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High-Affinity Transport of L-Glutamine by a Plasma Membrane Preparation from Rat Brain†

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ABSTRACT: Plasma membrane vesicles prepared from rat brain contain a saturable, high-affinity transport system for L-glutamine that exhibits the following characteristics: (1) The rate of L-glutamine transport is linear up to 200 μg/mL membrane protein. (2) Transport of [^{3}H]-L-glutamine is linear with time for at least 10 min, is significantly reduced by lowering the assay temperature to 4 °C, and is essentially abolished by the addition of excess unlabeled L-glutamine. (3) The transport rate is optimal in the range of pH 7.4–8.2. (4) The system exhibits a K_m for L-glutamine of ~1.7 μM and a V_{max} of ~46 pmol/(min·mg of protein). (5) The system is not highly dependent upon the addition of monovalent or divalent cations. (6) Inhibitor studies reveal that the amino acid amides exhibit the highest affinity for the system and that there is a high specificity for the L-isomers.

The amino acid L-glutamine serves as an important extracellular carrier of amino acid nitrogen in the central nervous system and also as a primary metabolic precursor of the neurotransmitter pool of L-glutamate (Kvamme, 1983). Because glutamine is inactive as a neurotransmitter, it is tolerated in the extracellular field of the central nervous system in relatively high (0.2–0.5 mM) levels, in contrast to amino acid neurotransmitters or modulators such as glutamate, aspartate, γ-aminobutyrate, and glycine, which occur at much lower concentrations (<0.01 mM). The initial metabolic step in glutamine catabolism involves the action of glutaminase, which is active in neurons and also in glial cells (Kvamme et al., 1985).

The transport of glutamine into brain cells has been investigated by using brain slices, cultured neurons and glia, and crude synaptosomal preparations. Because of its high extracellular concentration, there is no obvious need for high-affinity glutamine transport in the central nervous system. In keeping with this, a number of workers have detected glutamine transport system that exhibit low affinity (K_m = 0.2–1.0 mM) and relatively high capacity [1–10 nmol/(min·mg of protein)] in both glia and neuronal cell cultures (Cohen & Lajtha, 1972; Baldessarini & Yorke, 1974; Balcar & Hauser, 1978; Walum & Weiler, 1978; Schousboe et al., 1979; Weiler et al., 1979; Benjamin et al., 1980; Yu & Hertz, 1982; Minn & Besagni, 1983; Johansen et al., 1987). However, other investigators have identified high-affinity (K_m ~ 10–50 μM) glutamine transport systems that exhibit a relatively low capacity for glutamine (Roberts & Keen, 1974; Balcar & Johnston, 1975; Roberts, 1976; Shank & Campbell, 1982). These systems may represent minor carriers under physiological conditions.

An energy source of glutamine transport has not been determined, but energy input may be required at some level since the intracellular level of glutamine is at least 10 times higher than the extracellular levels (Kvamme, 1983). Most workers have concluded that low-affinity, high-capacity glutamine transport across the plasma membrane of CNS cells does not involve sodium ion symport (Cohen & Lajtha, 1972; Bal-

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dessarini & Yorke, 1974; Balcar & Hauser, 1978; Walum & Weiler, 1978; Schousboe et al., 1979). Nevertheless, stimulation of glutamine transport by sodium ion has sometimes been observed for the low-affinity systems (Minn & Besagni, 1983; Johansen et al., 1987), and sodium dependency has also been reported for some of the high-affinity systems (Roberts & Keen, 1974; Balcar & Johnston, 1975; Roberts, 1976).

We are unaware of any reports detailing the purification or reconstitution of glutamine transport proteins in the CNS. The availability of purified transport proteins would facilitate the characterization and localization of glutamine transport systems by immunological and molecular biological methodologies. In addition, purified membrane vesicles active for glutamine transport, or proteoliposomes exhibiting reconstituted glutamine transport, would also be useful in detailing the energetics of the transport process in the absence of endogenous enzymes, ions, or energy-coupling factors. As a first step toward characterizing glutamine transport proteins, we have investigated the uptake of glutamine by plasma membrane vesicles prepared from rat brain. These vesicle preparations exhibit a high-affinity, low-capacity uptake of L-glutamine that is independent of sodium ion.

Experimental Procedures

Materials

[3,4-^{3}H(N)]-L-Glutamine and other radiolabeled amino acids were obtained from New England Nuclear. β-Cyano-L-alanine and N^{α}-(chloroacetyl)-L-asparagine were purchased from Vega-Fox Biochemicals; ibotenate, quisqualate, and kainate were obtained from Cambridge Research Biochemicals; L-azaserine and L-isoglutamine were from Calbiochem. All other unlabeled amino acids used in this study were from Sigma.

Methods

Characteristics of the Purified Plasma Membranes. The membrane fractions used in this study were prepared by a modification of the method of Cotman and Taylor (Cotman & Taylor, 1972; Robinson et al., 1985). We used this procedure because the resulting membrane preparations are essentially free of mitochondria and are enriched for plasma membranes, especially those originating from the synaptic complex. Furthermore, electron micrographic studies have indicated that membranes prepared by this method exhibit extensive formation of plasma membrane vesicles. Holtzman male rats, 26–29 days old, were sacrificed by stunning and decapitation, and the forebrain was removed and homogenized (600 rpm, seven strokes) at 30% w/v (dilutions refer to wet weight) in a solution containing 50 μM $CaCl_2$, 0.2 mM HEPES–KOH, pH 7.4, and 0.32 M sucrose (solution A). The homogenate was diluted to 10% w/v with solution A and sedimented at 1500g for 4 min. (Except where noted, the preparation was maintained at 4 °C.) The supernatant solution was saved. The pellets were suspended to 20% w/v in solution A, homogenized, and sedimented as outlined above. The supernatant solutions were combined and sedimented at 17000g for 15 min. To lyse the membrane fraction, the pellets were suspended to 10% w/v in 50 μM $CaCl_2$ and 0.2 M HEPES–KOH, pH 7.4 (solution B), and homogenized (300 rpm, four strokes; all subsequent homogenizations were performed in this manner). This mixture was incubated for 15 min and homogenized again. Potassium phosphate buffer (pH 7.4) containing *p*-iodonitrotetrazolium violet and sodium succinate was added to produce final concentrations of 40, 1, and 15 mM, respectively. This mixture was incubated for 20 min, at 33 °C, and then sedimented at 50000g for 25 min. The pellet was suspended (3 mL/g wet weight) by homogenization in solution C [40 mM potassium phosphate buffer (pH 7.4) containing 0.32 M sorbitol], and the suspension was layered on a discontinuous density gradient composed of 0.85, 1.0, and 1.3 M sucrose and sedimented at 70000g for 100 min. The synaptic plasma membranes were isolated at the 1.0–1.3 M interface. To remove endogenous inhibitors of glutamine transport, the membranes were suspended in solution C and sedimented at 55000g for 15 min. After each wash, the membranes were homogenized in solution C and diluted 100-fold. The membranes were washed a total of three times. The pellet was then suspended in solution C to a concentration of 3–5 mg of protein/mL as determined by the Lowry protein assay using bovine serum albumin as a standard (Lowry et al., 1951). Prior to assay the membranes were pelleted by centrifugation at 55000g for 15 min.

Perchloric acid extracts of these preparations have been subjected to amino acid analysis to determine the amount of endogenous glutamine remaining. A typical preparation contains 100–150 pmol of L-glutamine/mg of membrane protein. Since residual endogenous glutamine may influence the rate and extent of radiolabeled glutamine uptake via an exchange process (see Figures 1 and 2 and Discussion), in some experiments we assayed membrane preparations that had been subjected to more extensive osmotic lysis to reduce the endogenous glutamine levels to below 10 pmol/mg of membrane protein. In these preparations the extent of glutamine uptake was reduced, but there were no detectable changes in the initial rate kinetics of the system.

Glutamine Transport Assays. The assays were performed in 50 mM Tris–acetate buffer (pH 7.1) containing 0.32 M sorbitol (solution D). The membrane pellets were diluted and homogenized in solution D immediately before use. The assays were initiated by the addition of a 100-μL sample of the radiolabeled substrate to 0.9 mL of the membrane preparation. (The standard assay contained 200 nM [^{3}H]-L-glutamine and 200 μg/mL membrane protein.) The solutions were mixed vigorously and then incubated in a shaker water bath at 34 °C for 10 min unless specified otherwise in individual experiments. The membrane-associated radioactivity was determined by filtration through 0.45-μm membrane filters according to a previously published method (Kanner, 1978) or by a rapid centrifugation method (Robinson et al., 1985). The two methods gave equivalent results. Under initial rate conditions, most of the membrane-associated radioactivity (>90%) could be extracted and identified by amino acid analysis as unreacted glutamine.

Results

Kinetics of L-Glutamine Transport. Kinetic analysis of L-glutamine transport by our membrane preparations revealed a significant level of L-glutamine uptake. The incorporation of [^{3}H]-L-glutamine into membrane fractions was linear up to a membrane protein concentration of 200 μg/mL. The rate was linear with time for at least 10 min, was highly diminished when the assay temperature was reduced from 34 to 4 °C, and was essentially abolished in the presence of excess unlabeled L-glutamine (Figure 1). The transport process was sensitive to variations in pH, with maximum rates observed in the range of pH 7.4–8.2 and half the maximum rate at pH 6.8 and 9.0.

Substrate saturation studies were conducted over a substrate range of 100 nM–1 mM. These experiments revealed a hyperbolic response with a small nonsaturable component which accounted for ca. 10% of the uptake at 8 μM substrate levels. After correction for this nonsaturable component, the data in the range of 0.2–8 μM indicated a classical Michaelis–Menten

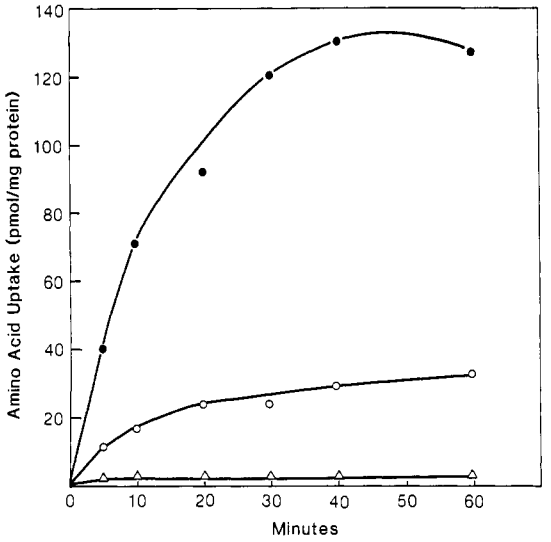

FIGURE 1: Time course for glutamine transport. The uptake of [^{3}H]-L-glutamine was measured as outlined under Methods under the following conditions: incubated at 34 °C (control) (●), incubated at 4 °C (○), and incubated at 34 °C with 1 mM unlabeled L-glutamine added at time 0 (△).

Table I: Effect of Cations on [^{3}H]-L-Glutamine Transport

cation	% control act.[a]	cation	% control act.[a]
10 mM NaCl	104 ± 6	1 mM $CaCl_2$	90 ± 3
50 mM NaCl	103 ± 7	5 mM $CaCl_2$	62 ± 8
100 mM NaCl	99 ± 10	10 mM $CaCl_2$	58 ± 6
10 mM KCl	97 ± 3	1 mM $MgCl_2$	94 ± 6
50 mM KCl	95 ± 3	5 mM $MgCl_2$	90 ± 6
100 mM KCl	102 ± 4	10 mM $MgCl_2$	86 ± 4

[a] Values represent the average of three experiments, each consisting of at least four assays, ± the standard error of the mean.

response, with a K_m of 1.7 μM and a V_{max} of ~46 pmol/(min·mg of protein) (Figure 2).

In some previous studies, glutamine uptake in the CNS was found to be stimulated by sodium ion (Roberts & Keen, 1974; Balcar & Johnston, 1975; Roberts, 1976; Minn & Besagni, 1983; Johansen et al., 1987). In addition, other amino acid transport systems in the brain are known to be dependent on sodium, potassium, or chloride ion (Kanner, 1978; Kanner & Sharon, 1978). We therefore tested the ion dependency of glutamine transport. In our system, none of the ions appear to be necessary for transport activity. We found that the addition of sodium, potassium, or magnesium chloride to the Tris–acetate buffer had no effect on the rate of glutamine transport and that calcium chloride was somewhat inhibitory (Table I).

Substrate Specificity of the Uptake System. The substrate specificity of [^{3}H]glutamine transport was determined by using a 500-fold excess of the unlabeled amino acids as inhibitors (Table II). We found that L-glutamine and L-asparagine were the most effective inhibitors. Other amino acids of similar size were also inhibitory, and there appeared to be little sensitivity to the net charge on the amino acid side chain since neutral, basic, and acidic amino acids were all effective, provided the side chain was not too elongated or bulky. In contrast, there appeared to be a stringent stereospecificity in that none of the D-isomers tested gave strong inhibition. Even though the system favors L-glutamine, D-glutamine was one of the weakest inhibitors.

We also compared the transport of several ^{3}H-amino acids directly. We were particularly interested in determining the transport rates for [^{3}H]-L-glutamate because of the potential

Table II: Inhibition of 200 nM [^{3}H]-L-Glutamine Transport by Amino Acids

inhibitor (100 μM)	% control act.[a]
L-glutamine	4 ± 1
L-asparagine	14 ± 4
β-chloro-L-alanine	18 ± 2
L-cysteine	20 ± 4
L-glutamate γ-hydroxamate	22 ± 4
L-glutamate	22 ± 7
L-aspartate	24 ± 7
β-cyano-L-alanine	26 ± 5
L-histidine	29 ± 7
L-aspartate β-hydroxamate	32 ± 3
L-serine	32 ± 6
L-valine	33 ± 4
L-alanine	35 ± 4
glycine	45 ± 7
L-glutamate β-hydrazide	50 ± 6
L-isoleucine	51 ± 7
L-leucine	51 ± 9
ibotenate	52 ± 7
L-threonine	57 ± 3
L-azaserine	57 ± 6
D-glutamate	62 ± 2
O-acetyl-L-serine	62 ± 6
L-methionine	70 ± 2
N^{α}-(chloroacetyl)-L-asparagine	72 ± 6
quisqualate	74 ± 6
kainate	78 ± 6
L-2-amino-4-phosphonobutanoate	81 ± 4
L-arginine	84 ± 3
L-tyrosine	84 ± 5
D-aspartate	86 ± 3
L-proline	87 ± 7
L-lysine	87 ± 3
L-tryptophan	87 ± 3
L-phenylalanine	90 ± 6
D-asparagine	94 ± 7
L-isoglutamine	94 ± 2
γ-aminobutyrate	97 ± 5
D-glutamine	98 ± 4

[a] Values represent the average of three experiments, each consisting of at least four assays, ± the standard error of the mean.

Table III: Effect of Inhibitors on [^{3}H]-L-Glutamine Transport

reagent	% control act.[a]
100 μM ouabain	103 ± 4
5 μM carbonyl cyanide *m*-chlorophenylhydrazone	86 ± 4
100 μM *p*-(hydroxymercuri)benzoate	85 ± 7
5 μM gramicidin D	92 ± 9
2.5 μM valinomycin	95 ± 4
0.1% saponin	12 ± 6

[a] Values represent the average of three experiments each consisting of at least four assays, ± the standard error of the mean.

metabolic breakdown of glutamine to glutamate during the transport assay. We found that glutamate transport activity was significantly lower than that of glutamine under our assay conditions (Figure 3). In addition, [^{3}H]glutamate uptake was most strongly inhibited by acidic amino acids, whereas glutamine uptake was most sensitive to the amino acid amides (data not shown). The data are thus consistent in suggesting that the system we are assaying is most effective with L-glutamine as the transport substrate.

Effect of Inhibitors. We tested various metabolic inhibitors, uncouplers, and ionophores for the ability to inhibit or stimulate glutamine transport (Table III). The proton conductors and ionophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), gramicidin D, and valinomycin had little effect at levels that cause inhibition or stimulation of other amino acid transport systems (Kanner, 1978; Kanner & Sharon, 1978). This is consistent with the lack of ion requirements that we observed for glutamine transport. The

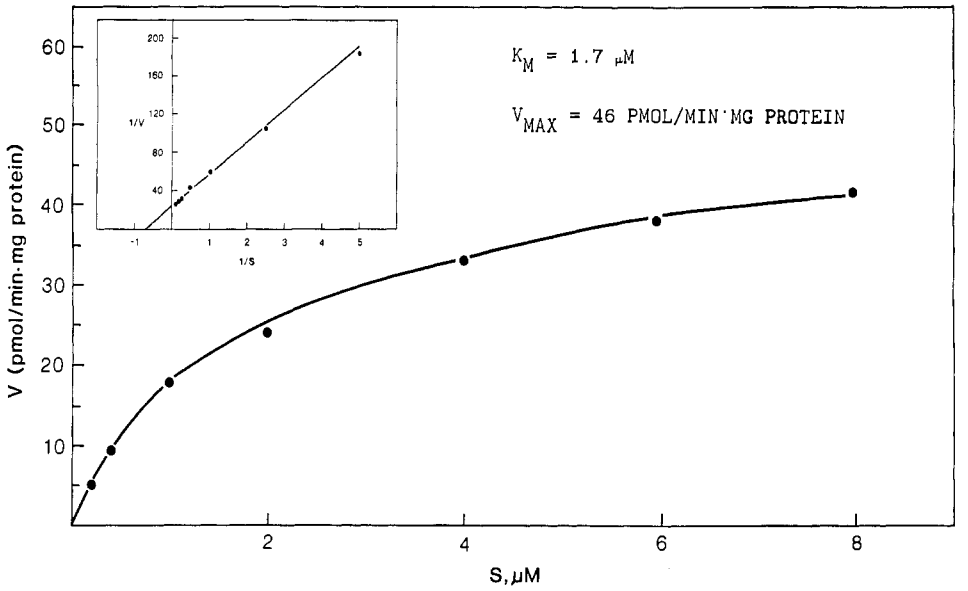

FIGURE 2: Substrate saturation response for glutamine. The assay conditions were those given under Methods except the [^{3}H]-L-glutamine levels were varied. Inset shows data plotted by the method of Lineweaver and Burk.

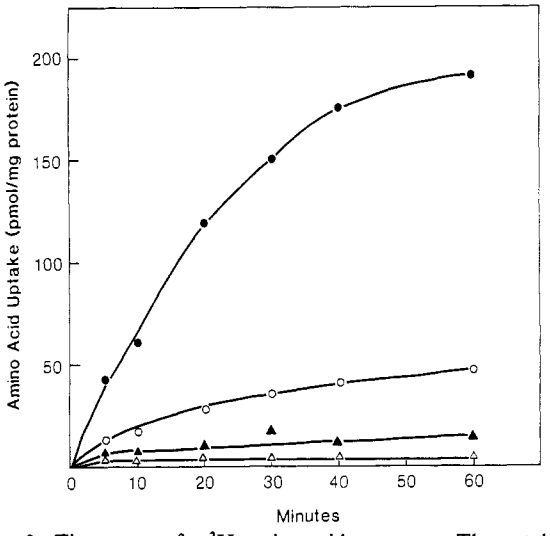

FIGURE 3: Time course for ^{3}H-amino acid transport. The uptake of various ^{3}H-amino acids (200 nM) was measured under conditions identical with those used for glutamine uptake: L-glutamine (●), L-glutamate (○), L-alanine (▲), and D,L-2-amino-4-phosphonobutyrate (△).

transport activity was inhibited by the detergent saponin, suggesting that intact vesicles were necessary for transport activity. Consistent with this, our findings show that the extent of glutamine uptake is responsive to the osmolarity of the transport media; i.e., the extent of glutamine uptake is reduced approximately 30% in experiments in which the preincubation buffer contains no sorbitol and the transport buffer contains either 0.3 M sorbitol or 0.3 M sucrose.

Discussion

We have characterized a transport system for [^{3}H]-L-glutamine using plasma membrane vesicles prepared from rat brain. This transport system exhibits a very high affinity for L-glutamine and is not significantly stimulated by sodium, potassium, or chloride ions. Inhibitor studies and assays conducted with alternate amino acid substrates suggest that L-glutamine is the preferred substrate and that there is a high degree of stereospecificity.

One question concerning our study is why we observed high-affinity, low-capacity glutamine transport in purified membrane vesicles but did not detect significant levels of low-affinity, high-capacity systems which other workers have detected using brain slices or crude synaptosomes (Cohen & Lajtha, 1972; Baldessarini & Yorke, 1974; Balcar & Hauser, 1978; Walum & Weiler, 1978; Schousboe et al., 1979; Weiler et al., 1979; Benjamin et al., 1980; Yu & Hertz, 1982; Minn & Besagni, 1983; Johansen et al., 1987). One possible explanation is that the preparation conditions and assay methods we utilized are not favorable for transport activity by the high-capacity system and thus facilitate the characterization of a low-capacity system. Consistent with this possibility, our results in preliminary experiments show that crude synaptosomal preparations from rat brain exhibit considerable low-affinity L-glutamine uptake activity and that much of this activity is lost when the synaptosomes are subjected to osmotic lysis and the membranes fractionated under the conditions used for our membrane vesicle preparations (data not shown). It seems likely that by selecting appropriate lysis conditions, fractionation procedures, buffer systems, and cofactor additions, it will be possible to retain activity for the high-capacity system in membrane vesicles.

The occurrence of both high- and low-affinity amino acid transport systems in the central nervous system is not without precedent. For example, two kinetically distinguishable systems have recently been described for γ-aminobutyrate transport (Keynan & Kanner, 1988). However, the physiological significance of high-affinity, low-capacity systems for glutamine is presently unclear. Because extracellular levels of L-glutamine are quite high in the CNS, there is no obvious need for high-affinity systems. These systems may be concentrated at particular cellular locations where glutamine is favorably accumulated. In order to determine whether the system that we have detected is enriched in specific regions of the CNS and/or at specific subcellular locations, it will probably be necessary to further characterize and purify the system so that specific immunological and molecular biological probes can be developed.

A second question that has arisen during our study concerns the apparent concentrative accumulation of glutamine within the vesicles. When 200 nM labeled glutamine is assayed, we find a typical steady-state accumulation of approximately 150 pmol/mg of membrane protein. By use of previously accepted values for the intravesicular volume (Kanner, 1978), this

corresponds to a concentration of labeled glutamine within the vesicles of about 20–30 μM (i.e., an approximate 100-fold enrichment). We have found no evidence for either membrane potential or ion gradient requirements to account for this concentrative uptake. External ions are not needed, and the usual preincubation buffer containing potassium can be replaced with no significant change in uptake kinetics. The concentrative effect we are seeing is probably due to an exchange reaction between the external labeled glutamine and unlabeled glutamine that has been retained within the vesicle preparation. Indeed, amino acid analysis of several vesicle preparations indicated that even after extensive washing L-glutamine is present at about 50 μM levels. This is not surprising given the high endogenous levels of glutamine in the CNS (3–5 mM) (Kvamme, 1983), and this level is sufficient to account for the observed concentration of the labeled glutamine by the system.

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Functional Modification of a Ca^{2+}-Activated K^+ Channel by Trimethyloxonium†

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ABSTRACT: Single Ca^{2+}-activated K^+ channels from rat skeletal muscle plasma membranes were studied in neutral phospholipid bilayers. Channels were chemically modified by briefly exposing the external side to the carboxyl group modifying reagent trimethyloxonium (TMO). TMO modification, in a "multi-hit" fashion, reduces the single-channel conductance without affecting ion selectivity. Modification also shifts the voltage activation curve toward more depolarized voltages and reduces the affinity of the channel blocker charybdotoxin (CTX). CTX, bound to the channel during the TMO exposure, prevents the TMO-induced reduction of the single-channel conductance. These data suggest that the high-conductance Ca^{2+}-activated K^+ channel has carboxyl groups on its external surface. These groups influence ion conduction, gating, and the binding of CTX.

Ion channels are integral membrane proteins that serve as the molecular basis for cellular electrical excitability. They perform the seemingly simple task of allowing ions to diffuse passively down their electrochemical gradients. However, to do this in a physiologically useful way, ion channels must exhibit several sophisticated properties. In response to external signals, such as transmembrane voltage changes or binding of specific ligands, they must be able to switch rapidly between nonconducting and conducting states. The open channel must be able to discriminate among various ions, allowing only one or a few types to permeate. Moreover, many channels are specifically altered in function by natural toxins or synthetic drugs which bind to receptor sites on the channel protein.

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