109 ± 4)	100 ± 1	(101 ± 3)	113 ± 6	113 ± 5
NEM (0.1 mM)	4 ± 2	1 ± 2	(3 ± 4)	0 ± 3	(3 ± 1)	7 ± 4	4 ± 3
Bafilomycin (1 μM)[d]	6 ± 4	ND	(2 ± 3)	4 ± 3	(7 ± 2)	8 ± 2	2 ± 3
Modulatory agents							
Bromocriptine (0.1 mM)[d]	44 ± 2	31 ± 3	(36 ± 3)	41 ± 2	(39 ± 2)	50 ± 5	30 ± 2
3-P (1 mM)	122 ± 6	130 ± 4	(109 ± 4)	158 ± 3	(160 ± 5)	132 ± 5	190 ± 4

Crude synaptic vesicles were isolated from the brains of various species as described in Materials and Methods. The vesicles were incubated with 0.5 mM ^{3}H-glutamate in the presence or absence of 2 mM ATP under standard conditions for 10 min, and the ATP-dependent uptake was determined for control as well as experimental conditions. The values presented are representative inhibition profiles of two duplicate experiments for goldfish and rat and of three experiments for frog, turtle, and pigeon. All numbers are mean ± SD of triplicate determinations, and the values represent ATP-dependent glutamate uptake activity found in the presence of the various agents, expressed as percent of control. ND, not determined.

[a] Concentration of test agents was 5 mM unless otherwise indicated.

[b] Vesicles were incubated with 0.05 mM ^{3}H-glutamate instead of 0.5 mM ^{3}H-glutamate.

[c] Control values are expressed as ATP-dependent uptake activity (nmol/mg protein/10 min).

[d] Both control and experimental conditions were tested in the presence of 1.0% dimethylsulfoxide, which reduced controls by 10–12%.

the strongest preference for glutamate as seen by weaker inhibition of uptake by D-glutamate, D- and L-aspartate, α-methyl glutamate, and γ-methylene glutamate, as well as the strongest stimulation by 3-PG. Second, the uptake of glutamate by pigeon vesicles was reduced 15–20% by both glutamine and the receptor agonists NMDA, kainate, and quisqualate. These variations in substrate–inhibitor interactions may denote minor structural differences in the active site of these glutamate transporters, when compared to the other vertebrate transporters.

The exclusion of glutamate receptor agonists, metabolic intermediates, and aspartate isomers by the vesicular glutamate transporter suggests that the recognition sites for the γ-carbonyl group, the α-amino group, and the five-carbon chain of glutamate are strictly conserved throughout vertebrate evolution. The structural conservation of the glutamate transporter is further demonstrated by the universal effect of 4-bromocriptine, a competitive inhibitor of ATP-dependent glutamate uptake (Carlson et al., 1989a), among vertebrate species tested.

Not only is the structural nature of the active site of this translocator conserved, but the translocator's affinity of glutamate is conserved, as well. Table 2 displays the kinetic parameters for glutamate uptake into the vesicles of various vertebrates studied. While some variation is seen in the K_m values, these numbers are rather similar when compared to the values reported for the sodium-dependent uptake system in the plasma membrane (3–20 μM; Logan and Snyder, 1972; Kanner and Sharon, 1978), or the K_d values reported for glutamate binding to its receptors (10 nM to 2.5 μM; Foster and Fagg, 1984). The V_{max} values for glutamate uptake generated from these experiments varied considerably among the species tested.

It is worth noting that there appears to be an approximate inverse relationship between the V_{max} for the initial rate of glutamate uptake (Table 2) and the amount of glutamate retained in the vesicle at steady state (Fig. 1). Species such as goldfish, which display a low steady-state level of glutamate, also display a high V_{max} for uptake. Conversely, species such as frogs show a high steady-state level of glutamate and a relatively low V_{max} value. These findings may suggest that evolution regulates the internal amount of glutamate by adjusting the vesicular capacity for glutamate and the initial rate of glutamate uptake into the vesicle.

Along with chloride stimulation, substrate/inhibitor specific-

Table 2. Kinetic parameters for glutamate uptake into crude synaptic vesicles

Species	K_m (mM)	V_{max} (nmol/mg protein/min)
Rat	1.65 ± 0.12	5.96 ± 0.11
Pigeon	0.71 ± 0.03	4.40 ± 0.06
Turtle	1.70 ± 0.24	1.67 ± 0.09
Frog	1.39 ± 0.54	3.76 ± 0.07
Goldfish	1.26 ± 0.12	7.02 ± 0.27

Crude synaptic vesicles from the brains of various species were incubated for 1 min with varying concentrations of ^{3}H-glutamate in the presence or absence of 2 mM ATP. The K_m, V_{max}, and error values were determined from single experiments and were assayed in triplicate by least-squares analysis as described under Materials and Methods. Variability is presented as calculated variance.

ity, and substrate affinity, it also appears that the type of ATPase responsible for providing the energy for glutamate uptake in brain synaptic vesicles is conserved among vertebrates. Only NEM and bafilomycin, inhibitors of V-type ATPases, eliminated vesicular glutamate uptake (Table 1), whereas inhibitors of F-type (oligomycin) and P-type ATPase (ouabain and orthovanadate) did not affect glutamate uptake. It should also be noted that the stimulation of 3-PG on ATP-dependent glutamate uptake is also conserved, though the degree of stimulation is variable among these species.

Attempts to demonstrate ATP-dependent glutamate uptake in synaptic vesicles from invertebrates such as crayfish and *Drosophila* were not successful. It is well documented that glutamate is a neurotransmitter at the invertebrate neuromuscular junction (Sansom and Usherwood, 1986), but its role in the invertebrate CNS has not been established. While glutamate receptors have been found in *Drosophila* heads (Fielder et al., 1986), it might be that the levels of glutamatergic neurons in the central nervous ganglia of invertebrates are too low for us to detect glutamate uptake with our transport assay. Alternatively, it is possible that invertebrate central nervous ganglia might utilize a different mechanism for the accumulation of glutamate into synaptic vesicles, if glutamate serves as a neurotransmitter in these ganglia. It is also possible that vesicles from invertebrates lack a glutamate transport system altogether.

The results presented in this study clearly show that the uptake of glutamate into brain synaptic vesicles is conserved throughout vertebrate classes. All of the major functional characteristics of vesicular glutamate transport, such as ATP dependence, chloride stimulation, substrate specificity, substrate affinity, and mode of energization, are similar among the vertebrate species tested. This suggests that the mechanism of vesicular glutamate transport has remained conserved for at least 350–400 million years, from the evolution of boney fish through the evolution of mammals. This well-conserved nature further supports the concept (Ueda, 1986) that the vesicular active transport system specific for glutamate plays a pivotal role in selecting glutamate for synaptic release as a neurotransmitter in the vertebrate CNS.

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