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Transport of the Synthetic Opioid Peptide DADLE ([D-Ala², D-Leu⁵]-Enkephalin) in Neuronal Cells

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Abstract

The Na⁺-coupled oligopeptide transporters SOPT1 and SOPT2 transport peptides consisting of 5 amino acids and show potential for the delivery of therapeutically relevant peptides/peptidomimetics. Here we examined the expression of these two transporters in the retinal neuronal cell line RGC-5. These cells showed robust uptake activity for the synthetic pentapeptide DADLE ([D-Ala², D-Leu⁵]-Enkephalin). The uptake was Na⁺-dependent and saturable (K_t , $6.2 \pm 0.6 \mu\text{M}$). A variety of oligopeptides inhibited DADLE uptake. The uptake of the competing oligopeptides was directly demonstrated with fluorescein isothiocyanate-labeled Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys in RGC-5 cells and primary mouse retinal ganglion cells. The characteristics of DADLE uptake matched those of SOPT2. We then examined the expression of SOPT1 in these cells with deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂) as the substrate and found that RGC-5 cells also expressed SOPT1. Since it is already known that SOPT1 is expressed in the neuronal cell line SK-N-SH, we investigated SOPT2 expression in these cells to determine whether the presence of both oligopeptide transporters is a common feature of neuronal cells. These studies showed that SK-N-SH cells also expressed SOPT2. This constitutes the first report on the functional characterization of SOPT1 and SOPT2 in retinal neuronal cells and on the expression of SOPT2 in non-retinal neuronal cells.

Keywords

Oligopeptide transport; Sodium-coupled oligopeptide transporter; Opioid peptides; Cell-penetrating peptides; Neuronal cells; Retinal ganglion cell; Drug delivery

INTRODUCTION

DADLE ([D-Ala², D-Leu⁵]-Enkephalin) is a fairly hydrolysis-resistant synthetic peptide, which is often considered as a specific agonist for the delta subtype of opioid receptors. It is a pentapeptide with the sequence Tyr-D-Ala-Gly-Phe-D-Leu. This peptide has a multitude of biological effects including decrease in heart rate and blood pressure, hibernation, induction of hypometabolic state, and neuroprotection.^{1–3} These effects are generally

considered to be mediated by the activation of the delta subtype of the opioid receptors on the cell surface of the target cells. Interestingly, the biological effects of DADLE are not restricted to its cell surface action. It enters the cytoplasm and the nucleus in certain cells and elicits profound intracellular actions including changes in gene expression and cell proliferation.^{4, 5} These intracellular effects are seen even in cells that do not express the opioid receptors on the cell surface,⁵ implying that transport of DADLE across the plasma membrane is obligatory for this process.

There is evidence in the literature for the involvement of the organic anion transporting polypeptide OATP1B1 (also known as OATP-C), coded by the *SLCO1B1* gene, in the transport of DADLE across the plasma membrane.⁶ OATP1B3 (also known as OATP8), coded by the *SLCO1B3* gene, has been shown to transport other synthetic opioid peptides such as DPDPE (Tyr-D-Penicillamine-Gly-Phe-D-Penicillamine) and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂)^{7, 8}, but the transport of DADLE by this transporter has not been investigated. Recent studies in our laboratory have identified two novel transport systems for oligopeptides, including opioid peptides, which appear to be distinct from the organic anion transporting polypeptides. These transport systems are the Sodium-coupled OligoPeptide Transporter 1 (SOPT1) and SOPT2.⁹ SOPT1 was discovered first in the human retinal pigment epithelial cell line ARPE-19,¹⁰ and later found to be expressed also in human and mouse primary retinal pigment epithelial cells,¹¹ the human neuronal cell line SK-N-SH,^{12, 13} the human intestinal epithelial cell line Caco-2,¹⁴ and the human colonic epithelial cell line CCD841.¹⁴ SOPT2, which is similar to SOPT1 in some features but distinct in other respects, was discovered first in the rabbit conjunctival epithelial cell line CJVE,¹⁵ but later found to be expressed also in retinal pigment epithelial cell lines, human and mouse primary retinal pigment epithelial cells as well as human intestinal and colonic epithelial cell lines.^{11, 14} DADLE is a preferred substrate for SOPT2 whereas deltorphin II is a preferred substrate for SOPT1; additionally, the involvement of the two transporters in the uptake process can be differentiated based on the influence of dipeptides and tripeptides on the uptake. The function of SOPT1 is markedly stimulated by these small peptides whereas the function of SOPT2 is markedly inhibited by the same small peptides.^{11, 14, 15} Even though there is convincing evidence at the functional level for the existence of SOPT1 and SOPT2 in mammalian cells, neither of the transport systems has been identified at the molecular level. The identities of both transport systems, both at the gene level and protein level, remain unknown.

The purpose of the present study was three-fold. First, we wanted to determine whether DADLE is taken up in retinal neuronal cells (rat retinal neuronal cell line RGC-5 and mouse primary retinal ganglion cells) because of convincing evidence in the literature for a role of δ -opiate receptor, for which DADLE is a selective agonist, in protection of retina from ischemic injury.^{16, 17} Second, if DADLE is taken up by retinal neuronal cells, we wanted to determine if the uptake occurs via SOPT1 and/or SOPT2, the oligopeptide transporters that are known to transport DADLE. Third, we wanted to characterize the transport of DADLE and the potential involvement of SOPT2 in the process in a non-retinal neuronal cell line (SK-N-SH cells). To date, only the expression of SOPT1 has been documented in neuronal cells. Here we report for the first time on the characteristics of DADLE transport and on the involvement of SOPT2 in the process in neuronal cells, in retinal and non-retinal neuronal cells. The data show clearly that these neuronal cells express robust uptake activity for DADLE and also provide evidence that neuronal cells express not only SOPT1 but also SOPT2.

MATERIALS AND METHODS

Materials

The synthetic opioid peptides DADLE ([D-Ala², D-Leu⁵]Enkephalin), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂), deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂), DAMGO, ([D-Ala², N-methyl-Phe⁵, Glyol⁵]Enkephalin), DSLET, ([D-Ser²]Leu-enkephalin-Thr⁶), and DALCE (Tyr-D-Ala-Gly-Phe-Leu-Cys) and the endogenous opioid peptides Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), Met-enkephalin (Tyr-Gly-Gly-Phe-Met), dynorphin A (1–7) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg), dynorphin B (1–9) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg), enkephalinamide (Tyr-Gly-Gly-Phe-Met-NH₂), endomorphin (Tyr-Pro-Trp-Phe-NH₂), Arg⁶, Phe⁷-Met-enkephalin (Tyr-Gly-Gly-Phe-Met-Arg-Phe), and dynorphin (1–13) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys) and the HIV-1 Tat peptides Tat_{47–57} (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg), and Tat_{49–55} (Arg-Lys-Lys-Arg-Arg-Gln-Arg) were obtained from National Institute on Drug Abuse Research Resources (Bethesda, MD, USA), Bachem Americas, Inc. (Torrance, CA, USA) or the American Peptide Company, Inc. (Sunnyvale, CA, USA). The tripeptides Gly-Gly-Ile, Gly-Gly-Phe, Gly-Gly-His, and Leu-Gly-Gly and the non-peptide opioid antagonists naloxone and naltrexone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Poly-Arg₍₉₎ was obtained from Bachem Americas. Pep1 (Acetyl-Lys-Glu-Thr-Trp-Trp-Glu-Thr-Trp-Trp-Thr-Glu-Trp-Ser-Gln-Pro-Lys-Lys-Lys-Arg-Lys-Val-cysteamine) was from GenWay Biotech, Inc. (San Diego, CA, USA) and FITC-conjugated Tat peptide Tat_{47–57} was from AnaSpec, Inc. (San Jose, CA, USA).

[tyrosyl-3,5-³H(N)]DADLE (sp. radioactivity, 45.7 Ci/mmol) and [tyrosyl-3,5-³H(N)]Deltorphin II (sp. radioactivity, 38.5 Ci/mmol) were from PerkinElmer (Waltham, MA, USA).

Cell Culture

The rodent retinal neuronal cell line RGC-5 was provided by Dr. Neeraj Agarwal (National Eye Institute, Bethesda, MD, USA). They were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The human neuronal cell line SK-N-SH was obtained from the American Type Culture Collection (ATCC, Manassas, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures of both cell lines were maintained at 37 °C in a humidified chamber of 95% O₂/5% CO₂. Culture medium was replaced with fresh medium every other day, and uptake measurements were made in confluent cells on third day in culture.

Primary ganglion cells were isolated from the retinas of 1- to 5-day-old C57BL/6 mice, according to our published method.¹⁸ Verification of cell purity has been reported.^{19, 20} Care and use of mice adhered to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee.

Uptake Measurements

Uptake of [³H]DADLE and [³H]deltorphin II in RGC-5 cells and SK-N-SH cells was measured as described previously.^{10–15} The medium was removed by aspiration and the cells washed with uptake buffer once. Uptake was initiated by adding 0.25 ml of uptake buffer containing 0.1 – 0.25 µCi of [³H]DADLE or [³H]deltorphin II. Concentration of these peptides during uptake was 10 – 25 nM depending on the experiment. Initial experiments were carried out to determine the time course of uptake. Subsequent uptake measurements were made with either 15 min or 30 min incubation, both representing initial

uptake rates. Uptake was terminated by aspiration of the uptake buffer from the wells. The cell monolayers were quickly washed twice with ice-cold uptake buffer without the radiolabeled substrates. Cells were then lysed in 0.5 ml of 1% sodium dodecylsulfate/0.2N NaOH and the radioactivity associated with the cells was quantified. The composition of the uptake buffer in most experiments was: 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose. When Na^+ -free buffer was used, NaCl in the uptake buffer was replaced iso-osmotically with *N*-methyl-D-glucamine chloride (NMDGCl). Non-carrier-mediated diffusional component of uptake was determined by measuring the uptake of [^3H]DADLE and [^3H]deltorphan II in the presence of excess (1 mM) of unlabeled DADLE and deltorphan II, respectively. For both peptides, the diffusional component represented less than 3% of measured total uptake. Saturation kinetics was analyzed by measuring the uptake at increasing concentrations of the substrate. The Michaelis constant and the maximal velocity were determined by fitting the Michaelis-Menten equation describing a single saturable transport system to the data: $v = V_{\max} \cdot S / (K_t + S)$ where v is the uptake rate, S is the substrate concentration, K_t is the Michaelis constant, and $V_{\max}$ is the maximal velocity. The IC_{50} values (i.e., concentrations of various peptides necessary to cause 50% inhibition of DADLE uptake) were calculated from dose-response experiments. To determine the influence of ouabain, an inhibitor of Na^+/K^+ ATPase, on DADLE uptake, RGC-5 cells were first pre-incubated with 1 mM ouabain for 30 min at 37 °C and then used for measurement of DADLE uptake; ouabain (1 mM) was present also during the uptake measurement. As a control, RGC-5 cells were pre-incubated for 30 min at 37 °C in the absence of ouabain and then used for DADLE uptake in the absence of ouabain.

Analysis of FITC-Tat₄₇₋₅₇ Uptake

Uptake of FITC-labeled Tat₄₇₋₅₇ peptide into RGC-5 cells and primary mouse retinal ganglion cells was monitored as follows. Cells were seeded in chamber slides (Nalge Nunc International, Chicago, IL, USA) at a density of 5000 cells/chamber and cultured for 24 h (RGC-5 cells) or 72 h (primary mouse retinal ganglion cells). Cells were then washed with phosphate-buffered saline twice and subsequently incubated with or without 250 μM DADLE for 15 min. Fluorescein isothiocyanate-conjugated Tat₄₇₋₅₇ (FITC-Tat; final concentration, 25 nM) was then added to the cells (DADLE concentration was maintained at 250 μM) and the incubation continued for another 15 min. Cells were washed with phosphate-buffered saline and then fixed with 4% paraformaldehyde for 5 min at room temperature. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min. Cells were then washed with water and the slides were mounted with Gel Mount (Sigma-Aldrich). Green fluorescence, as a measure of FITC, associated with the cells was detected using the Zen 2008 software (version 5.0) and a Zeiss laser-scanning confocal microscope (LSM 510).^{11, 14, 21}

Data Analysis

The kinetic parameters (K_t and $V_{\max}$) were determined using the computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL, USA). These determinations were made by non-linear regression analysis and the values confirmed by linear regression analysis according to the Eadie-Hofstee transformation of the Michaelis-Menten equation. Statistical analysis was done using the paired Student's t test. A $p < 0.05$ was taken as statistically significant. Experiments were repeated at least three times and measurements were made in duplicate for each experimental condition. Data are presented as means $\pm$ S. E.

RESULTS

Time Course and Saturation Kinetics of DADLE Uptake in RGC-5 Cells

Fig. 1A describes the time-dependent uptake of DADLE (25 nM) in RGC-5 cells in the presence and absence of Na⁺. The uptake was linear under both conditions at least up to 30 min, and the presence of Na⁺ had a marked stimulatory effect on the uptake. At 30 min of incubation, the uptake in the presence of Na⁺ was 2–3 times the uptake in the absence of Na⁺. In all subsequent experiments, uptake measurements were made with either 15 min or 30 min incubation to represent linear uptake rates. The uptake process was saturable both in the presence and absence of Na⁺, and the data fit well with the Michaelis-Menten equation describing a single saturable system (Fig. 1B). In the presence of Na⁺, the Michaelis constant (K_t) for DADLE uptake was $6.2 \pm 0.6 \mu\text{M}$ and the maximal velocity (V_{max}) was $2.25 \pm 0.09 \text{ nmol/mg of protein/15 min}$. The corresponding values for uptake in the absence of Na⁺ were $5.5 \pm 1.0 \mu\text{M}$ and $1.43 \pm 0.10 \text{ nmol/mg of protein/15 min}$. The values for the Michaelis constant were not statistically different, indicating that the affinity of the transport system for DADLE is not affected by the presence or absence of Na⁺. In contrast, the maximal velocity was significantly lower in the absence of Na⁺ than in its presence, showing that the Na⁺-dependent stimulation of DADLE uptake is primarily due to an increase in the maximal velocity. We then examined the effect of ouabain on DADLE uptake in these cells. When treated with 1 mM ouabain, the uptake was inhibited $34 \pm 5\%$ ($p < 0.01$), demonstrating the relevance of transmembrane Na⁺ gradient for the uptake process.

Involvement of SOPT2 in DADLE Uptake in RGC-5 Cells

The two recently identified oligopeptide transporters SOPT1 and SOPT2 can be differentiated based on the influence of dipeptides and tripeptides on their uptake activity. These small peptides stimulate the activity of SOPT1 but inhibit the activity of SOPT2. To determine whether the uptake of DADLE observed in RGC-5 cells is mediated by SOPT1 or SOPT2, we studied the influence of several tripeptides on the uptake (Table 1). All of the tripeptides examined inhibited DADLE uptake, leading to the conclusion that DADLE uptake in these cells occurs via SOPT2.

Substrate Selectivity of DADLE Uptake System in RGC-5 Cells

We examined the influence of a variety of endogenous opioid peptides, synthetic opioid peptides, and non-opioid antagonists (naloxone and naltrexone) on the uptake of [³H]DADLE in RGC-5 cells, and the data are presented in Table 2. At a concentration of 1 mM, all endogenous opioid peptides examined inhibited DADLE uptake markedly. The inhibition was >90% in all cases except for endomorphin which caused only 78% inhibition. Among the synthetic opioid peptides examined, deltorphin I and deltorphin II caused about 50% inhibition. However, DAMGO, DSLET, and DALCE were able to inhibit [³H]DADLE uptake greater than 95%. Interestingly, the non-peptide opioid antagonists naloxone and naltrexone stimulated the uptake. The stimulation was 33% for naloxone and 50% for naltrexone. In both cases, the difference was statistically significant ($p < 0.001$). We determined the IC_{50} values for four endogenous opioid peptides (Fig. 2A). The values were $205 \pm 42 \mu\text{M}$ for endomorphin, $14.9 \pm 2.5 \mu\text{M}$ for Leu-enkephalin, $15.8 \pm 3.2 \mu\text{M}$ for Met-enkephalin, and $5.5 \pm 0.8 \mu\text{M}$ for dynorphin A (1–7).

An important aspect of SOPT1 and SOPT2 is their ability to transport the HIV-1 Tat peptide (Tat_{47–57}), widely known as a “cell-penetrating” peptide.^{11, 14, 21} Since RGC-5 cells appear to transport DADLE via SOPT2, we examined the influence of four “cell-penetrating” peptides (HIV-1 Tat_{47–57}, HIV-1 Tat_{49–55}, Pep1, and poly-arginine) on DADLE uptake in these cells (Fig. 2B). Each of these peptides caused marked inhibition of DADLE uptake.

The IC_{50} values were $6.4 \pm 1.1 \mu\text{M}$ for poly(Arg)₉, $3.2 \pm 0.4 \mu\text{M}$ for Pep1, $1.7 \pm 0.2 \mu\text{M}$ for HIV-1 Tat₄₉₋₅₅, and $0.9 \pm 0.1 \mu\text{M}$ for HIV-1 Tat₄₇₋₅₇.

Uptake of FITC-Labeled HIV-1 Tat₄₇₋₅₇ in RGC-5 Cells and Primary Mouse Retinal Ganglion Cells

The findings that the “cell-penetrating” peptides inhibited the uptake of DADLE in RGC-5 cells indicates that these oligopeptides interact with the DADLE uptake system and compete with DADLE for the uptake process, but do not show whether or not the competing peptides themselves are actually transported into cells via the DADLE uptake system. It is possible that these oligopeptides compete with DADLE for the substrate-binding site, thus causing inhibition of DADLE uptake, but may not themselves be transported into cells. To address this issue, we examined the uptake of fluorescein isothiocyanate (FITC)-labeled HIV-1 Tat₄₇₋₅₇ peptide in RGC-5 cells by using cell-associated fluorescence as a measure of cellular uptake of the peptide and tested the ability of DADLE to inhibit this process. The rationale was that if FITC-Tat₄₇₋₅₇ enters RGC-5 cells via the DADLE uptake system, DADLE would be expected to compete with FITC-Tat₄₇₋₅₇ for the uptake process and thus inhibit the entry of the Tat peptide. The results shown in Fig. 3A demonstrate that FITC-labeled HIV-1 Tat₄₇₋₅₇ enters RGC-5 cells and that DADLE blocks this process, providing evidence for the cellular uptake of the HIV-1 Tat peptide via the DADLE uptake system. We also examined the uptake of FITC-Tat₄₇₋₅₇ in primary mouse retinal ganglion cells (Fig. 3B). The fluorescent peptide was taken up into these cells and the uptake was effectively blocked by DADLE, indicating that the oligopeptide transport system is expressed not only in the retinal neuronal cell line RGC-5 but also in primary retinal ganglion cells. Primary retinal ganglion cells do not divide and do not proliferate in culture; therefore, uptake of DADLE could not be studied directly in these cells. Since the uptake of FITC-labeled peptide substrates can be monitored even with single cells, FITC-Tat₄₇₋₅₇ was used to demonstrate the functional expression of the oligopeptide transport system in primary retinal ganglion cells. The cells were cultured for one week to allow differentiation and then used for the experiments. The differentiated cells contain the cell body and also axonal/dendritic processes,¹⁸⁻²⁰ but these processes are too thin compared to the cell body, thus giving the appearance that the fluorescence signals are present only in the cell body.

Evidence for Expression of SOPT1 in RGC-5 Cells

The experiments described thus far in this study show for the first time that the sodium-dependent oligopeptide transport system is expressed in the retinal neural cell line RGC-5 and that the observed uptake of DADLE in this cell line is mediated by SOPT2. Previous studies from our laboratory have shown that retinal pigment epithelial cells (ARPE-19 as well as human and mouse primary RPE cells) express SOPT1 and SOPT2 whereas the conjunctival epithelial cell line CJVE expresses predominantly SOPT2.^{10, 11, 15} Therefore, we wanted to determine if the retinal neuronal cell line RGC-5 expresses SOPT1. For this, we studied the uptake characteristics of another synthetic opioid peptide deltorphin II, a preferred substrate for SOPT1, in these cells and also assessed the effect of tripeptides on the uptake process. The dependence of deltorphin II uptake by Na⁺ and the stimulation of the uptake process by tripeptides are the hallmarks of SOPT1.¹¹⁻¹⁴ Our studies showed that RGC-5 cells possess robust uptake activity for deltorphin II and that the activity was dependent on Na⁺. The uptake of deltorphin II at the concentration of 25 nM was 3.6 ± 0.2 pmol/mg of protein/30 min in the presence of Na⁺ and the uptake decreased to 1.1 ± 0.1 pmol/mg of protein/30 min in the absence of Na⁺. Several tripeptides stimulated deltorphin II uptake; the stimulation was in the range of 2- to 3-fold (Table 3). These data show that RGC-5 cells express not only SOPT2 but also SOPT1.

Evidence for Expression of SOPT2 in SK-N-SH Cells

We then wanted to determine if the expression of SOPT2 also occurs in non-retinal neuronal cells. For this, we studied the uptake characteristics of DADLE in SK-N-SH cells, a human catecholaminergic neuronal cell line. The uptake of DADLE in these cells was time-dependent and Na^+ -dependent, and the uptake was linear at least up to 30 min both in the absence and presence of Na^+ (data not shown). The uptake process was saturable irrespective of whether or not the uptake was measured in the presence or absence of Na^+ (Fig. 4). In the presence of Na^+ , the Michaelis constant (K_i) for DADLE uptake was $2.7 \pm 0.3 \mu\text{M}$ and the maximal velocity (V_{max}) was $3.15 \pm 0.13 \text{ nmol/mg of protein/30 min}$. The corresponding values in the absence of Na^+ were $1.8 \pm 0.3 \mu\text{M}$ and $0.84 \pm 0.03 \text{ nmol/mg of protein/30 min}$. These data show that the stimulation of DADLE uptake by Na^+ observed in SK-N-SH cells was primarily due to an increase in the maximal velocity and that Na^+ has little or no effect on substrate affinity. The uptake was inhibited by the tripeptide Gly-Gly-Ile and also by the dipeptide L-kyotorphin (L-Tyr-L-Arg) (Fig. 5). D-Kyotorphin (L-Tyr-D-Arg) showed a much lower inhibitory effect than L-kyotorphin, indicating that the presence of a D-amino acid in the dipeptide markedly compromises its inhibitory potency. The observed inhibition with L-kyotorphin was however nothing to do with the peptide's ability to function as an opioid peptide because L-Arg-L-Tyr, a dipeptide with no opioid activity, also inhibited DADLE uptake as much as L-kyotorphin did. Since the uptake of DADLE was partially dependent on Na^+ , we analyzed the substrate selectivity features specifically for the Na^+ -independent uptake component and for the Na^+ -dependent uptake component. For this, we monitored the influence of Gly-Gly-Ile and L-kyotorphin on DADLE uptake in the presence and absence of Na^+ . We then analyzed the data separately for the Na^+ -independent component (i.e., uptake measured in the absence of Na^+) and the Na^+ -dependent component, which was determined by subtracting the Na^+ -independent component from total uptake measured in the presence of Na^+ . The inhibitory profiles were similar for both components. Gly-Gly-Ile and L-kyotorphin inhibited the Na^+ -dependent DADLE uptake and the Na^+ -independent DADLE uptake to the same extent. Similarly, both uptake components were less sensitive to inhibition by D-kyotorphin than by L-kyotorphin (data not shown).

These data that dipeptides and tripeptides inhibited DADLE uptake provide functional evidence for the expression of SOPT2 in SK-N-SH cells. As seen in RGC-5 cells, SOPT2 in SK-N-SH cells also accepts large peptides (deltorphan II, dynorphin 1–13, and $\text{Arg}^6, \text{Phe}^7$ -Met-enkephalin) as substrates. The ability of these peptides to compete with DADLE for uptake via SOPT2 was seen both in the presence and absence of Na^+ (Fig. 6). The IC_{50} values in the presence of Na^+ were $0.7 \pm 0.1 \mu\text{M}$ for dynorphin (1–13), $3.6 \pm 0.4 \mu\text{M}$ for $\text{Arg}^6, \text{Phe}^7$ -Met-enkephalin, and $87 \pm 12 \mu\text{M}$ for deltorphan II. The corresponding values in the absence of Na^+ were $0.25 \pm 0.01 \mu\text{M}$, $2.1 \pm 0.3 \mu\text{M}$, and $105 \pm 21 \mu\text{M}$. When analyzed the Na^+ -dependent component of DADLE uptake separately, the IC_{50} values for the three competing peptides were $1.1 \pm 0.1 \mu\text{M}$, $5.7 \pm 0.8 \mu\text{M}$, and $146 \pm 20 \mu\text{M}$, respectively.

DISCUSSION

The results of the present study are summarized as follows. The rodent retinal neuronal cell line RGC-5 expresses robust activity for the uptake of the synthetic opioid peptide DADLE. The uptake occurs in a Na^+ -dependent manner and with high affinity. The functional characteristics of DADLE uptake in this cell line, particularly the inhibition of the transport system by tripeptides, are similar to those of SOPT2, suggesting that DADLE uptake is mediated by SOPT2. Even though the transport system interacts with a variety of endogenous and exogenous opioid peptides, the process is not exclusive to opioid peptides. Several non-opioid peptides are also recognized as substrates for SOPT2 in this cell line. In addition to SOPT2, RGC-5 cells also express SOPT1, evidenced by the robust uptake

activity for the synthetic opioid peptide deltorphin II and its stimulation by tripeptides. This constitutes the first report of the expression of the Na⁺-coupled oligopeptide transporters SOPT1 and SOPT2 in retinal neuronal cells. The expression of these novel transporters is not restricted to the cell line as evidenced from the uptake activity demonstrable in primary cultures of retinal ganglion cells. Though RGC-5 cell line has been widely used as a model for retinal ganglion cells, recent studies have indicated that this cell line most likely represents retinal neuronal precursor cells rather than retinal ganglion cells.²² SK-N-SH is a neuronal cell line widely used as a model for noradrenergic and dopaminergic neurons in the central nervous system. This cell line has already been shown to express SOPT1. Here we showed that these cells also express SOPT2. Thus we conclude that neuronal cells of the retina and central nervous system express both isoforms of the recently discovered Na⁺-coupled oligopeptide transporters. Previous reports from our laboratory have shown that these two transport systems are distinct from OATPs based on their interaction with naloxone, bile salts, and estrone-3-sulfate.^{10, 14}

The uptake of DADLE observed in RGC-5 cells represents transmembrane transport rather than cell-surface adsorption. This is evidenced from its Na⁺-dependence. There is no evidence for any cell-surface receptor in mammalian cells that binds DADLE in a Na⁺-dependent manner. δ -Opiate receptor has high affinity for DADLE, but the binding to this receptor is Na⁺-independent and also is inhibitable by naloxone and naltrexone, which are non-peptide opiate antagonists. The uptake of DADLE observed in RGC-5 cells is not inhibited, but instead stimulated, by these compounds, arguing against the possibility that the binding to cell-surface opiate receptor is responsible for DADLE uptake measured in these cells. The partial dependence of the uptake process on Na⁺ is interesting. Notably, the Na⁺-dependent component and the Na⁺-independent component of the uptake exhibit similar features in terms of substrate affinity and substrate selectivity. Generally, Na⁺-dependent solute transporters show marked differences in substrate affinity when measured in the presence and absence of Na⁺ because Na⁺ binds to the transporter first and as a consequence of conformational change resulting from this binding the affinity of the transporter to its substrates increases. This does not appear to be the case with SOPT2. Therefore, the exact role of extracellular Na⁺ in the uptake process remains to be determined. Nonetheless, it is evident that the uptake of DADLE observed in RGC-5 cells in the absence of Na⁺ does not represent passive diffusion because this component also exhibits substrate selectivity and allosteric modulation by small peptides.

It is surprising that the two uptake systems SOPT1 and SOPT2 possess similar substrate specificity. Both can transport DADLE as well as deltorphin II. However, there are notable differences between them. First, the Na⁺-dependence is more robust for SOPT1 than for SOPT2. Second, dipeptides and tripeptides stimulate SOPT1 but the same peptides inhibit SOPT2. Even though both DADLE and deltorphin II are transported by SOPT1 as well as SOPT2, DADLE uptake is inhibited by dipeptides and tripeptides whereas deltorphin II uptake is stimulated by dipeptides and tripeptides. This suggests that DADLE is taken up into RGC-5 cells preferentially via SOPT2 while deltorphin II is taken up preferentially via SOPT1. These differences in the relative contributions of the two transport systems for the uptake of DADLE and deltorphin II may be due to differences in substrate affinity and/or translocation rate which could differ between the two transporters depending on the substrate. Another possibility, though very unlikely, is that a single transporter mediates the uptake of both DADLE and deltorphin II but dipeptides and tripeptides allosterically modulate the uptake of these substrates differentially. Though it is difficult to explain this conceptually, it does remain a possibility. Without any information on the molecular nature of the protein(s) responsible for the uptake of DADLE and deltorphin II in these cells, we are not able to distinguish between the two possibilities, namely two distinct transporters versus a single transporter.

The present studies also provide evidence for the expression of SOPT2 in two different neuronal cell lines, RGC-5 and SK-N-SH. The general characteristics of the transporter in both cell lines are comparable in terms of Na⁺-dependence, substrate specificity, and allosteric modulation by small peptides. However, the substrate affinities appear to be different between the two cell lines. In RGC-5 cells, deltorphin II at a concentration of 1 mM caused ~50% inhibition of DADLE uptake, indicating that the *IC*₅₀ value for the inhibition by deltorphin II is approximately 1 mM. In contrast, the corresponding value in SK-N-SH cells is ~100 μM. This represents a 10-fold difference and it is unlikely to be due to experimental variations. One possible explanation for this variation is the species difference. RGC-5 cells are of rodent origin whereas SK-N-SH cells are of human origin. It is likely that the substrate affinities of SOPT2 vary depending on the species.

Apart from the functional characteristics, little is known at present on the biological roles of these transporters. Since endogenous opioid peptides are transported with high affinity by SOPT1 and SOPT2, it is possible that the transporters play a role in opioid biology by modulating the extracellular levels of these peptides and consequently their interaction with the cell surface opioid receptors. Whether the observed rates of transport with these transporters are suitable for the rapid clearance of endogenous opioid peptides from the synapse remains to be determined. Furthermore, there is no information on the exact location of these transporters in neurons. For the transporters to function in the clearance of opioid peptides from the synapse, they need to be located at the synaptic terminals. Thus, additional studies are needed to elucidate the physiological role of these transporters. There is also evidence for a nuclear receptor for certain opioid peptides such as enkephalins.²³ This receptor, often identified as Opioid Growth Factor receptor (OGFr), is distinct from the classical cell surface opioid receptors in ligand specificity, signaling, and biological functions. SOPT1 and SOPT2, with their ability to transport enkephalins into cells with high affinity, may possibly play a role in providing extracellular enkephalins access to the intracellular OGFr.

Another potential role of SOPT1 and SOPT2 is in drug delivery. SOPT1 and SOPT2 exhibit broad substrate selectivity and are expressed robustly in several cell types, including neuronal cells, corneal, conjunctival, and retinal pigment epithelial cells, and intestinal and colonic epithelial cells. They transport a broad spectrum of endogenous and synthetic oligopeptides. To date, the longest peptides transported by SOPT1 and SOPT2 include hepcidin (a 25-amino-acid peptide hormone).²¹ An important aspect of SOPT1 and SOPT2 is their ability to transport the HIV-1 Tat peptide (Tat₄₇₋₅₇), widely known as a “cell-penetrating” peptide.^{24, 25} A variety of compounds (e.g., siRNAs, oligonucleotides, and nanoparticles) can be linked to this peptide for delivery into cells.^{26, 27} The mechanism of membrane translocation of the Tat peptide and other “cell-penetrating” peptides has not been elucidated unequivocally. The discovery of SOPT1 and SOPT2 as the active transport systems for the HIV-1 Tat₄₇₋₅₇ peptide constitutes a major breakthrough in drug delivery. We have already tested fluorescein isothiocyanate (FITC) as a model ‘payload’ for delivery via SOPT1 and SOPT2. It is very likely that these transporters also transport other “cell-penetrating” peptides, making it possible to utilize these transporters for the delivery of a wide variety of cargo by ‘piggy-backing’ them on the backbone of these peptides.

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Abbreviations

SOPT	Sodium-coupled oligopeptide transporter
DADLE	[D-Ala ² , D-Leu ⁵]Enkephalin
NMDG	<i>N</i> -methyl-D-glucamine
FITC	fluorescein isothiocyanate
DAPI	4', 6-diamidino-2-phenylindole

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Figure 1.

Time-dependence and saturation kinetics of DADLE uptake in RGC-5 cells. (A) Uptake of [³H]DADLE (25 nM) was measured in confluent cultures of RGC-5 cells for varying periods of time in the presence (NaCl) and absence (NMDG Cl) of Na⁺. (B) Uptake of DADLE was measured at increasing concentrations (1–25 μM) in the presence of 25 nM [³H]DADLE as the tracer. Uptake measurements were made in the presence and absence of Na⁺. Data are means ± S. E. for six determinations.

Figure 2.

Dose-dependent inhibition of DADLE uptake in RGC-5 cells by endogenous opioid peptides (A) and “cell-penetrating” peptides (B). Uptake of [³H]DADLE (25 nM) was measured in confluent cultures of RGC-5 cells in the presence of increasing concentrations of various peptides using the NaCl-containing medium. Uptake value obtained in the absence of any competing peptide was taken as 100% (control). Uptake values measured in the presence of competing peptides is given as percent of this control uptake. Data represent means ± S. E. for four determinations.

Figure 3.

Uptake of FITC-labeled HIV-1 Tat₄₇₋₅₇ peptide in RGC-5 cells (A) and primary mouse retinal ganglion cells (B). Cells, cultured on coverslips (24 h for RGC-5 cells; 72 h for primary mouse retinal ganglion cells), were incubated first in the absence or presence of 250 μ M DADLE for 15 min. Following this, FITC-labeled Tat peptide was added to the cells to a final concentration of 25 nM. DADLE concentration was maintained at 250 μ M. Cells were incubated for another 15 min. These experiments were performed in a NaCl-containing buffer. At the end of this incubation, cells were washed and then fixed in 4% paraformaldehyde. Green fluorescence, as a measure of FITC, associated with the cells was detected by confocal microscopy.

Figure 4. Saturation kinetics of DADLE uptake in SK-N-SH cells. Uptake of DADLE was measured in confluent cultures of SK-N-SH cells at increasing concentrations of DADLE (1–25 μM) in the presence of 25 nM [^3H]DADLE as the tracer. Uptake measurements were made in the presence (NaCl) and absence (NMDG Cl) of Na^+ . Data are means $\pm$ S. E. for six determinations.

Figure 5.

Evidence for the involvement of SOPT2 in DADLE uptake in SK-N-SH cells. Uptake of [^3H]DADLE (10 nM) was measured in confluent cultures of SK-N-SH cells in a NaCl-containing medium for 15 min in the absence or presence of various peptides (1 mM). Uptake measured in the absence of these peptides was taken as the control value (100%). Uptake values obtained in the presence of these peptides are presented as percent of this control value. GGI, Gly-Gly-Ile; L-Kyo, L-Kyotorphin (L-Tyr-L-Arg); D-Kyo, D-Kyotorphin (L-Tyr-D-Arg). Data are from two independent experiments, each performed in duplicate.

Figure 6.

Inhibition of DADLE uptake in SK-N-SH cells by endogenous opioid peptides. Uptake of [³H]DADLE (25 nM) was measured for 15 min in the absence or presence of increasing concentrations of three different endogenous opioid peptides. Uptake measurements were made both in the presence (NaCl) and absence (NMDG Cl) of Na⁺. Uptake value obtained in the absence of competing peptides was taken as control (100%) and the uptake values obtained in the presence of competing peptides are presented as percent of this control value. Data are means ± S. E. for two experiments, each performed in duplicate.

Table 1

Influence of tripeptides on DADLE uptake in RGC-5 cells

Tripeptide	[³ H]DADLE uptake	
	pmol/mg of protein/30 min	%
None (control)	1.31 ± 0.04	100
Gly-Gly-Ile	0.26 ± 0.11 *	20
Gly-Gly-Phe	0.13 ± 0.00 *	10
Gly-Gly-His	0.54 ± 0.03 *	41
Leu-Gly-Gly	0.23 ± 0.00 *	18

Uptake of [³H]DADLE (10 nM) was measured in confluent cultures of RGC-5 cells with a 30 min incubation in the presence or absence of various tripeptides (1 mM). Results are presented as means ± S.E. for 6 independent experiments.

* significantly different from the control value ($p < 0.001$).

Table 2

Influence of endogenous and synthetic opioid peptides as well as non-peptide opioid antagonists on DADLE uptake in RGC-5 cells

Compound	[³ H]DADLE uptake	
	pmol/mg of protein/30 min	%
None (control)	1.24 ± 0.12	100
<u>Endogenous opioid peptides</u>		
Leu-Enkephalin	0.03 ± 0.00 *	2
Met-Enkephalin	0.02 ± 0.00 *	2
Endomorphin	0.28 ± 0.03 *	22
Dynorphin A (1–7)	0.05 ± 0.02 *	4
Dynorphin B (1–9)	0.02 ± 0.00 *	2
Enkephalinamide	0.08 ± 0.02 *	6
<u>Synthetic opioid peptides</u>		
Deltorphan I	0.60 ± 0.33 *	48
Deltorphan II	0.65 ± 0.18 *	53
DADLE	0.06 ± 0.01 *	5
DAMGO	0.08 ± 0.02 *	6
DSLET	0.03 ± 0.00 *	2
DALCE	0.02 ± 0.00 *	2
<u>Non-peptide opioid antagonists</u>		
Naloxone	1.64 ± 0.15 *	133
Naltrexone	1.86 ± 0.22 *	150

Uptake of [³H]DADLE (10 nM) was measured in confluent cultures of RGC-5 cells with a 30 min incubation in the presence or absence of various compounds (1 mM). Results are presented as means ± S.E. for 6 independent experiments.

* significantly different from the control value ($p < 0.001$).

Table 3

Influence of tripeptides on deltorphin II uptake in RGC-5 cells

Tripeptide	[³ H]Deltorphin II uptake	
	pmol/mg of protein/30 min	%
None (control)	3.39 ± 0.07	100
Gly-Gly-Ile	10.40 ± 0.11 *	307
Gly-Gly-Phe	5.86 ± 0.11 *	173
Gly-Gly-His	8.17 ± 0.09 *	241
Leu-Gly-Gly	6.77 ± 0.26 *	199

Uptake of [³H]deltorphin II (25 nM) was measured in confluent cultures of RGC-5 cells with a 30 min incubation in the presence or absence of various tripeptides (1 mM). Results are presented as means ± S.E. for 6 independent experiments.

* significantly different from the control value ($p < 0.001$).