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Low-Affinity γ-Aminobutyric Acid Transport in Rat Brain†

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ABSTRACT: The low-affinity (K_m = 100–200 μM) γ-aminobutyric acid (GABA) transporter from membrane vesicles from rat brain has been characterized and found to be in many aspects similar to the well-known sodium- and chloride-coupled high-affinity γ-aminobutyric acid transporter (K_m = 2–4 μM). Influx by this system is sodium and chloride dependent and stimulated by an interior negative membrane potential. Steady-state levels obtained by both systems are lowered by the sodium channel openers veratridine and aconitine. However, while the channel blocker tetrodotoxin fully reverses this inhibition with the high-affinity system, this is not the case for its low-affinity counterpart. Furthermore, the toxin from the scorpion *Androctonus australis* Hector inhibited high-affinity transport only. Efflux of γ-aminobutyric acid taken up by the high-affinity system displayed a K_m of about 100 μM. Exchange catalyzed by the low-affinity system was observed in the absence of external sodium and chloride. Furthermore, both activities copurified in the fractionation procedure developed to purify the high-affinity transporter. All these observations are consistent with the idea that both activities are manifestations of only one γ-aminobutyric acid transporter. The high-affinity binding site represents the extracellular and the low-affinity site the cytosolic aspect of the transporter. In addition, it was found that right-side-out synaptosomes also contain a low-affinity GABA transporter. This apparently represents a different transport protein.

The role of high-affinity neurotransmitter transport is to terminate the overall process of synaptic transmission by removing the transmitters from the synaptic cleft (Iversen, 1975; Kuhar, 1973). These transporters are in fact sodium ion–neurotransmitter cotransporters and are able to accumulate the neurotransmitter against considerable concentration gradients by using the electrochemical gradient of sodium ions. Furthermore, many of these transporters are also absolutely

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dependent on other ions, such as chloride or potassium, and are apparently coupled to them as well [reviewed in Kanner (1983) and Kanner and Schuldiner (1987)]. One of the important and abundant transporters in rat brain is the one for γ-aminobutyric acid (GABA).[1] It is absolutely dependent on sodium and chloride ions (Kanner, 1978) and has been demonstrated to be an electrogenic sodium–chloride–GABA cotransporter (Radian & Kanner, 1983; Keynan & Kanner, 1988). The high-affinity transporter has been reconstituted (Radian & Kanner, 1985) and purified to an apparent homogeneity (Radian et al., 1986). It is a glycoprotein with an apparent molecular weight of 80 000. This polypeptide contains all the information necessary to catalyze electrogenic, sodium- and chloride-coupled GABA transport (Radian et al., 1986).

Recently it has been observed that in addition to the high-affinity GABA transporter there are—at least kinetically—low-affinity transporter(s) for this transmitter in synaptosomes (Wood & Sidhu, 1986), in synaptic plasma membrane vesicles, and in reconstituted systems (Keynan & Kanner, 1988). In the latter system it has been observed that the transport is also dependent on both sodium and chloride (Keynan & Kanner, 1988).

The aim of this paper is to establish if there is a relationship between high- and low-affinity GABA transporters from rat brain.

Experimental Procedures

Materials

[2,3-^{3}H]GABA (specific radioactivity, 36.9–50.0 Ci/mmol) was purchased from the Nuclear Research Center, Negev. Sephadex G-50–80 was from Pharmacia, DEAE-cellulose (DE-52) from Whatman, and wheat germ agglutinin–Sepharose 4B-CL was from Makor Chemicals. Aconitine, veratridine, valinomycin, cholic acid, and asolectin were from Sigma. The cholic acid was recrystallized and asolectin was purified by acetone extraction, both as described (Kagawa & Racker, 1971). Crude lipids were extracted from bovine brain (Folch et al., 1957). Triton X-100 was from Packard, and β-octyl glucoside and nigericin were from Calbiochem.

MT_{II} purified from crude venom from the scorpion was a gracious gift of Professor Eliahu Zlotkin, Department of Zoology, The Hebrew University. MT_{II} was stored in the presence of 1 mg/mL of BSA for stability. ACHC was synthesized for us by Dr. Erwin Gross from the Pilot Plant at the School for Applied Science of the Hebrew University according to the method of Johnston et al. (1977). Membrane filters were from Schleicher and Schuell or from Millipore. All other reagents were of the purest commercially available.

Methods

Preparations. Crude and Ficoll gradient purified synaptosomes were prepared as described, and membrane vesicles were prepared from the latter upon osmotic shock (Kanner, 1978). Synaptosomes were used immediately, and membrane vesicles were quick-frozen in liquid air and stored at −70 °C. The sodium- and chloride-coupled high-affinity GABA transporter was solubilized from rat brain membranes, purified, and reconstituted as described (Radian & Kanner, 1985; Radian et al., 1986).

[1] Abbreviations: GABA, γ-aminobutyric acid; TTX, tetrodotoxin; MT_{II}, mammal toxin II, purified from the scorpion *Androctonus australis* Hector; BSA, bovine serum albumin; ACHC, *cis*-3-aminocyclohexanecarboxylic acid.

Table I: Effects of Sodium, Chloride, and Ionophores on Low-Affinity GABA Transport[a]

external medium	addition	GABA (nmol/mg of protein)
150 mM NaCl		9.9 ± 1.0
150 mM LiCl		1.4 ± 0.1
150 mM sodium glucuronate		0.8 ± 0.2
150 mM NaCl	nigericin, 5.0 μM	1.9 ± 0.5
150 mM NaCl	valinomycin, 2.5 μM	13.2 ± 0.3

[a] Low-affinity GABA transport was measured as described under Experimental Procedures by using the indicated influx media. Transport was measured for 7 min with 80 μg of membrane vesicles per time point. Each value was obtained from triplicate determinations.

High- and Low-Affinity GABA Transport. The high-affinity process was measured as described (Kanner, 1978), except that 10 μL of potassium-loaded membrane vesicles was diluted in 100 μL of the influx solution containing 150 mM NaCl and 1 μCi of [2,3-^{3}H]GABA. Low-affinity transport was done as described (Keynan & Kanner, 1988) by using 4 μCi of [2,3-^{3}H]GABA and 1 mM unlabeled GABA in the above influx solution. The values were corrected for binding by subtracting results obtained in identical incubations in the presence of 50 mM unlabeled GABA. It can be calculated by using the known values of K_m and V_{max} for the high-affinity system (Kanner, 1978; Kanner et al., 1983) that the contribution of high-affinity transport to the low-affinity process—due to incomplete suppression of the high-affinity process by 1 mM GABA—was never more than 10–20%. The measurement of the two transport processes in synaptosomes was as above except that the temperature was 37 °C and the influx solution contained 100 mM NaCl, 6 mM KCl, 30 mM Tris–P_i, pH 7.3, and 10 mM glucose instead of the 150 mM NaCl. Influx in proteoliposomes was measured as described (Radian & Kanner, 1985). Efflux and exchange of GABA from membrane vesicles were done as previously reported (Kanner & Kifer, 1981; Kanner et al. 1983).

Protein Determination. Protein was determined by using the method of Lowry et al. (1951) or, in the case of diluted solutions of purified GABA transporter preparations, the method of Peterson (1977).

Results

Ion Dependence of Low-Affinity GABA Transport. GABA influx via the low-affinity system in membrane vesicles from rat brain is measured by preloading the vesicles in potassium phosphate and diluting them into sodium chloride. Thereby, artificial sodium and chloride ion gradients (both inward) are generated, and optimal transport is observed under these conditions. With 1 mM GABA in the external medium, this uptake is linear for 3 min, and a steady-state level of 8–10 nmol of GABA/mg of protein is attained. The low-affinity system has a K_m of 150 ± 50 μM as compared with a K_m of 2–4 μM for the high-affinity system (Keynan & Kanner, 1988). Low-affinity uptake has a V_{max} of 3.2 ± 1.0 nmol min^{-1} (mg of protein)$^{-1}$ and was sensitive to the osmolarity of the external medium (data not shown). This implicates that the uptake process represents transport into an osmotically sensitive space. Like the high-affinity system, its low-affinity counterpart is dependent on both sodium and chloride (Table I). Using the approach of ion substitutes (Shank et al., 1987), we found that addition of 100 mM lithium chloride or 100 mM sodium glucuronate to the standard uptake medium—150 mM NaCl—had only a slight (10%) inhibitory effect on low- as well as high-affinity GABA uptake. The same effect was also observed when 200 mM sucrose was used instead. Thus, the

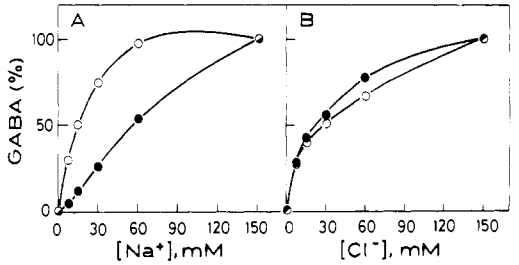

FIGURE 1: Dependence of the initial rates of high- and low-affinity GABA transport on the external sodium and chloride concentration. Transport was carried out for 1 min. (A) Sodium dependence. The membrane vesicles, 60 μg of protein per time point, were diluted into influx solution containing the indicated sodium chloride concentrations plus amounts of lithium chloride, such that the sum was 150 mM. The influx solution also contained 1 μCi of [2,3-^{3}H]GABA and 20 μM unlabeled GABA, (solid symbols) high-affinity transport, or 6 μCi of [2,3-^{3}H]GABA and 500 μM GABA, (open symbols) low-affinity transport. (B) Chloride dependence. The membrane vesicles, 75 μg of protein per time point, were diluted into influx medium containing the indicated sodium chloride concentrations plus amounts of sodium glucuronate to bring the sum to 150 mM. The influx solution was supplemented as above with labeled and unlabeled GABA. (Solid symbols) High-affinity transport; (open symbols) low-affinity transport.

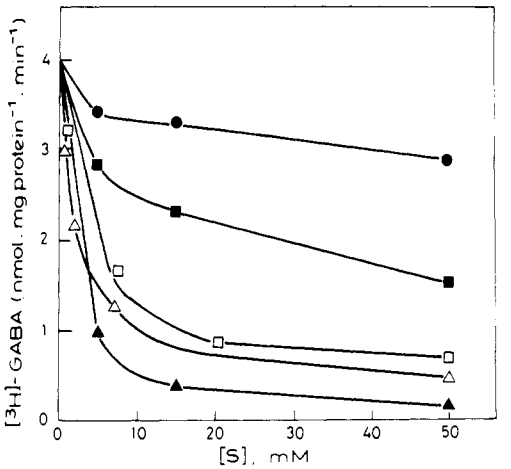

FIGURE 2: Effect of substrate analogues on low-affinity GABA transport. Transport was done with 70 μg of membrane protein per time point as indicated under Experimental Procedures by using the indicated concentrations of analogue: (▲) GABA; (■) β-alanine; (□) ACHC; (△) nipecotic acid; (●) L-glutamic acid.

substituents appear to be inert, and the processes exhibit a true dependence on sodium and chloride. Valinomycin, the potassium ionophore, is expected under the above conditions (outwardly directed potassium gradient) to increase the membrane potential. Its presence results in a stimulation of transport (Table I), indicating that also this system is electrogenic. Nigericin, which is expected to collapse the sodium ion gradient by exchanging external sodium for internal potassium, strongly inhibits the process (Table I). The sodium dependence of the low-affinity process is somewhat different from that of the high-affinity one. While the latter is a sigmoidal function of the sodium concentration, the dependence of the former appears to be hyperbolic (Figure 1A). On the other hand, the dependence of both processes on the chloride concentration is very similar (Figure 1B).

Inhibitor Sensitivity of Low-Affinity GABA Transport. The sensitivity of the low-affinity system to a variety of established inhibitors—substrate analogues—of the high-affinity system was tested with the objective to differentiate between the two systems. Of all compounds, GABA itself had the highest affinity for the low-affinity system. L-Glutamic acid, which is not a substrate for the high-affinity system, did not have much effect (Figure 2). The slight inhibition observed may be due to trace amounts of GABA present in commercial sources of L-glutamic acid. Nipecotic acid and β-alanine, two well-known inhibitors of high-affinity GABA transport, are also inhibitors of the low-affinity system (Figure 2). Another potent inhibitor of high-affinity transport is *cis*-3-aminocyclohexanecarboxylic acid (ACHC). This inhibitor, which is thought to be specific for neuronal GABA transport, has been reported to exhibit a single class of binding sites on brain membranes (Neal & Bowery, 1977). Thus, it was anticipated that ACHC might differentiate between both systems. However, as shown in Figure 2, ACHC also inhibits the low-affinity process. Thus, all known substrate analogues for the high-affinity system also interact with the low-affinity system. All these analogues—just like GABA itself—have an affinity that is lower by almost 2 orders of magnitude than that to the high-affinity system.

There is a class of inhibitors of high-affinity GABA transport that act indirectly. These are compounds that have the ability to open action potential sodium channels, like

Table II: Effects of Veratridine, Aconitine, and TTX on GABA Transport[a]

expt	addition	GABA (nmol/mg of protein) high affinity	GABA (nmol/mg of protein) low affinity
1	none	0.182 ± 0.004	9.9 ± 0.6
	veratridine, 50 μM	0.133 ± 0.002	7.3 ± 0.1
	TTX, 1 μM	0.194 ± 0.003	9.8 ± 0.1
	veratridine, 50 μM, + TTX, 1 μM	0.187 ± 0.003	8.5 ± 0.2
2	none	0.261 ± 0.004	18.9 ± 0.4
	veratridine, 50 μM	0.210 ± 0.004	12.2 ± 0.3
	TTX, 1 μM	0.275 ± 0.008	19.5 ± 0.2
	veratridine, 50 μM, + TTX, 1 μM	0.263 ± 0.002	14.3 ± 0.8
3	none	0.364 ± 0.012	16.8 ± 0.7
	aconitine, 100 μM	0.213 ± 0.006	11.5 ± 0.3
	TTX, 1 μM	0.399 ± 0.019	16.9 ± 0.5
	aconitine, 100 μM, + TTX, 1 μM	0.370 ± 0.004	13.2 ± 0.4

[a] High-affinity transport and low-affinity transport were measured as described under Experimental Procedures, in the presence of the inhibitors at the indicated final concentrations. Time points were taken at 7 min with 70, 85, or 100 μg of vesicle protein for experiments 1, 2, and 3, respectively. Quadruplicate determinations were done.

veratridine. The inhibition takes place apparently via dissipation of the electrochemical gradient for sodium ions by way of sodium channels located in the same vesicles as the transporter. The effect of veratridine is reversed by TTX, which closes these channels—even in the presence of veratridine. We have examined the effects of sodium channel ligands on low-affinity GABA transport and compared them with those on its high-affinity counterpart. As shown in Table II, an inhibition of high-affinity transport of about 25% by 50 μM veratridine is observed, and this inhibition is fully reversed by 1 μM TTX. In the neuron veratridine inhibits since it is hydrophobic and readily crosses the membrane; TTX, on the other hand, is impermeant and only inhibits from the outside (Narahashi et al., 1966). These results therefore confirm our previous conclusion that high-affinity transport is catalyzed by membrane vesicles having the same orientation of the neuronal cell (Kanner, 1980, 1983). Low-affinity GABA transport is also inhibited by veratridine to about the same extent as above; however, TTX is able to reverse only 10–50% of this inhibition (Table II). This indicates that at least 50% of the low-affinity transport is residing in inside-out membranes. This number may even be higher since about 10–20% of the observed low-affinity transport is contributed

Table III: Effects of MT_{II} and TTX on GABA Transport[a]

addition	GABA (nmol/mg of protein) high affinity	low affinity
none	0.280 ± 0.008	13.9 ± 1.1
MT_{II}, 1.25 μg/mL	0.238 ± 0.008	13.7 ± 0.8
TTX, 1 μM	0.273 ± 0.004	13.5 ± 0.6
MT_{II}, 1.25 μg/mL, + TTX, 1 μM	0.277 ± 0.008	13.5 ± 0.5

[a] Membrane vesicles were preincubated with MT_{II} at 1.25 μg/mL for 15 min at 37 °C. Since the MT_{II} stock solution also contained BSA, so that its final concentration was 25 μg/mL, the controls were incubated with this BSA concentration alone. Subsequently high-affinity transport and low-affinity transport were done by using 100 μg of membrane vesicles per time point. Values were computed from quadruplicate determinations.

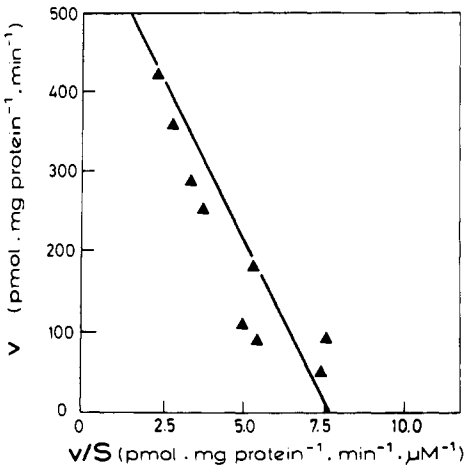

FIGURE 3: Kinetics of efflux of GABA, previously taken up by the high-affinity system. Membrane vesicles (5 μL, 25 μg of protein), preloaded in 10 mM KP_i, pH 6.8, and 140 mM KCl, were diluted into 45 μL of influx medium containing 150 mM NaCl and 0.015–0.045 μCi of [2,3-^{3}H]GABA, such that the external concentration ranged from 0.033 to 1.6 μM. By use of the value of 3.8 μL/mg of protein for the internal volume, the internal GABA concentrations were found to be between 6.5 and 172 μM. After 7 min of influx, 1 mL of efflux medium (10 mM KP_i, pH 6.8, 80 mM KCl, 60 mM NaCl, and 5 μM nigericin) was added. After 10 s of efflux, reactions were terminated.

by residual high-affinity transport—not completely suppressed by the 1 mM unlabeled GABA present under the conditions of low-affinity transport. Similar data are also obtained by using aconitine instead of veratridine (Table II). The potent toxin MT_{II} purified from the crude venom of the scorpion *Androctonus australis* Hector is also an opener of the sodium channel. But unlike veratridine and aconitine, the scorpion toxins act from the outside (Narahashi et al., 1972). Indeed, as predicted MT_{II} inhibits high-affinity transport in a TTX-reversible manner (Table III). However, no effect of the toxin was seen on the low-affinity transport (Table III), entirely consistent with the idea that low-affinity transport is catalyzed by inside-out membrane vesicles and high-affinity transport by right-side-out ones.

Efflux and Exchange. The experiments above suggest that the sodium- and chloride-coupled GABA transporter has a high affinity to GABA at its extracellular side and a low affinity to it at its cytoplasmic side. Thus, GABA taken up with high affinity by the right-side-out vesicles should exit the vesicles in a manner showing low affinity for internal GABA. This is indeed the case as shown in the experiment depicted in Figure 3. The membrane vesicles are actively loaded with radioactive GABA at external concentrations ranging from 0.033 to 1.6 μM by using an inwardly directed sodium gradient and an internal negative membrane potential.

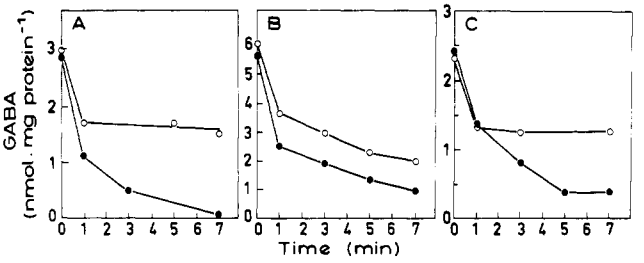

FIGURE 4: Efflux and exchange of GABA, previously taken up by the low-affinity system. Membrane vesicles (3 μL, 27 μg of protein) were diluted into 22 μL of the standard low-affinity transport medium. After 7 min, efflux was initiated by addition of 1 mL of (A) 90 mM NaCl plus 10 mM NaP_i, pH 6.8, (B) 90 mM LiCl plus 10 mM LiP_i, pH 6.8, or (C) 100 mM NaP_i, pH 6.8. These solutions were (solid symbols) or were not (open symbols) supplemented by 5 mM unlabeled GABA.

When the steady state was reached, dilution-induced efflux was initiated by diluting the mixture 20-fold in a sodium-containing medium without the radioactive GABA. Nigericin was present to "clamp" the internal sodium to a controlled level—that of the dilution medium. This sodium concentration was chosen, such that efflux, which is dependent on internal sodium and chloride, would be relatively high—not limited by internal ion concentrations, unlike efflux under more physiological conditions (Kanner & Kifer, 1981; Kanner et al., 1983)—and linear for enough time to determine initial rates of efflux. The Eadie–Hofstee plot of this experiment, where the rate of efflux was measured as a function of the internal GABA concentration [by use of a value of 3.8 μL/mg of protein for the value of the volume to which GABA has access (Kanner, 1978)], yields a K_m of 90 μM and a V_{max} of 0.62 nmol min^{-1} (mg of protein)$^{-1}$. This K_m is almost 50-fold higher than the K_m for influx and is similar to that of the low-affinity uptake. Although K_m and V_{max} values for influx are somewhat dependent on sodium and chloride concentrations (Radian & Kanner, 1983), this is certainly not sufficient to account for the large difference in K_m. However, it can explain the about 4-fold difference between the V_{max} for efflux (Figure 3) and the V_{max} of low-affinity influx, which was determined under optimal conditions.

The ability of external GABA to stimulate dilution-induced GABA efflux via the high-affinity system (by converting it to exchange) has been used to obtain information on the binding order of the substrates sodium, chloride, and GABA (Kanner et al., 1983). According to the model, on the inside sodium and chloride inside bind first followed by GABA. If the inside-out vesicles are catalyzing low-affinity uptake, the model would predict that exchange of radioactive GABA previously taken up by the low-affinity process for unlabeled external GABA would take place in the absence of external sodium or chloride. It can be seen that the biggest stimulation of dilution-induced efflux by external GABA is obtained in the presence of both sodium and chloride (Figure 4A). However, a clear stimulation is also observed in the absence of sodium (Figure 4B) or chloride (Figure 4C). These results are different from the exchange data with the high-affinity system. In the latter case external sodium is required (Kanner & Kifer, 1981).

Copurification of High- and Low-Affinity GABA Transport. If the same sodium- and chloride-coupled GABA transporters exhibit high- and low-affinity GABA transport, the two activities should copurify. We have solubilized, purified, and reconstituted the high-affinity GABA transporter (Radian & Kanner, 1985; Radian et al., 1986). During the process the transporter is partly inactivated, but enough activity

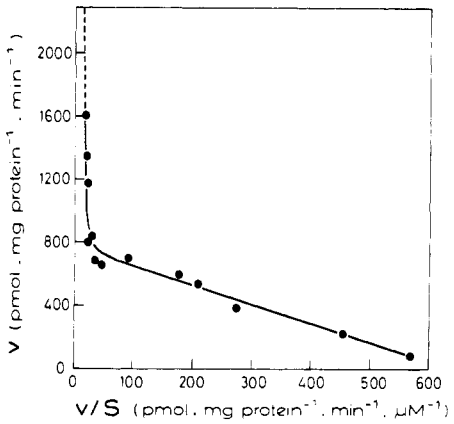

FIGURE 5: Kinetics of GABA uptake in proteoliposomes reconstituted with the DEAE peak fraction. Transport with proteolysosomes that were reconstituted with the DEAE peak fractions (10 μL, 2 μg of protein) was carried out by using 100 μL of a 150 mM NaCl containing influx solution that was supplemented with [2,3-^{3}H]GABA (1.5–7 μCi), unlabeled GABA (0.9–100 μM), and valinomycin (2.5 μM). Reactions were stopped after 3 min.

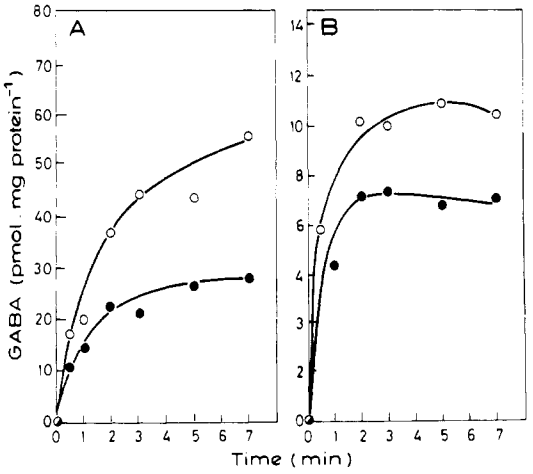

FIGURE 6: Inhibition of high- and low-affinity GABA transport in synaptosomes by ouabain. Synaptosomes were resuspended in a buffer containing 100 mM NaCl, 6 mM KCl, 30 mM Tris–P_i, pH 7.3, and 10 mM glucose with (solid symbols) or without (open symbols) 1 mM ouabain and incubated for 10 min at 37 °C. Subsequently, 5 μL of the synaptosomal suspension (30 μg of protein) was diluted into 190 μL of the buffer with (solid symbols) or without (open symbols) 1 mM ouabain and also 1 μCi of [2,3-^{3}H]GABA (A, high-affinity transport) or 8 μCi of [2,3-^{3}H]GABA and 1 mM unlabeled GABA (B, low-affinity transport).

remains to identify it (Radian et al., 1986). Low-affinity transport is much more difficult to monitor because the 1 mM unlabeled GABA present—to suppress the high-affinity process—reduces the sensitivity. Therefore, the activity of the pure GABA transporter, which is about 1000-fold purified as compared to the crude extract, was not high enough to monitor low-affinity transport. However, we noted that the two activities copurified, and we were able to monitor both activities until the stage of the DEAE chromatography. At that stage, the high-affinity transporter is purified about 100-fold. It can be seen in Figure 5 that the DEAE peak fractions exhibit both activities.

Low-Affinity GABA Transport in Synaptosomes. It has been reported recently that in synaptosomes there is low-affinity GABA transport as well (Wood & Sidhu, 1986). Synaptosomes are thought to be right-side-out, and therefore this low-affinity process could not represent the GABA transporter in its inside-out conformation. We have confirmed the presence of ouabain-sensitive high- and low-affinity GABA

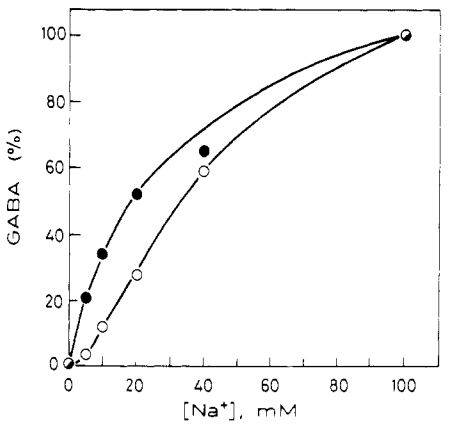

FIGURE 7: Sodium dependence of high- and low-affinity GABA transport in synaptosomes. Synaptosomes (10 μL, 60 μg of protein) were diluted in uptake medium of the composition indicated in Figure 6, except that sodium chloride was present at the indicated concentrations and lithium chloride was added so that the sum was 100 mM. For high-affinity transport (open symbols), the medium was supplemented with 1 μCi of [2,3-^{3}H]GABA and 20 μM unlabeled GABA; for low-affinity transport (solid symbols), the medium was supplemented with 4 μCi of [2,3-^{3}H]GABA and 500 μM unlabeled GABA. Transport was for 1 min.

Table IV: Effects of Veratridine and TTX on GABA Transport in Synaptosomes[a]

addition	GABA (nmol/mg of protein) high affinity	low affinity
none	0.093 ± 0.004	10.9 ± 0.6
veratridine, 50 μM	0.051 ± 0.006	7.4 ± 0.3
TTX, 1 μM	0.108 ± 0.008	10.7 ± 0.3
veratridine, 50 μM, and TTX, 1 μM	0.099 ± 0.010	10.3 ± 0.1

[a] Transport was done for 3 min as described, using triplicate time points.

transport in synaptosomes (Figure 6). The sensitivity of both processes to the inhibitor is only partial, probably due to the well-known low sensitivity of the ($Na^+ + K^+$)-ATPase from rat brain to it. In the synaptosomes, ouabain inhibits the ($Na^+ + K^+$)-ATPase and thereby the generation of the major driving force for GABA transport, the electrochemical sodium gradient. At a concentration of 1 mM it does not inhibit GABA transport in membrane vesicles (data not shown). Thus, low-affinity ouabain-sensitive GABA transport in synaptosomes represents an additional low-affinity process. This process is sodium dependent (Figure 7), and this dependency is reminiscent of that observed in membrane vesicles (Figure 1A). Consistent with the idea of an additional low-affinity process, but in the right-side-out conformation, is the fact that it can be inhibited by veratridine and fully reversed by TTX (Table IV), in contrast to the low-affinity process in membrane vesicles (Table II).

Discussion

Low-affinity GABA transport has been demonstrated recently in a variety of preparations from rat brain including synaptosomes (Wood & Sidhu, 1986), synaptic plasma membrane vesicles, and reconstituted preparations (Kanner & Keynan, 1988). In this paper we have characterized the low-affinity process and investigated if it is related to the well-known sodium- and chloride-coupled high-affinity GABA transporter [reviewed in Kanner (1983) and Kanner and Schuldiner (1987)].

Two types of low-affinity processes have been observed. In membrane vesicles the low-affinity transport is primarily due

to an inverted population, exposing the cytosolic face of the transporter to the outside. This is supported by the mode of action of compounds exerting indirect effects on GABA transport that act on sodium channels located in the same vesicles as the GABA transporter. Some of them, namely, TTX and MT_{II}, act in intact cells only from the outside of the sodium channel, and thus their effects on transport can be used to monitor the orientation of the transporter. These experiments (Tables II and III) indicated that high-affinity GABA transport is observed in right-side-out vesicles, whereas the low-affinity process is taking place in inside-out ones. This conclusion is supported by efflux (Figure 3) and exchange data (Figure 4). Furthermore, when high-affinity GABA transport is fractionated and subsequently reconstituted, the low-affinity process was found to cofractionate with it (Figure 5).

The phenomenon of greatly differing affinities of cotransporters for their solute is not unique to the sodium- and chloride-coupled GABA transporter. Other examples include the proton-coupled lactose transporter from *Escherichia coli* (Kaback, 1985) and the glutamate/aspartate exchanges from mitochondria (Dierks & Kramer, 1988). It is of interest to note that in all cases the affinity from the outside is substantially higher than that from the inside.

In intact synaptosomes, which are right-side-out, there is an additional low-affinity GABA transporter (Figure 6, Table IV). Most of this activity is lost when the crude synaptosomes are fractionated on Ficoll gradients subjected to osmotic shock to obtain the membrane vesicles. The ratio in high- to low-affinity transport under standard assay conditions expressed in counts per minute is 14 for synaptosomes and 100 for the membrane vesicles. The latter primarily contain the activity of low affinity due to inverted vesicles. The membrane vesicles probably contain only residual amounts of the synaptosomal low-affinity transporter, and this may help to explain the ability of TTX to restore part of the inhibition by veratridine and aconitine (Table II). The function of this transporter is not clear. Neither is it known if it is related to the high-affinity GABA transporter. A possible function could be that there exists some regulatory mechanism that can convert high- into low-affinity GABA transport. This would effectively inhibit reuptake and could be operative under certain conditions. In this regard it is of interest to mention that we have tried to phosphorylate the partially pure high-affinity GABA transporter with [^{32}P]ATP and the catalytic subunit of protein kinase A. Although in these experiments some polypeptides were phosphorylated, the 80-kDa polypeptide that constitutes the GABA transporter was not (B. I. Kanner, unpublished experiments). This does not, of course, rule out the existence of other covalent modifications, which may regulate GABA transport.

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