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48. Simulated delays were measured between the $[Ca^{2+}]_i$ jump and the time when the integral of the release rate equaled one vesicle. Delays predicted by the model were about 250 μs faster than the measured delays. Ca^{2+} uncaging is too fast (10 to 20 μs) to contribute substantially to this additional delay (*11*, *12*). Therefore, it probably originates from processes downstream of Ca^{2+} binding, including vesicle fusion, glutamate diffusion, and activation of AMPA receptors.

49. We thank C. J. Meinrenken, A. D. G. de Roos, and C. C. H. Petersen for comments on an earlier version of the manuscript; A. Roth for advice on simulations; and F. Helmchen for help with early flash photolysis experiments. J.G.G.B. was supported by a Pionier program of Netherlands Organization for Scientific Research (NWO).

12 May 2000; accepted 17 July 2000

Uptake of Glutamate into Synaptic Vesicles by an Inorganic Phosphate Transporter

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Previous work has identified two families of proteins that transport classical neurotransmitters into synaptic vesicles, but the protein responsible for vesicular transport of the principal excitatory transmitter glutamate has remained unknown. We demonstrate that a protein that is unrelated to any known neurotransmitter transporters and that was previously suggested to mediate the Na^+-dependent uptake of inorganic phosphate across the plasma membrane transports glutamate into synaptic vesicles. In addition, we show that this vesicular glutamate transporter, VGLUT1, exhibits a conductance for chloride that is blocked by glutamate.

Synaptic transmission involves the regulated exocytotic release of neurotransmitter. Because most classical transmitters are synthesized in the cytoplasm, they require transport into the secretory compartment for exocytotic release, and synaptic vesicles exhibit multiple distinct transport activities (*1*, *2*). All of these active transport processes depend on the proton electrochemical gradient ($\Delta\mu_{H+}$) across the vesicle membrane generated by the vacuolar H^+-dependent adenosine triphosphatase (H^+-ATPase) (*3*) and involve the exchange of lumenal protons for cytoplasmic transmitter. In particular, the transport of monoamines and acetylcholine (ACh) depends primarily on the chemical component (ΔpH) of $\Delta\mu_{H+}$ (*4*, *5*), whereas the transport of glutamate depends predominantly on the electrical component ($\Delta\Psi$) (*6*, *7*). Accumulation of the inhibitory transmitters γ-aminobutyric acid (GABA) and glycine relies on both ΔpH and $\Delta\Psi$ (*8*, *9*). Consistent with the observed differences in mechanism, the vesicular transporters for monoamines and ACh belong to a family of proteins distinct from the vesicular GABA transporter (VGAT) (*2*). VGAT shows greater dependence on $\Delta\Psi$ than do the vesicular monoamine and ACh transporters (*10*), suggesting that the vesicular glutamate transporter, which depends predominantly on $\Delta\Psi$, might belong to the same family of proteins defined by VGAT. Although several other proteins related to VGAT appear to have a role in the recycling of glutamate through glutamine at excitatory synapses (*11*–*14*), none have been implicated in vesicular glutamate transport.

The brain-specific Na^+-dependent inorganic phosphate transporter (BNPI) belongs to a family of proteins that use the inwardly directed Na^+ gradient across the plasma membrane to cotransport inorganic phosphate (P_i). Originally identified as a sequence upregulated by the exposure of cerebellar granule cells to subtoxic concentrations of *N*-methyl-D-aspartate, BNPI mediates the Na^+-dependent accumulation of P_i in *Xenopus* oocytes (*15*). Additional work has implicated BNPI in adenosine 5′-triphosphate (ATP) production by neurons and protection against excitotoxic injury (*16*, *17*). However, BNPI is only expressed by glutamatergic neurons (*18*), militating against a general metabolic role in all neuronal populations. In *Caenorhabditis elegans*, genetic screens for multiple behavioral defects have identified mutants in the BNPI ortholog *eat-4* (*19*, *20*), and recent studies indicate a specific role for *eat-4* in glutamatergic neurotransmission (*21*). The glutamatergic defect in *eat-4* mutants appears to be presynaptic, consistent with the localization of BNPI to excitatory nerve terminals (*21*, *22*). The accumulation of cytoplasmic P_i mediated by BNPI may activate the phosphate-activated glutaminase responsible for biosynthesis of the bulk of glutamate released as a neurotransmitter (*22*–*25*). However, the family of proteins including BNPI/EAT-4 may have functions in addition to P_i transport.

BNPI shows sequence similarity to type I but not type II Na^+/P_i cotransporters. In contrast to the type II transporters that exhibit robust Na^+-dependent P_i uptake, the accumulation of P_i by type I transporters is less striking (*26*-*28*). Rather, the type I transporter NaPi-1 transports organic anions, including phenol red and penicillin G, with substantially higher apparent affinity than P_i (*28*). Human genetic studies have shown that mutations in another protein closely related to BNPI and NaPi-1 account for disorders of sialic acid storage (*29*). In these conditions, sialic acid accumulates in lysosomes because of a defect in proton-driven export (*30*–*33*). Although the sialin protein (*29*) has not been demonstrated to mediate sialic acid transport, these observations together with the report that NaPi-1 accumulates organic anions with high apparent affinity suggest that BNPI might also transport organic anions. Localization to glutamatergic nerve terminals raises the possibility that it transports glutamate. In addition, BNPI is localized to synaptic vesicles in the brain (*22*) and to intracellular membranes in transfected cells (*34*), suggesting a role for BNPI in the transport of glutamate into synaptic vesicles for regulated exocytotic release. To determine whether BNPI mediates the transport of glutamate into synaptic vesicles, we transfected the rat BNPI cDNA into rat pheochromocytoma PC12 cells (*35*), which lack detectable endogenous BNPI protein (*34*). We then prepared a population of light membranes, including synaptic-like microvesicles, from the transfected and untransfected cells (*10*) and tested their ability to accumulate ^{3}H-glutamate in the presence of 4 mM chloride and ATP (*36*), conditions that optimize glutamate accumulation by native synaptic vesicles (*6*, *7*). Membranes from the transfected cells exhibited an uptake of glutamate that was two to four times the uptake by membranes from

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untransfected cells (Fig. 1A), a signal very similar to that obtained for GABA transport by VGAT (*10*).

Glutamate transport into synaptic vesicles exhibits a number of properties that distinguish it from glutamate uptake by other transport systems. First, in contrast to plasma membrane glutamate uptake (*37, 38*), the accumulation of glutamate in synaptic vesicles does not rely on a Na^+ electrochemical gradient. Consistent with this, glutamate was transported by BNPI in the absence of Na^+ (Fig. 1). Second, vesicular glutamate transport has a substantially lower apparent affinity (K_m of ~1 mM) than the plasma membrane excitatory amino acid transporters (K_m of ~10 to 100 μM). Glutamate transport by BNPI is saturated with a K_m of ~2 mM (Fig. 1B), in the same range as transport by synaptic vesicles. The slightly lower apparent affinity relative to synaptic vesicles may reflect a reduced driving force for transport in the PC12 membranes. Third, plasma membrane glutamate transporters recognize both aspartate and glutamate as substrates, whereas vesicular glutamate transport does not recognize aspartate (*6, 7*). Although 10 mM L-glutamate inhibited the uptake of ^{3}H-glutamate by membranes from BNPI-expressing cells, 10 mM L-aspartate did not (Fig. 2). D-Glutamate partially inhibited the transport of ^{3}H-glutamate, and L-glutamine had no effect, also consistent with prior work (*39*). Fourth, low micromolar concentrations of the dye Evans blue inhibited the transport of glutamate into both synaptic vesicles (*40*) and membranes expressing BNPI (Fig. 2).

Vesicular glutamate transport has a biphasic dependence on chloride concentration that may reflect the presence of an anion binding site distinct from the site of substrate recognition (*41*). Chloride concentrations of ~4 to 10 mM appear optimal for transport, with substantially lower activity detected at higher and lower levels (*6, 7*). Thus, we tested the chloride dependence of glutamate transport mediated by BNPI. Maximal uptake of ^{3}H-glutamate by BNPI was conferred by 2 to 4 mM chloride, with much less activity detected at 0 and 25 mM (Fig. 3A). Variation in the chloride concentration, as well as other manipulations including the addition of L-glutamate and Evans blue, had little effect on the background glutamate uptake observed in membranes from untransfected cells (Fig. 3A). In addition, the anion transport blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) inhibits glutamate transport by synaptic vesicles with a median inhibitory concentration of ~0.7 μM, apparently by interacting with the distinct chloride recognition site; here, 1 μM DIDS inhibited ~60% of the ^{3}H-glutamate transport mediated by BNPI (Fig. 2). The dependence of BNPI on the proton electrochemical gradient also strongly resembled that observed for glutamate transport into synaptic vesicles.

Glutamate transport into synaptic vesicles relies primarily on the electrical component $\Delta\Psi$ of the $\Delta\mu_{H+}$ across the vesicle membrane (*6, 7, 42, 43*). To assess the dependence of transport mediated by BNPI on $\Delta\Psi$, we used membranes preloaded with 4 mM KCl and the K^+ ionophore valinomycin. With 4 mM KCl in the reaction solution as well as in the membranes, valinomycin dissipates $\Delta\Psi$ (*43*) and substantially reduces glutamate uptake by BNPI (Fig. 3B). The ionophore nigericin, which exchanges K^+ for H^+ and dissipates ΔpH, increases both $\Delta\Psi$ and glutamate uptake by synaptic vesicles at low chloride concentrations and subsaturating amounts of glutamate (*43, 44*). Under these same conditions, nigericin increased the uptake of ^{3}H-glutamate by BNPI (Fig. 3B). The addition of both nigericin and valinomycin essentially abolished glutamate uptake by native synaptic vesicles and BNPI (Fig. 3B), presumably because the increase in $\Delta\Psi$ produced by nigericin is eliminated by valinomycin. The residual activity present in valinomycin that was abolished by the addition of nigericin also suggests that the transporter can use the outwardly directed ΔpH to drive transport in the absence of $\Delta\Psi$.

Native synaptic vesicles acidify during glutamate uptake (*6*). Glutamate presumably serves as a counterion for protons and dissipates $\Delta\Psi$, enabling the vacuolar H^+-ATPase to generate a larger ΔpH, but cotransport of H^+ with glutamate remains an alternative possibility. To determine whether acidification also accompanies glutamate transport by BNPI, we used the quenching of acridine orange fluorescence to measure vesicle pH (*45*). In the presence of ATP and 4 mM KCl, the addition of 10 mM glutamate reduced the lumenal pH of membranes expressing BNPI but not of membranes from untransfected cells (Fig. 4A). However, we also noted that 90 mM KCl produced acidification of the BNPI-expressing membranes that was considerably greater than that of the control membranes, suggesting that BNPI exhibits a conductance for chloride as well as transport activity for glutamate. To characterize further the chloride conductance, we examined the effect of a range of chloride concentrations on vesicle pH in the absence of glutamate. Membranes from multiple cell lines expressing BNPI all exhibited substantially greater acidifi-

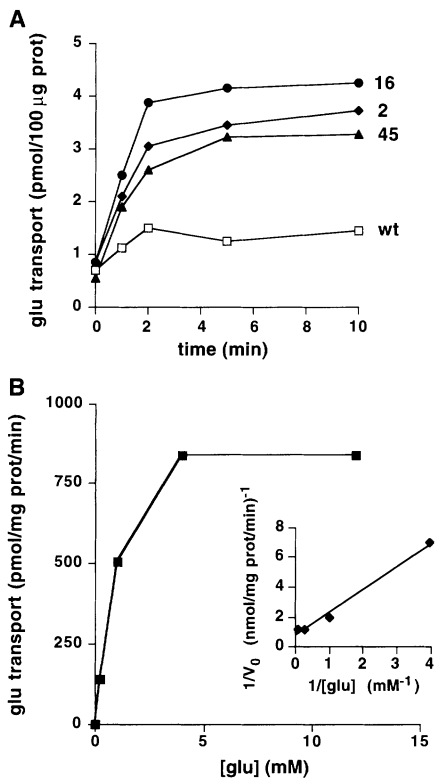

Fig. 1. BNPI expression confers saturable glutamate uptake in PC12 cell membranes. (**A**) Membranes prepared from transfected PC12 cells (lines 2, 16, and 45, solid symbols) accumulate two to four times the ^{3}H-glutamate (glu) that membranes from untransfected cells accumulate [wild type (wt), open squares; prot, protein]. (**B**) The initial maximal rate of transport by BNPI at 1 min (V_o) saturates with increasing concentrations of ^{3}H-glutamate. The uptake by untransfected cell membranes was subtracted as background uptake. (Inset) Lineweaver-Burke analysis indicates a K_m of ~2 mM.

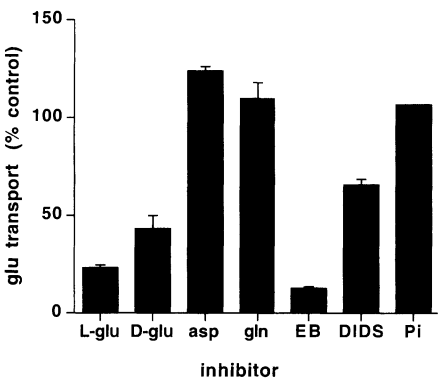

Fig. 2. Inhibition of BNPI transport by amino acids and other compounds. L-Glutamate (L-glu), but not L-aspartate (asp) or L-glutamine (gln) (all 10 mM), markedly inhibits the uptake of ^{3}H-glutamate at 5 min by membranes expressing BNPI. D-Glutamate (D-glu) (10 mM) partially inhibits the uptake. Evans blue (EB) (5 μM) also inhibits BNPI activity dramatically, whereas 1 μM DIDS blocks transport only partly, and 10 mM P_i has little effect. Uptake by membranes from untransfected PC12 cells (also for 5 min) was subtracted from that observed in the transfected cell membranes, and the results were normalized to uptake by transfected cell membranes in the absence of inhibitors. The compounds had no significant effect on the background uptake by untransfected cell membranes. The results represent the average from at least two experiments performed in duplicate, and the error bars represent the standard deviation.

cation than wild-type membranes did, particularly at higher chloride concentrations (Fig. 4, B and D). To assess the relation of the chloride conductance to glutamate transport, we pretreated the membranes with 10 mM glutamate and found that this decreased the acidification produced by chloride (Fig. 4, C and D). Because the membranes evidently express chloride conductances in addition to BNPI, the increase in conductance due to BNPI appears large, and the inhibition by glutamate is substantial. The nontransported amino acid aspartate (10 mM) failed to reduce the acidification by chloride (Fig. 4, C and D), supporting the relevance of the glutamate effect on chloride-induced acidification for glutamate transport. Further, glutamate had no effect on the acidification produced by chloride in nontransfected cell membranes (Fig. 4D), supporting the specificity of the interaction with BNPI.

BNPI transports glutamate with all of the functional characteristics previously reported for glutamate transport into native synaptic vesicles from the brain. It also localizes to synaptic vesicles (*22*), and the *C. elegans* mutation *eat-4* reduces glutamate release (*21*). BNPI thus functions as a vesicular glutamate transporter, VGLUT1. Only a subset of glutamate neurons expresses VGLUT1 (*18, 22*), but a closely related sequence has recently been identified and appears to be expressed in brain regions that lack VGLUT1 (*46*). The two isoforms together may therefore account for the uptake of glutamate by synaptic vesicles from all glutamatergic neurons.

The structural similarity of VGLUT1 to sialin and NaPi-1 may reflect similarities in the ionic dependence of transport. The extrusion of sialic acid from lysosomes, which appears to be defective in sialic acid storage diseases, depends on cotransport with H^+ (*30–33*), and vesicular glutamate transport may involve a proton exchange mechanism (*44*). The expression of NaPi-1 in *Xenopus* oocytes confers a large chloride conductance blocked by the organic anions that are recognized as substrates for transport (*28*). We found that the closely related VGLUT1 exhibits a substantial chloride conductance in addition to the unusual biphasic dependence on chloride previously reported for glutamate transport into synaptic vesicles. Glutamate blocks this chloride conductance, suggesting that the two anions compete for permeation. It remains unknown whether glutamate and chloride share a common pathway for permeation through VGLUT1, but a channel-like mode or anion exchange mechanism may enable the rapid filling necessary to keep up with the high rates of transmitter release and synaptic vesicle recycling observed at glutamatergic synapses.

The relation between glutamate and P_i transport by VGLUT1 remains unclear. High concentrations of P_i did not inhibit glutamate transport by VGLUT1 (Fig. 2). In addition, the related sialin protein apparently functions to transport sialic acid rather than P_i (*29*). NaPi-1 also recognizes organic acids with higher affinity than P_i (*28*). Further, P_i transport does not correlate with other activities, such as the chloride conductance induced by NaPi-1 expression in *Xenopus* oocytes, and has properties similar to endogenous oocyte P_i uptake (*47*). Nonetheless, previous work has demonstrated the Na^+-dependent uptake of P_i by NaPi-1 and BNPI (*15, 27*). VGLUT1 may thus function as both a phosphate transporter, presumably at the plasma membrane, and a glutamate transporter in synaptic vesicles. The localization of VGLUT1 to synaptic

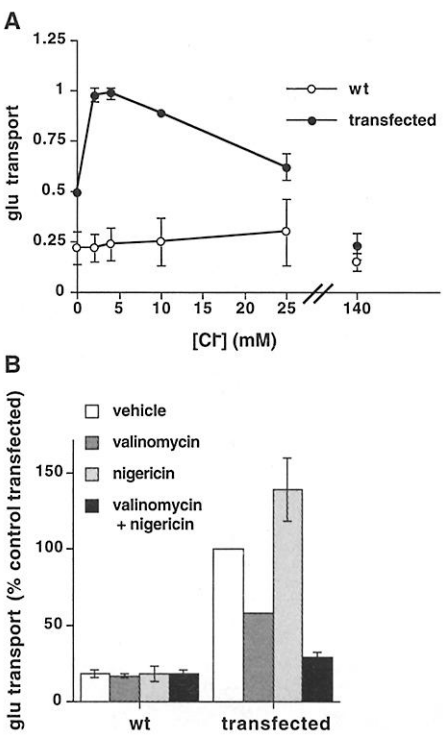

Fig. 3. Ionic dependence of transport by BNPI. (**A**) Transport of ^{3}H-glutamate at 5 min by membranes expressing BNPI shows a strong dependence on chloride concentration, with an optimum of ~2 to 4 mM. The experiments were performed with varying proportions of 0.14 M potassium gluconate and 0.14 M KCl in the standard reaction buffer (*35*) without sucrose to produce the different concentrations of chloride and to maintain constant osmolarity. The uptake by untransfected as well as transfected cells was normalized to maximal uptake by the transfected cells. (**B**) With membranes preloaded with 4 mM KCl, the proton ionophore nigericin (5 μM) increases the uptake of ^{3}H-glutamate by membranes from transfected cells, whereas valinomycin (20 μM), which eliminates the membrane potential ($\Delta\Psi$), reduces uptake; the combination essentially eliminates transport mediated by BNPI. The results are presented as a percentage of uptake by transfected cells in the absence of ionophore and represent the average of at least two experiments performed in duplicate on different membrane preparations. The error bars indicate the standard deviation.

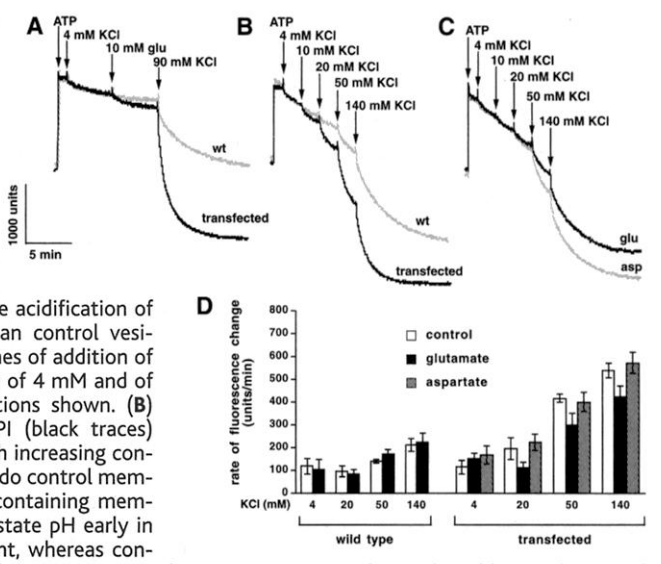

Fig. 4. Role of BNPI in acidification by glutamate and chloride. (**A**) The quenching of acridine orange fluorescence shows that glutamate (10 mM) acidifies membranes expressing BNPI (black traces) but not membranes from untransfected cells (wt, gray traces). A high concentration of chloride (90 mM) also produces more acidification of BNPI-expressing vesicles than control vesicles. Arrows indicate the times of addition of ATP to a final concentration of 4 mM and of KCl to the final concentrations shown. (**B**) Membranes expressing BNPI (black traces) show more acidification with increasing concentrations of chloride than do control membranes (gray traces). BNPI-containing membranes also reach a steady-state pH early in the course of the experiment, whereas control membranes show a declining pH, even at late times. Arrows indicate the addition of ATP and KCl as described in (A). (**C**) Glutamate (10 mM) (black traces), but not aspartate (10 mM) (gray traces), reduces the acidification of BNPI-expressing membranes by chloride. Arrows indicate the additions, as in (A). (**D**) Quantitation of the chloride conductance. Acridine orange fluorescence quenching shows that transfected cell membranes (open bars, right) exhibit a substantially greater rate of chloride-dependent acidification than untransfected cell membranes exhibit (open bars, left) at higher concentrations of KCl (20, 50, and 140 mM). Glutamate (10 mM) (black bars) reduces the rate of acidification in transfected cell membranes (right) but not in untransfected cell membranes (left). In contrast, the nontransported aspartate (10 mM) (gray bars) has little effect on the acidification of membranes expressing BNPI (right). The rate of fluorescence quenching was determined by averaging the change in fluorescence units over 12 consecutive 5-s intervals beginning 15 s after each addition of KCl. Error bars indicate the standard error of the mean.

vesicles, the phenotype of the *eat-4* mutant, and the biochemical evidence presented here, however, strongly suggest that vesicular glutamate transport is its primary role.

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35. The rat BNPI cDNA was stably expressed in PC12 cells, and immunofluorescence for BNPI was used to identify the most highly expressing cell clones. Membranes were prepared by homogenizing the washed cells in SH buffer [0.32 M sucrose and 10 mM Hepes-KOH (pH 7.4)] containing protease inhibitors and by pelleting the nuclei at 1000*g* for 5 min and the heavier membranes at 27,000*g* for 35 min. The supernatant containing lighter membranes was then pelleted at 210,000*g* for 1 hour and resuspended in SH buffer with protease inhibitors at a final concentration of ~10 μg protein/μl.
36. To initiate the transport reaction, we added 10 μl of membranes to 190 μl of SH buffer containing 4 mM KCl, 4 mM $MgSO_4$, 4 mM ATP, and 50 μM ^{3}H-glutamate, with other additions noted in the text and figure legends. The reaction mixture was incubated at 29°C for varying times, and the uptake was terminated by rapid filtration, followed by immediate washes with 6 ml of cold 0.155 M potassium tartrate and 10 mM Hepes (pH 7.4). Background uptake was determined using membranes prepared from untransfected cells that were incubated for the same amount of time at the same temperature. Transport measurements were performed in duplicate with at least two different preparations of membranes. The K_m was calculated by averaging the results of two separate determinations.
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45. Membranes (200 μg of protein) were diluted into 2 ml of 0.3 M sucrose and 10 mM tris-Mops (pH 7.4) containing 4 mM $MgSO_4$ and 5 μM acridine orange and incubated at 37°C with continuous stirring. Fluorescence was monitored with a Hitachi F4500 spectrofluorimeter at an excitation wavelength of 492 nm and emission of 530 nm. Separate measurements were made with at least three different membrane preparations for wild-type and transfected cells; representative tracings are shown in Fig. 4.
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48. We thank S. Paul for the BNPI cDNA; L. Avery, R. Lee, D. Julius, C. Bargmann, R. Tsien, A. Schinder, M.-M. Poo, G. Davis, and members of the Davis lab for stimulating discussions; B. Harris, K. Prehoda, and B. Rebres for help with the fluorimetric measurements; the members of the Edwards lab for helpful suggestions; and the National Institute of Mental Health (R.H.E.) and the National Institute of Neurological Disorders and Stroke (NINDS) (R.T.F. and R.H.E.) for support. E.E.B. was supported by a fellowship from the Howard Hughes Medical Institute and R.J.R. was supported by a career award from NINDS.

23 June 2000; accepted 14 July 2000