NEOTROFIN IS TRANSPORTED OUT OF BRAIN BY A SATURABLE MECHANISM: POSSIBLE INVOLVEMENT OF MULTIDRUG RESISTANCE AND MONOCARBOXYLIC ACID TRANSPORTERS

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ABSTRACT:
Neotrofin (AIT-082; leteprinim potassium) is transported out of brain by a saturable mechanism and in this study the mechanisms mediating this efflux were evaluated. Intracerebroventricular co-administration of [14C]Neotrofin with verapamil, a P-glycoprotein inhibitor, probenecid, an organic anion transporter inhibitor, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-[2-dimethylcarboxamoyethylsulfanyl]methylsulfanyl] propionic acid (MK571), a multidrug resistance-associated protein inhibitor, and salicylate or benzoate, both monocarboxylic acid transporter substrates, inhibited the efflux of [14C]Neotrofin. Additionally, Neotrofin inhibited the efflux of [3H]quinidine from brain. Compounds can diffuse from cerebrospinal fluid (CSF) into extracellular fluid of brain parenchyma and thus, efflux of [14C]Neotrofin after intracerebroventricular administration may indicate active transport across choroid plexus epithelium, brain capillary endothelium, or both. To determine whether [14C]Neotrofin efflux occurs at the brain capillary endothelium, experiments were performed in which [14C]Neotrofin was administered intraparenchymally. The t1/2 for [14C]Neotrofin disappearance from brain after intraparenchymal administration was significantly lower than that for [3H]sucrose and the efflux of Neotrofin was inhibited by 600-fold excess of unlabeled Neotrofin, verapamil, MK571, and salicylate. Together, these data suggest that a saturable mechanism for the efflux of Neotrofin is located at the blood-brain barrier and possibly the blood-CSF barrier. It is likely that multiple transporters are involved in the efflux of Neotrofin and these may include multidrug resistance and monocarboxylic acid transporters. These data are discussed in detail with respect to the site of transporter expression, the recent identification of numerous multidrug resistance-associated protein and monocarboxylic acid transporter homologs, the existence of other potential brain efflux transporters, and the availability of specific pharmacological agents with which to distinguish these transporters.

Efflux mechanisms at the blood-brain barrier are a limiting factor in the penetration of drugs from blood into the central nervous system (CNS); Taylor, 2002). Drugs including antiviral, chemotherapeutic, anticonvulsant, and antibiotic compounds are actively transported out of brain, thus limiting the achievable concentration of these compounds in brain.

To date, the best-characterized efflux transporters are the multidrug resistance transporters P-glycoprotein (P-gp, mdr1a; Gottesman et al., 1996) and multidrug resistance-associated protein (MRP) 1 (Borst et al., 1999), and gene knockout studies have provided compelling evidence for a role of these transporters in efflux across the blood-brain and blood-CSF barriers. P-glycoprotein knockout mice (mdr1a−/−) show increased blood-brain barrier permeability to digoxin, cyclosporin A, dexamethasone, vinblastine, ondansetron, and loperamide and increased sensitivity to the neurotoxic effects of ivermectin (Schinkel, 1999), clearly indicating that P-gp restricts the entry of drugs into the CNS. Although MRPI-deficient (mrp1−/−) mice do not demonstrate any blood-brain barrier-related deficits (Lorico et al., 1997; Wijnholds et al., 1997), a role for MRP1, specifically at the blood-CSF barrier, is indicated by studies using mdr1a/mdr1b triple knockouts (TKOs; Wijnholds et al., 2000). Although mdr1a/mdr1b double knockouts (DKOs) and TKOs had similar total brain levels of [3H]etoposide after intravenous administration, TKOs had increased CSF etoposide levels compared with DKO, indicating that MRP1 may mediate etoposide efflux at the blood-CSF barrier. In addition to studies with knockout animals, putative P-gp and MRP inhibitors have been shown to enhance the blood-brain barrier penetration of drugs, including dideoxynosine (Galinsky et al., 1991), zidovudine (Takasawa et al., 1997), cyclosporin A (Didier and Loor, 1995), quinidine (Koehler-Stec et al., 1997), colchicine (Drion et al., 1996), and vinblastine (Drion et al., 1996).

Less well characterized transporters are also implicated in efflux from brain to blood. The expression of monocarboxylic acid transporter (MCT) 1 at the blood-brain barrier has been demonstrated (Koehler-Stec et al., 1998) and this transporter may be involved in the efflux of monocarboxylic acids, including aluminum citrate (Ackley

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and Yokel, 1997, 1998), salicylate, and benzoate (Deguchi et al., 1997) from brain in vivo. Additionally, based on their expression profile and substrate specificity, organic anion transporters (OATs), organic anion transport proteins, organic cation transporters, and organic cation/carnitine transporters may play a role in the transport of drugs from brain to blood. Experimental evidence to support this hypothesis is beginning to emerge (Taylor, 2002).

In this study we have focused attention on the multidrug resistance and monocarboxylic acid transporters, the substrate specificities of which are broad. P-glycoprotein traditionally transports hydrophobic cationic or neutral compounds (Gottesman et al., 1996); however, it has been shown to transport hydrophilic acids such as methotrexate (De Graaf et al., 1996). MRP1 is known to transport organic anions, glutathione conjugates, and peptidyl leukotrienes (Borst et al., 1999), whereas MCT1 transports a wide range of monocarboxylic acids (Poole and Halestrap, 1993). Previously, we demonstrated that Neotrofin (AIT-08-2; letepinrim potassium), a cognitive enhancer and neuroprotective agent in development as a therapy for Alzheimer’s disease (Rathbone et al., 1999), is transported out of brain by a saturable efflux mechanism (Taylor et al., 2000). Neotrofin is a small organic anion that contains a single aromatic carboxylate, thus making it a potential substrate for MRP1, MCT1, and given its ever-widening substrate profile, P-gp. Thus, the aim of this study was to examine the role of multidrug and monocarboxylic acid transporters in the efflux of Neotrofin.

**Experimental Procedures**

**Animals.** Male Swiss-Webster CFW mice were supplied by Charles River Laboratories (Holister, CA) and all experiments were conducted according to the National Institutes of Health Guide on Care and Use of Laboratory Animals. Mice were 2 to 3 months old at the time of use.

**Materials.** Neotrofin (99.5% pure) was synthesized by Eprova (Schaffhausen, Switzerland) and [14C]Neotrofin (51.5 mCi/mmol; ≥98% pure) was synthesized by Chemsyn Laboratories (Lenexa, KS). [3H]Sucrose (5–15 Ci/mmol) and [3H]quinidine (10–20 Ci/mmol) were from Amersham Biosciences, Inc. (Piscataway, NJ) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Probenecid, verapamil hydrochloride, sodium salicylate, and sodium benzoate were purchased from Sigma Chemical Co. (St. Louis, MO). MK571 was purchased from Alexis Biochemicals (San Diego, CA).

**Intracerebroventricular Efflux Experiments.** These experiments were conducted according the method of Banks et al. (1997) with minor modifications. After mice were anesthetized with 2.25 g/kg i.p. urethane, the skull was exposed and a hole was made with a 25-gauge needle at 1 mm anterior-posterior and 1 mm left lateral, relative to bregma, and 3.5 mm dorsal-ventral, with respect to the skull. Using a 1-μl Hamilton syringe (25 gauge), 1 μl of phosphate-buffered saline (PBS) containing [14C]Neotrofin (5 × 10^4 dpm/μl), [3H]quinidine (−3 × 10^4 dpm/μl), or [3H]quinidine (−3 × 10^4 dpm/μl) was injected i.c.v. After injection and when withdrawing the needle, there was often backflux of fluid and this was collected. At 0, 2, 5, 8, 15, 23, and 30 min after injection brains were removed.

**Intraparenchymal Efflux Experiments.** Mice were anesthetized with 2.25 g/kg i.p. urethane and then immobilized in a stereotaxic apparatus with a mouse adaptor coupled to a microinjection unit (Kopf, Tujunga, CA) as described by Banks et al. (1994). A small hole was made in the skull with a Dremel drill (model 770; 2.4-mm drill bit, model 107; Racine, WI) at 1 mm anterior-posterior and 1 mm left lateral relative to bregma. Using a 0.5-μl Hamilton syringe (25 gauge), 0.1 μl of PBS containing [14C]Neotrofin (−5 × 10^4 dpm/μl) or [3H]quinidine (−5 × 10^4 dpm/μl) was injected i.p. at 3.5 mm dorsal-ventral, with respect to the skull. Backflux of injection fluid was collected and then at 0, 5, 8, 15, 23, 30, and 45 min after injection brains were removed.

**Measurement of Radioactivity and Calculations.** Brains were solubilized at 50°C overnight in 2 ml of BTS-450 (Beckman Coulter, Inc., Fullerton, CA). Backflux and samples of injection stock were solubilized in 1 ml of BTS-450 at room temperature overnight. Scintillation fluid (15 ml; Ready Organic, Beckman, CA) was added, samples were mixed well by inversion, and radioactivity was measured using a Beckman Coulter LS6500 scintillation counter. Radioactivity in brain was corrected for backflux using the following equation: (disintegrations per minute in brain)/(disintegrations per minute per microliter of injectate)/(disintegrations per minute per microliter of injectate − disintegrations per minute in backflux) = corrected disintegrations per minute. The log of corrected disintegrations per minute was plotted against time, and a line was fitted to the data using Delsagraph 4.0.5 (SPSS, San Rafael, CA). The t_{1/2} for disappearance of [14C]Neotrofin, [3H]sucrose, or [3H]quinidine was the inverse of the slope of the line multiplied by 0.693 (Banks et al., 1997).

**Transport Inhibitors.** To examine the mechanisms mediating efflux, [14C]Neotrofin was coadministered with a series of transporter substrates and inhibitors. Intracerebroventricular experiments: 1) probenecid (organic anion transport inhibitor): 350 mM in PBS containing 370 mM NaOH and 20 mM HCl, pH 7.4; 2) verapamil (P-glycoprotein inhibitor): 200 mM in PBS containing 4.2% ethanol, pH 7.4; 3) MK571 (MRP inhibitor): 1, 10, or 100 mM in PBS; 4) sodium salicylate (MCT substrate): 4 M in PBS; and 5) sodium benzoate (MCT substrate): 3 M in PBS. Intraparenchymal experiments: 1) verapamil: 2 mM in water with less than 0.05% ethanol; 2) MK571: 10 mM in PBS; and 3) sodium salicylate: 40 mM in PBS. In all experiments, animals in a control group were given [14C]Neotrofin in PBS. In i.c.v. experiments in which probenecid or verapamil were used, additional control groups received [14C]Neotrofin in PBS containing 370 mM NaOH and 370 mM HCl, pH 7.4, or [14C]Neotrofin in PBS with 4.2% ethanol, pH 7.4, respectively. In i.p.c. experiments in which verapamil was used a second control group received [14C]Neotrofin in water containing 4% ethanol and 20 mM NaOH.

All concentrations given are the concentration in the stock injectate. Due to dilution of injectate in CSF and extracellular fluid and clearance from brain, the effective concentration of each compound is assumed to be at least 100-fold less than the stock concentration quoted above in the lateral ventricle and brain parenchyma, respectively. Similar concentrations of each of these compounds have been used by others to inhibit efflux of known P-gp, MRP, and MCT substrates from brain in vivo (Ackley and Yokel, 1997; Deguchi et al., 1997; Kusuhara et al., 1997; Takasawa et al., 1997).

**Statistical Analysis.** All data are presented as mean ± S.E. Unpaired Student’s t test and ANOVA, coupled to Scheffe’s post hoc analysis, were used to compare 2 and 3 means, respectively. In all tests, p < 0.05 indicated significance.

**Results**

After both i.c.v. and i.p.c. administration, Neotrofin was cleared from brain in an exponential manner with a t_{1/2} of 20 ± 1.0 (Fig. 1) and 35 ± 1 min (Fig. 2), respectively, and the t_{1/2} of efflux of sucrose was significantly higher (i.e., efflux of sucrose is slower). In both cases, 600-fold excess of unlabeled Neotrofin significantly increased the t_{1/2} (Fig. 3). Verapamil, a P-gp inhibitor; probenecid, an organic anion transporter inhibitor; MK571, an MRP inhibitor; and salicylate and benzoate, both monocarboxylic acid transporter substrates, significantly inhibited efflux of [14C]Neotrofin when coadministered i.c.v. (Fig. 4) and Neotrofin significantly increased t_{1/2} for [3H]quinidine efflux (Fig. 5). With regard to the latter observation, the use of Neotrofin to inhibit efflux of quinidine was examined because, due to solubility of quinidine, it was not possible to study the inhibition of Neotrofin efflux by quinidine. Similarly, verapamil, MK571, and sodium salicylate significantly increased the t_{1/2} of [14C]Neotrofin clearance after i.p.c. coinjection (Fig. 6). The t_{1/2} for [14C]Neotrofin clearance from brain after i.p.c. administration was significantly reduced to 27 ± 3 min when [14C]Neotrofin was administered in verapamil vehicle (4% ethanol and 20 mM NaOH in water) compared with 33 ± 4 min when administered in PBS. Because this does not impact the conclusions drawn from the observations described above, these data are not shown. No other vehicle affected basal rates of Neotrofin efflux (data not shown).
Discussion

After both i.c.v. and i.p.c. administration, [14C]Neotrofin is transported out of brain in an exponential manner. In both cases the clearance of [14C]Neotrofin was significantly faster than for [3H]sucrose, indicating that [14C]Neotrofin is transported out of brain by an active mechanism. Indeed, efflux of [14C]Neotrofin is inhibited by excess unlabeled Neotrofin, thus confirming the presence of a saturable transport mechanism for this molecule. It is important to note that [14C]Neotrofin is minimally degraded in vivo and therefore the data do represent transport of [14C]Neotrofin and not a 14C-metabolite (Taylor et al., 2000). After i.c.v. administration, Neotrofin may be transported out of brain by bulk flow of CSF; transport across the choroid plexus epithelium; and/or, after diffusion into the parenchyma surrounding the ventricle, transport across the capillary endothelial cell barrier. However, after i.p.c. administration, given the slow rate of diffusion of compounds through the interstitial space and the distance of the site of injection from the ventricle, bulk flow of CSF and transport across the choroid plexus epithelial barrier are unlikely to contribute to the efflux rate. Thus, the demonstration that efflux of [14C]Neotrofin occurs after both i.c.v. and i.p.c. administration suggests that the saturable mechanism is present at the brain capillary endothelium and may also be present at the choroid plexus epithelial cell.

The data presented suggest that efflux of Neotrofin may be mediated, at least in part, by multidrug resistance and monocarboxylic acid transporters may play a role in the efflux of Neotrofin from brain. Evidence supporting this conclusion is provided by data obtained using at least two pharmacological agents per transporter. Verapamil, a known P-gp inhibitor, reduced efflux of Neotrofin and Neotrofin reduced the efflux of quinidine, a known P-gp substrate. Probenecid, an organic anion transporter inhibitor, and MK571, both known to inhibit MRP1, decreased transport of Neotrofin out of brain, and two MCT substrates, salicylate and benzoate, also diminished Neotrofin efflux. Probenecid has also been shown to be a substrate for MCT1. All inhibitors decreased the [14C]Neotrofin efflux rate when administered i.c.v. or i.p.c., suggesting that either the same brain capillary endothelium mechanisms are operating under both conditions in the absence of any choroid plexus mechanisms or that similar mechanisms are functioning at both the capillary endothelium and the choroid plexus epithelium.

Both possibilities are consistent with the sites of P-gp and MCT1 expression. Both transporters have been localized to brain capillary endothelial cells and choroid plexus epithelial cells (Takanaga et al.,

Fig. 1. Efflux of [14C]Neotrofin and [3H]sucrose after i.c.v. administration. [14C]Neotrofin (■) or [3H]sucrose (○) were injected i.c.v. Data shown in A are representative of four or more experiments. The mean ± S.E. is shown in B.* p < 0.0001 as indicated by Student’s unpaired t test. These data were previously published (Taylor et al., 2000) and are shown here to enhance the clarity of this article.

Fig. 2. Efflux of [14C]Neotrofin and [3H]sucrose after i.p.c. administration. [14C]Neotrofin (■) or [3H]sucrose (○) were injected i.p.c. Data shown in A are representative of four or more experiments. The mean ± S.E. is shown in B.* p < 0.0001 as indicated by Student’s unpaired t test.

MULTIPLE TRANSPORTERS MEDIATE NEOTROFIN EFFLUX OUT OF BRAIN
1995; Koehler-Stec et al., 1998; Leino et al., 1999; Rao et al., 1999; Schinkel, 1999). It is unclear whether the data are consistent with the expression profile for MRP1. The expression of MRP1 in brain capillary endothelial cells has been demonstrated using isolated microvessels, tissue sections, and cultured brain capillary endothelial cells (Huai-Yun et al., 1998; Kushuhara et al., 1998; Regina et al., 1998; Gutmann et al., 1999; Decleves et al., 2000; Zhang et al., 2000), although the latter may be a culture-dependent phenomenon (Seetharaman et al., 1998; Gutmann et al., 1999). However, others have found little or no staining for MRP1 in brain (Flens et al., 1996; Seetharaman et al., 1998; Gutmann et al., 1999; Wijnholds et al., 2000) and recently, it has been suggested that MRP1 expression is confined to the choroid plexus epithelium (Rao et al., 1999). This conclusion is supported by the work of Wijnholds et al. (1997, 2000a) using mrp1/H11002/H11002 single knockout and mrp1/mdr1a/mdr1b/H11002/H11002 TKO mice. They demonstrated that MRP1 gene ablation caused no change in the levels of etoposide in brain and concluded that MRP1 did not therefore function to limit entry of etoposide into brain. However, they showed that TKO mice had increased levels of etoposide in CSF compared with DKO animals and concluded that MRP1 acts as an efflux pump at the choroid plexus. The rationale for comparing DKO and TKO mice was that etoposide is also a substrate for P-gp and thus only in the absence of P-gp (mdr1a and mdr1b) could a role for MRP1

Fig. 3. Saturability of [14C]Neotrofin efflux after i.c.v. or i.p.c. administration.

To examine the saturability of Neotrofin efflux, 600-fold molar excess of unlabeled Neotrofin was coadministered with [14C]Neotrofin. Data shown are the mean ± S.E. of four separate experiments. *, p < 0.01 compared with appropriate control as indicated by one-way ANOVA with Scheffé’s post hoc analysis. The data from i.c.v. experiments were previously published (Taylor et al., 2000) and are shown here to enhance the clarity of this article.

Verapamil (2 mM), 350 mM probenecid, 10 mM MK571, 3 M salicylate, or 4 M benzoate was coadministered with [14C]Neotrofin i.c.v. Data shown are mean ± S.E. of four to seven separate experiments. The effect of each inhibitor was examined in a separate experiment with appropriate controls. For ease of presentation, all experiments have been presented together and the PBS control shown is an average from all experiments. Statistical analysis was conducted using only the appropriate control data for each inhibitor. *, p < 0.01 compared with control as indicated by one-way ANOVA with Scheffé’s post hoc analysis.

Verapamil (2 mM), 350 mM probenecid, 10 mM MK571, 3 M salicylate, or 4 M benzoate was coadministered with [14C]Neotrofin i.c.v. Data shown are mean ± S.E. of four to seven separate experiments. The effect of each inhibitor was examined in a separate experiment with appropriate controls. For ease of presentation, all experiments have been presented together and the PBS control shown is an average from all experiments. Statistical analysis was conducted using only the appropriate control data for each inhibitor. *, p < 0.01 compared with control as indicated by one-way ANOVA with Scheffé’s post hoc analysis.

Fig. 4. Effect of inhibitors on efflux of [14C]Neotrofin after i.c.v. or i.p.c. administration.

Verapamil (2 mM), 350 mM probenecid, 10 mM MK571, 3 M salicylate, or 4 M benzoate was coadministered with [14C]Neotrofin i.c.v. Data shown are mean ± S.E. of four to seven separate experiments. The effect of each inhibitor was examined in a separate experiment with appropriate controls. For ease of presentation, all experiments have been presented together and the PBS control shown is an average from all experiments. Statistical analysis was conducted using only the appropriate control data for each inhibitor. *, p < 0.01 compared with control as indicated by one-way ANOVA with Scheffé’s post hoc analysis.

Fig. 5. Effect of Neotrofin on [3H]quinidine efflux after i.c.v. coadministration.

To further examine the interaction of Neotrofin with P-gp the $t_{1/2}$ of [3H]quinidine disappearance from brain after i.c.v. administration was measured in absence and presence of 100-fold molar excess of Neotrofin. Data shown are mean ± S.E. of four separate experiments. *, p < 0.05 as indicated by Student’s unpaired t test.

Seetharaman et al., 1998; Gutmann et al., 1999; Wijnholds et al., 2000) and recently, it has been suggested that MRP1 expression is confined to the choroid plexus epithelium (Rao et al., 1999). This conclusion is supported by the work of Wijnholds et al. (1997, 2000a) using mrp1−/− single knockout and mrp1/mdr1a/mdr1b−/− TKO mice. They demonstrated that MRP1 gene ablation caused no change in the levels of etoposide in brain and concluded that MRP1 did not therefore function to limit entry of etoposide into brain. However, they showed that TKO mice had increased levels of etoposide in CSF compared with DKO animals and concluded that MRP1 acts as an efflux pump at the choroid plexus. The rationale for comparing DKO and TKO mice was that etoposide is also a substrate for P-gp and thus only in the absence of P-gp (mdr1a and mdr1b) could a role for MRP1

Fig. 6. Effect of inhibitors on efflux of [14C]Neotrofin after i.p.c. coadministration.

Verapamil (2 mM), 350 mM probenecid, 10 mM MK571, 3 M salicylate, or 4 M benzoate was coadministered i.c.v. with [14C]Neotrofin. Data shown are mean ± S.E. of four separate experiments. The effect of each inhibitor was examined in a separate experiment with appropriate controls. For ease of presentation, all experiments have been presented together and the PBS control shown is an average from all experiments. Statistical analysis was conducted using the appropriate control data for each inhibitor. *, p < 0.05 as indicated by Student’s unpaired t test.
be determined. However, etoposide is also a substrate for other efflux transporters, including MRP2 and MRP3, and therefore it is possible that even in TKO mice loss of MRP1 activity at the blood-brain barrier may be masked by activity of other efflux transporters. Thus, although these studies are superficially convincing of a role for MRP1 in choroid plexus alone, the use of an MRP1-specific substrate is required to definitively confirm this hypothesis.

If this hypothesis is confirmed then the data presented herein may indicate that i.p.c. administration does not completely separate the injectate from nonendothelial routes of efflux or that MK571 is not a specific MRP1 inhibitor. With regard to the former possibility, this is unlikely due to the distance of the injection site from the ventricle and the slow rates of diffusion through the interstitial space. However, without autoradiography or microdialysis to examine diffusion of the compound away from the site of injection this possibility cannot be completely ruled out. With respect to the latter possibility, it is becoming clear that MK571 may not be a specific MRP1 inhibitor. The development of MK571 as a specific inhibitor of MRP1 predated the identification of six MRP1 homologs (Gekeler et al., 1995; Borst et al., 1999) and it is now known to inhibit MRP2 (Chen et al., 1999; Leier et al., 2000). It is not known whether MK571 inhibits other members of the MRP family; however, based on their expression and substrate profiles it is conceivable that at least some of them may be responsible for the transport of Neotrofin out of brain. Analysis of human brain tissue by Northern blot and RNase protection assay demonstrated that MRP5 was highly expressed in brain (Kool et al., 1997, 1999; McAleen et al., 1999). More recently, MRP2, 4, 5, and 6 have been detected in endothelial cells of isolated brain capillaries and in cultured brain capillary endothelial cells (Miller et al., 2000; Zhang et al., 2000). MRP4 and MRP5 can transport nucleoside and nucleotide analogs, respectively (Schuetz et al., 1999; Jellitshsky et al., 2000; Wijnholds et al., 2000b) and, of particular interest, MRP5 can transport 6-mercaptopurine (Wijnholds et al., 2000b), which is remarkably similar in structure to Neotrofin. Finally, it is possible that if MRP1 expression is indeed restricted to the choroid plexus, the inhibition of Neotrofin efflux by MK571 could be due to an increase in the efflux from astrocytes in which it is expressed. This would increase the concentration of Neotrofin in extracellular fluid and increase the availability of Neotrofin to blood-brain barrier efflux transporters, including P-gp and MCT1.

The possible role of MCT1 in the efflux of Neotrofin raises the question of a role for MCT1 in the influx of Neotrofin into brain. MCTs are expressed on both the luminal and abluminal surfaces of capillary endothelial cells (Gerhart et al., 1997) and have been implicated in both the influx and efflux of monocarboxylic acids from blood to brain (Terasaki et al., 1991; Gerhart et al., 1997), although there is evidence that efflux by MCTs is more efficient than influx (Ackley and Yokel, 1997, 1998; Deguchi et al., 1997). In our previous study, we were unable to demonstrate a saturable influx mechanism (Taylor et al., 2000). However, it is possible that a MCT-mediated influx mechanism for Neotrofin may be masked by efflux mechanisms. It is becoming apparent that many efflux transporters, other than the well characterized P-gp and MRP1, are capable of transporting small molecules such as Neotrofin and likely function in the CNS. In addition to the six MRP1 homologs mentioned above, there are seven MCT family members (Halestrap and Price, 1999) and multiple members of the organic anion and organic cation families and many of these are expressed in brain (Taylor, 2002). Although the role of these efflux transporters in brain is not known, it is likely that at least some of them operate as efflux transporters. Importantly, the substrate/inhibitor profiles of these transporters overlap considerably (Taylor, 2002). With particular respect to this study, verapamil inhibits both P-gp and organic cation/carnitine transporter 1; quinidine is a substrate for organic cation transporter 1 as well as P-gp; probenecid is an inhibitor of MRP1, MRP5, OAT1, OAT3, MCT1, MCT, and organic anion transport protein 1; and, as mentioned above, MK571 inhibits MRP2 and MRP3 as well as MRP1. Thus, at this time, availability of pharmacological agents that are specific for each protein within each family of efflux transporters is the limiting factor in identifying the exact proteins involved in Neotrofin transport out of brain.

In conclusion, Neotrofin is transported out of brain by mechanism that likely comprises, at least in part, multidrug resistance and monocarboxylic acid transporters. This efflux presumably limits the concentration of Neotrofin in brain and, although this may increase the minimal effective dose, it may also reduce accumulation of Neotrofin in brain in patients that require long-term treatment, such as those suffering from Alzheimer’s disease.

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