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In Situ Entry of Oligonucleotides into Brain Cells Can Occur through a Nucleic Acid Channel

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ABSTRACT

Brain tissue has become a challenging therapeutic target, in part because of failure of conventional treatments of brain tumors and a gradually increasing number of neurodegenerative diseases. Because antisense oligonucleotides are readily internalized by neuronal cells in culture, these compounds could possibly serve as novel therapeutic agents to meet such a challenge. In previous *in vitro* work using cell culture systems, we have demonstrated that intracellular delivery requires a vector such as cationic liposomes since free oligonucleotides remain largely trapped in the endocytic pathway following cellular uptake. Here we studied the cellular uptake properties of oligonucleotides by explants of rat brain (brain slices), and by *in vivo* brain tissue after administration of oligonucleotides by bolus injection. In contrast to *in vitro* uptake, we show that in brain slices oligonucleotides were taken up by neuronal and nonneuronal cells, irrespective of their assembly with cationic liposomes. In either case, a diffuse distribution of oligonucleotides was seen in the cytosol and/or nucleus. Uptake of oligonucleotides by brain slices as a result of membrane damage, potentially arising from the isolation procedure, could be excluded. Interestingly, internalization was inhibited following treatment of the tissue with antibody GN-2640, directed against a nucleic acid channel, present in rat kidney cells. Our data support the view that an analogous channel is present in brain tissue, allowing entry of free oligonucleotides but not plasmids. Indeed, for delivery of the latter and accomplishment of effective transfection, cationic lipids were needed for gene translocation into both brain slices and brain tissue *in vivo*. These data imply that for antisense therapy to become effective in brain, cationic lipid-mediated delivery will only be needed for specific cell targeting but not necessarily for delivery *per se* to accomplish nuclear deposition of oligonucleotides into brain cells and subsequent down-regulation of disease-related targets.

INTRODUCTION

ANTISENSE OLIGONUCLEOTIDES offer an interesting potential as effective therapeutic agents. By virtue of their ability to hybridize to complementary mRNA, anti-

sense oligonucleotides can induce its effective degradation, thereby impeding *de novo* biosynthesis of target proteins. Antisense sequence, stability, and cellular uptake, among others, determine the antisense efficiency. *In vitro*, the uptake properties of antisense oligonucleotides

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have been well characterized. When added to cultured cells, free antisense oligonucleotides are endocytosed but remain largely trapped in the endocytic compartments. Their translocation from endosomal compartments is therefore essential, and can be promoted by oligonucleotide complexation with cationic lipids. The latter facilitate transfer of oligonucleotides across the endosomal membrane, and via transport through the cytosol, the oligonucleotides passively accumulate in the nuclei. Here they acquire access to target mRNAs via interaction with the nuclear matrix, leading to an inhibition of the synthesis of target proteins (Lorenz et al., 1998; Shi et al., 2003). Not only do cationic liposomes greatly enhance the antisense effect by promoting endocytic escape of oligonucleotides *in vitro* (DeLong et al., 1999; Shi et al., 2001), but they are also used to prolong the plasma half-lives of oligonucleotides *in vivo* (Blume et al., 1993; Maruyama et al., 1997).

The rapid development of *in vivo* models and pharmacokinetic distribution studies suggest that oligonucleotide delivery displays a wide spectrum of tissue distribution, oligonucleotides being taken up in particular by organs of the reticuloendothelial system (RES) such as liver, spleen, kidney, and lungs. However, brain tissue has proven to be essentially inaccessible for oligonucleotides, following systemic administration. Accordingly, the brain is a particularly challenging organ for drug targeting, especially in light of the need for improved treatment of neurodegenerative diseases and brain tumors. Although oligonucleotides hardly ever cross the blood–brain barrier, they readily accumulate in neuronal tissue when injected directly into brain (Chauhan, 2002). Furthermore, it has also been claimed that oligonucleotides, complexed with liposomes conjugated with transferrin receptor antibodies OX-26, could cross the blood–brain barrier and distribute over a wide area in the brain (Penichet et al., 1999; Shi et al., 2000). It has also been shown that following a bolus injection of fluorescein isothiocyanate (FITC)-oligonucleotides into intracerebroventricles, the fluorescein isothiocyanate (FITC) signal is primarily localized to the ventricle, whereas continuous infusion of FITC-oligonucleotides into the ventricle causes a wide distribution in the brain, except in the white matter (Zhang et al., 1996). However, little insight is available as to how oligonucleotides enter cells in the brain, and whether delivery vectors will facilitate oligonucleotides in penetrating the extracellular matrix or plasma membranes, as observed *in vitro*.

Hence, on the basis of previous work on *in vitro* delivery of oligonucleotides into neuronal cells (Shi et al., 2003), we examined here whether and how oligonucleotides could acquire access to brain cells, using an *ex vivo* brain slice model. The data were verified by investigating directly the fate of a bolus injection into the brain *in vivo*. In contrast to the *in vitro* findings, we observed

that oligonucleotides were taken up through the whole slice without the need of carriers. We demonstrate that facilitated diffusion of oligonucleotides through a brain cell-localized nucleic acid channel can account for the high uptake of oligonucleotides in brain slices. In comparison, a green fluorescent protein (GFP) reporter plasmid did not acquire access via the channel, but rather, required cationic liposomes for cellular entry and subsequent expression. Similar properties of oligonucleotide uptake characteristics, as observed in brain slices, were apparent *in vivo* following a bolus injection of oligonucleotides in rat brain.

MATERIALS AND METHODS

Materials

The cationic lipid *N*-methyl-4(dioleoyl)methylpyridiniumchloride (SAINT-2) was synthesized as described in detail elsewhere (Meekel et al., 2000). Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL), and Lipofectamine 2000™ was purchased from Invitrogen (San Diego, CA). Antisense oligonucleotides, complementary to CRF-R mRNA (sequence see GenBank, accession no. L25438; Shi et al., 2003), targeted to base pairs 474–490 with the sequence 5'-GGA TGA AAG CCG AGA TG-3', as well as the GC-mismatched randomized-sequence oligonucleotide with the sequence 5'-ACT ACG ACC TAC GTG AC-3' and a fluorescein-labeled randomized-sequence oligonucleotide with the sequence 5'-ACT ACG ACC TAC GTG AC-3', were designed and manufactured by Biognostik (Göttingen, Germany). All oligonucleotides were thioated and purified by high-performance liquid chromatography, cross-flow dialysis, and ultrafiltration. Polyclonal antibodies GN-2640 raised against a peptide of the 45-kDa pore-forming subunit of the rat kidney nucleic acid channel were a kind gift from Dr. Basil Hanss (Mt. Sinai School of Medicine, NY). The plasmid pEGFP-N1, containing the gene encoding for GFP under the control of the cytomegalovirus (CMV) promoter, was obtained from Clontech (Mountain View, CA). Rhodamine-labeled plasmids were obtained from GTS INC (San Diego, CA). FITC-dextran (M.W. 10,000 Da) was purchased from Molecule Probes (Eugene, OR). All chemicals were from Sigma (St. Louis, MO), unless stated otherwise.

Preparation of brain slices

Eight-day-old Sprague-Dawley rat pups were decapitated and the brains were removed aseptically. The cerebellum was dissected in ice-cold preparation medium consisting of minimum essential medium (MEM) containing 2 mM glutamax I pH 7.3, and the meninges were

carefully removed. Sagittal slices of 400 μm were cut using a McIlwain tissue chopper, separated with fine forceps, and transferred onto humidified transparent membranes (Millicell-CM, Millipore). They were cultured on a liquid layer of MEM containing Hanks' balanced salt solution (HBSS; 25%), horse serum (25%), glutamax I (2 mM) and NaHCO_3 (5 mM), HEPES (10 mM) pH7.3 in a humidified atmosphere with 5% CO_2 at 37°C. Cultures were maintained for up to 12 days *in vitro* (DIV). Medium was changed every 2–3 days.

Uptake of oligonucleotides and plasmids, and transfection of pGFP in brain slices

One nanomole oligonucleotides or 5 μg of pGFP in 50 μL Dulbecco's-modified Eagle medium (DMEM) were added on top of the mounted brain slices for the indicated time. Then the brain slices were washed and supplemented with fresh medium. Alternatively, the same amount of oligonucleotides or pGFP was mixed with 75 nmol ($\pm$ charge ratio 2.5:1) SAINT-2/DOPE or 15 μL of lipofectamine in 10 μL of 5% glucose, and after 20 minutes the lipoplexes were added on top of the mounted brain slice at 4°C or 37°C. After 4 hours, lipoplexes were removed and the medium was refreshed. The delivery efficiency was determined at 2, 4, and 24 hours, and the transfection efficiency was analyzed after 2 and 6 days by (epi-)fluorescence microscopy (Olympus, Tokyo, Japan) or by TCS Leica SP2 confocal laser scanning microscopy (Leica, Wetzlar, Germany). The integrity of the plasma membranes of the cells in the brain slices was verified by Hoechst Blue staining (20 $\mu\text{g}/\text{mL}$, Molecular Probe). The nuclei were counterstained with propidium iodide (2.5 $\mu\text{g}/\text{mL}$) in the fixed brain slices.

FITC-dextran (2 mg/mL) was added onto brain slices, and after 4 hours of incubation, brain slices were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) and the uptake of dextran was analyzed by confocal microscopy. Nuclei were counterstained with Hoechst Blue staining (20 $\mu\text{g}/\text{mL}$). To monitor their uptake and intracellular trafficking by fluorescence microscopy, oligonucleotides labeled with FITC, plasmids labeled with rhodamine, and SAINT-2/DOPE traced with rhodamine (Rh) phosphatidylethanolamine (PE) were used, as previously described (Shi et al., 2001, 2003).

Western blotting of the nucleic acid channel

The expression of the nucleic acid channel was examined by western immunoblot. Cerebellar samples and kidney at P8 (8 days after birth) were prepared from fresh rat tissue, lysed in sample buffer [3 mL/mg, 5% (SDS) 5% mercaptoethanol, 8 M urea, 6.25 mM Tris-HCl pH 6.8, and 0.01% bromophenol blue]. A total of 30 μg of cerebellum or kidney samples were separated on 12.5% SDS polyacrylamide gel electrophoresis (PAGE) (Bio-

Rad, Hercules, CA), blotted on a pure nitrocellulose membrane (Bio-Rad, Hercules, CA) and probed with rabbit GN-2640 (1:75) or nonimmune rabbit serum. The blot was then probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:3000, Amersham Biosciences, Buckinghamshire, England), and processed using enhanced chemiluminescence (ECL) plus (Amersham Pharmacia Biotech) according to the manufacturer's instruction.

Inhibition of nucleic acid channel activity in brain slices

Brain slices were prepared as above. The nucleic acid-conducting channels in the brain slices were blocked either with specific polyclonal antibody GN-2640 or with preimmune serum at 4°C for 2 hours at the indicated concentration. Alternatively, the channels were blocked with heparan sulfate or L-malate acid at a concentration range of 20–200 $\mu\text{g}/\text{mL}$ for 1 hour. Then 1 nmol oligonucleotides, suspended in a volume of 50 μL , was added to the brain slices in 1 mL of culture medium, and after 30 minutes the uptake of oligonucleotides was analyzed by confocal microscopy.

Bolus injection of oligonucleotides into rat brain in vivo

A guide cannula was implanted into the right nucleus accumbens of Wistar rats. FITC-oligonucleotides (0.1 mM) diluted with 4.5% NaCl (4:1; final concentration 0.9 % NaCl) were infused for 4 minutes into the nucleus accumbens (0.75 $\mu\text{L}/\text{minute}$) through a cannula after a brief anesthesia with isoflurane. After 4 or 24 hours, the animals were decapitated, and the brain was dissected and frozen on dry ice. Subsequently, cryosections (20 μm) were cut, collected, and evaluated by fluorescence microscopy. Alternatively, the cannula was inserted into the lateral ventricle of adult Wistar rats under anesthesia. Then 5 μg pGFP alone or complexed with 100 nmol SAINT-2/DOPE in a volume of 15 μL were bolus injected into intracerebroventricular (ICV) via a cannula (2 animals). GFP expression was examined after 48 hours.

RESULTS AND DISCUSSION

Uptake of oligonucleotides by brain slices does not depend on cationic lipids

Free oligonucleotides can be endocytosed by cultured cells, and most of them end up in endocytic compartments. By contrast, when complexed with cationic lipids, the uptake of oligonucleotides is not only enhanced, but in this case translocation of oligonucleotides from the endosomes into the cytosol occurs, which leads to nuclear

uptake, a prerequisite for down-regulation of protein targets. To verify our previous data on the cellular uptake of oligonucleotide lipoplexes in neuronal cells *in vitro* (Shi et al., 2003), the next challenge was to investigate biological consequences of a similar approach *in vivo*. Therefore, we studied the uptake properties of oligonucleotides by brain slices in the presence and absence of cationic lipids. In preliminary experiments, using freshly prepared brain slices, we observed a substantial internalization of free FITC-labeled oligonucleotides, i.e., without their packaging into SAINT-2/DOPE lipoplexes, and their diffuse distribution in cytosol and nucleus. Accordingly, because our previous data, as obtained in cell cultures, showed a need for carrier-mediated entry of oligonucleotides to acquire access into the cytosol, these observations raised concern as to the integrity of the plasma membrane of the relevant cells. To exclude that oligonucleotide uptake could be due to damage of the plasma membrane during slice preparation, the integrity of the membrane was verified by Hoechst staining. As shown in Fig. 1A, when added to brain slices after their fixation with 3% paraformaldehyde, which is used as a positive control, Hoechst permeated into virtually every

cell in the slice. Importantly, when stained immediately after isolation (without fixation), damage of the brain slices is apparent particularly at the periphery of the slices (Fig. 1B). However, after 4 days of recovery in culture, the damage to the brain slices was essentially minimal and only occasional penetration of the Hoechst dye could be detected (Fig. 1C), as verified in three independent experiments. Therefore, all further experiments on the brain slices were carried out with preparations that had been cultured for 4 days. When such slices were treated with FITC-labeled oligonucleotides and oligonucleotides complexed with cationic lipids (SAINT-2/DOPE, 1:1) for 4 hours, qualitative analysis of the specimen by confocal microscopy revealed that the uptake of oligonucleotides *per se* (Fig. 1D) or oligonucleotides complexed with cationic lipids (Fig. 1E) was almost identical. Furthermore, oligonucleotides alone or complexed with cationic lipids were taken up through the whole slice, as readily determined by serial scanning by confocal microscopy (data not shown), whereas the uptake of oligonucleotides continued to increase when monitored over a time interval of 24 hours (Fig. 1F). Hence, these data, typical for the data obtained in three

FIG. 1. Uptake of oligonucleotides by brain slices. Brain slices ($400\ \mu\text{m}$) were prepared as described in Materials and Methods. To evaluate their plasma membrane integrity, the slices were incubated with the membrane permeating dye Hoechst ($20\ \mu\text{g/mL}$) for 10 minutes at various conditions. (A) The brain slice was fixed and permeabilized, and stained with Hoechst, the sample serving as a positive control. (B) A brain slice was stained with Hoechst immediately after preparation, without fixation. (C) A brain slice was kept in culture for 4 days and then stained with Hoechst. Note that after 4 days in culture, only few plasma membrane-damaged cells were apparent. (D) Free FITC-oligonucleotides ($1\ \text{nmol/mL}$) were incubated with the brain slice for 4 hours. (E) FITC-oligonucleotides ($1\ \text{nmol/mL}$), complexed with cationic lipids ($75\ \text{nmol/mL}$), were incubated with the brain slice for 4 hours. Note that visual inspection reveals that the uptake of free oligonucleotides or oligonucleotides complexed with cationic lipids is very similar, and substantial internalization of oligonucleotides can be seen in D and E, as detected by confocal microscopy. (F) Free FITC-oligonucleotides ($1\ \text{nmol}$) were incubated with brain slices for 24 hours and a gradual increase in uptake of oligonucleotides was apparent. Scale bar, $100\ \mu\text{m}$. The data as shown were typically obtained in three independent experiments.

independent experiments, not only imply that cationic lipids did not significantly improve the uptake of oligonucleotides by the brain slices, but rather, that oligonucleotides were internalized by cells present in the slices without a need for a vector for internalization and, presumably, for their release from endocytic compartments. *A priori*, having demonstrated previously (Shi et al., 2001) that endocytosed oligonucleotides remain trapped within the endocytic compartments, these data could suggest that oligonucleotides taken up by the cells in the brain slices did not substantially enter the cells by endocytosis. Rather direct access into the cytosol might have been acquired by direct translocation across the plasma membrane. Having thus acquired access to the cytosol, the oligonucleotides would then readily accumulate into the nucleus via diffusion through the nuclear pore (Shoeman et al., 1997; Hartig et al., 1998). Therefore, it was important to define more carefully the intracellular localization of internalized oligonucleotides, incubated with the slices as such or when complexed with cationic lipids.

Free oligonucleotides are internalized by brain cells and acquire access to the nucleus

As demonstrated in Fig. 2, showing results typically obtained in three independent experiments, when FITC-oligonucleotides alone (Fig. 2A, green) or complexed with cationic lipids (Fig. 2B,C) were applied to the brain slices, more than 50% of the cells showed high uptake of oligonucleotides in the cytosol and/or nuclei. Accumulation of free oligonucleotides in the nuclei (yellow) could be clearly discerned when the latter had been counterstained with propidium iodide (red) (Fig. 2A). Occasionally, an almost exclusive localization in the cytosol was seen, while the oligonucleotides had not yet reached the nucleus (insert, Fig. 2A). Overall, the uptake of oligonucleotides did not show a cell type-specific preference, as inferred from oligonucleotide internalization versus morphology of the cells in the brain slices.

When complexed with cationic lipids, efficient delivery of labeled oligonucleotides into cells in the brain slices and their localization into cytosol and nucleus, were also readily observed (Fig. 2C). N-Rh-PE-labeled cationic liposomes were rapidly taken up by the brain slices, and they appeared as fine dots in intracellular compartments without showing any diffuse appearance in the cytoplasm or nucleus (Fig. 2B). This would suggest that the cells have most likely internalized the liposomes via an endocytic internalization mechanism, the particles becoming entrapped in endosomal/lysosomal compartments, consistent with previous observations (Shi et al., 2001; Zuhorn et al., 2002). Note that FITC-labeled oligonucleotides do escape from the complexes and reach the cytosol prior to reaching the nucleus, with-

FIG. 2. Cellular uptake of FITC-oligonucleotides and cationic lipids, detected at high magnification. Free FITC-labeled oligonucleotides (1 nmol/mL) or complexed with cationic liposomes (75 nmol/mL), labeled with the fluorescent lipid analogue N-Rh-PE, or FITC-dextran (M.W.10,000) of comparable size were incubated with brain slices as described and examined by fluorescence microscopy. (A) Free FITC-labeled oligonucleotides were incubated with brain slices for 4 hours. The nuclei were counterstained with propidium iodide (2.5 μ g/ μ L, red) after fixation. Note that a prominent localization of oligonucleotides was seen in both the cytosol (green) and nuclei (yellow), whereas in some cells oligonucleotides appeared only in the cytosol as diffuse fluorescence (insert in A; nuclei were not counterstained). (B) Localization of cationic liposomes, traced with N-Rh-PE (red), following incubation with brain slices for 4 hours. The liposomes, marked by N-Rh-PE fluorescence, appeared as fine dots in the cytosol, presumably reflecting their localization in the endocytic pathway, without any significant diffuse fluorescence in the cytoplasm. (C) FITC-oligonucleotides were complexed with N-Rh-PE-labeled cationic liposomes and were subsequently incubated with brain slices for 4 hours at 37°C. Note that the oligonucleotides (green) accumulated in the nuclei, while the cationic lipids (red) remained in the cytosol; some intact complexes (yellow) remaining clustered at the extracellular matrix. (D) FITC-dextran was incubated with brain slices for 4 hours, and the nuclei were counterstained with Hoechst, after fixation of the brain slices. No diffuse dextran could be observed in the cytosol or nuclei in the cells. Occasionally punctate staining was seen in the cytosol. Scale bar, 20 μ m. The data were typical, as obtained in three independent experiments. (For color, see online version.)

out the lipids gaining access to this organelle, as red fluorescence could not be detected in association with the nucleus (Fig. 2B,C; cf. Shi et al., 2003). Taken together, these data, in conjunction with those presented in Fig. 1 clearly indicate that oligonucleotides, irrespective of

their complexation with cationic lipid complexes, readily acquire access to cells present in brain slices to very similar extents. Treatment with oligonucleotides alone or complexed with cationic lipids did not cause an increase of Hoechst staining in the brain slices, indicating that the slices are tolerant to oligonucleotide and cationic lipid treatment (data not shown). Furthermore, when FITC-dextran of a size (10 kDa) comparable to that of oligonucleotides was incubated with brain slices for 4 hours at 37°C, no substantial uptake could be observed by confocal microscopy (Fig. 2D). Occasionally punctate instead of diffuse fluorescence could be seen in the cells, consistent with dextran's entry into the cells via endocytosis rather than via nonspecific leakiness across the plasma membrane (cf. Shi et al., 2003). Moreover, free oligonucleotides do not become entrapped in the endocytotic pathway, but rather show a very similar distribution, i.e., in the cytosol and nucleus (Fig. 2), as that observed when delivered via cationic lipids. Because oligonucleotides, present in endosomes do not substantially leak into the cytosol, the data strongly support the view that free oligonucleotides, when incubated with brain slices, must acquire cytosolic access by direct translocation across the plasma membrane, which is in marked contrast to an almost exclusive endocytic uptake of oligonucleotides, when added to cells in culture.

For oligonucleotides to translocate across the plasma membrane, a membrane-localized transporter, present in cells that constitute the brain slices, would be a likely mechanism, given that permeation across perturbed plasma membranes can be excluded (see above). In fact, recently, the presence of such nucleic acid channels has been demonstrated to be localized in the plasma membrane of rat kidney cells (Hanss et al., 1998, 2002). Therefore, the potential presence of such a channel in brain cells was examined.

Entry of oligonucleotides into cells in brain slices can be accomplished via nucleic acid channels

If direct uptake across the plasma membrane of brain cells would occur, and if endocytosis would not significantly contribute to oligonucleotide uptake, as characterized in the previous section, the effect of temperature might discriminate between either mechanism, endocytosis showing a prominent temperature dependence. Interestingly, when brain slices were incubated with oligonucleotides at 4°C for 2 hours, a substantial uptake could still be seen (Fig. 3A), but less than that at 37°C (Fig. 3B). By quantitative analysis of fluorescence from three different experiments, it was determined that at 4°C, approximately 30% of the oligonucleotide uptake was inhibited, compared to the uptake at 37°C. Furthermore, cationic liposomes and lipoplexes, which are known to enter cells via clathrin-mediated endocytosis (Zuhorn et

al., 2002), only associated with cells but were not internalized by cells at 4°C (Fig. 3C,E). This is in marked contrast to the high uptake at 37°C (Fig. 3D,F). The uptake of FITC-dextran, which is also known to enter cells via endocytosis, could be totally blocked at 4°C even though the uptake of FITC-dextran at 37°C was rather low (Fig. 2D) (data not shown). Taken together, the experiments show oligonucleotide uptake occur at conditions, i.e., at 4°C, where endocytosis is effectively inhibited, implying that entry of a substantial fraction of free oligonucleotides into brain cells can occur via direct passage across the plasma membrane.

Further support for the involvement of a nonendocytic pathway of entry of free oligonucleotides by brain slices was derived from inhibition experiments using heparan sulfate. This large polyanion is similar in molecular weight and valency to oligonucleotides, and can compete with oligonucleotides for binding sites on the cell surface, causing an inhibition of endocytosis of oligonucleotides. A dose dependent inhibition of the uptake of oligonucleotides by heparan sulfate could be seen (Fig. 5E,F), although the inhibition was modest. Thus, at the highest dose used in this study (200 µg/mL; Fig. 5F), the inhibition of uptake was approximately 30% ($n = 3$). Together, the uptake at low temperature and the (modest) inhibition of oligonucleotide internalization by brain slices at 37°C in the presence of heparan sulfate are in line with the notion that a nonendocytic entry pathway contributes to the internalization of free oligonucleotides in brain cells. Also taking into account the intracellular distribution, a direct crossing of oligonucleotides across the plasma membrane appeared the most likely path of entry. Therefore, it was of interest to investigate whether such oligonucleotide transfer could occur via a plasma membrane-localized channel. Recently, such channels have been identified for the first time in rat kidney cell membranes (Hanss et al., 1998, 2002), but whether such channels are ubiquitously distributed in tissues, is unknown.

The nucleic acid channel is a heteromultimeric complex, which consists of at least two protein subunits, i.e., a 45-kDa pore-forming subunit (p45) and a 36-kDa regulatory subunit (p36). The latter has been identified as a cytosolic malate dehydrogenase (cMDH), which seems to confer the selectivity of the pore protein to oligonucleotides. Both phosphodiester and phosphothioate oligonucleotides can be translocated via this channel (Hanss et al., 1998). To investigate the potential presence of this channel in rat brain cells, a cellular lysate of rat brain was prepared and the sample was analyzed by western blotting, using an antibody, GN-2640, raised against a peptide of the 45-kDa pore-forming subunit, as detected in rat kidney. As shown in Fig. 4, the antibody staining clearly revealed the presence of one protein band, showing a molecular weight of at 83 kD (Fig. 4C),

FIG. 3. Uptake of free oligonucleotides can occur at 4°C. The internalization of FITC-oligonucleotides, N-Rh-PE-labeled cationic liposomes or FITC-oligonucleotide lipoplexes by brain slices was carried out at 4°C or 37°C, as described in Materials and Methods, for an incubation period of 2 hours. Note that a prominent internalization of (free) oligonucleotides could be seen at 4°C (A), although it was less than at 37°C (B). The data imply that a mechanism other than endocytosis is involved in oligonucleotide internalization in brain slices. Note that the internalization of cationic liposomes (C) and lipoplexes (E) was completely blocked at 4°C and only some association could be seen, as compared to extensive internalization of liposome (D) and lipoplexes (F) at 37°C. Scale bar, 100 μ m, $n = 3$.

and nonimmune serum failed to interact with brain or kidney samples (Fig. 4A). Thus, it is tempting to suggest that this band likely represents heterodimers of the putative oligonucleotide channel protein, which may bear a remarkable similarity with the heteromultimeric complex identified in rat kidney (Fig. 4B).

To obtain further support for this notion, we next examined whether the channel's activity could be blocked upon binding of the antibody. Interestingly, an antibody concentration dependent inhibition of the uptake of oligonucleotides was observed (Fig. 5C, D). As shown in Fig. 5B, relative to control uptake of oligonucleotides, no

FIG. 4. Expression of the nucleic acid channel in rat brain. The expression of the nucleic acid channel was examined with immuno-Western blot using the specific antibody GN-2640, as described in Materials and Methods. No immune interaction was observed with lysate of rat brain and kidney when nonimmune serum was used (A), whereas with the specific antibody two bands were detected at 83 KDa and 175 KDa in kidney lysate (30 μ g, B), and one band at 86 kDa in the lysate of rat brain (30 μ g, C).

effect was seen when the brain slices had been preincubated with preimmune serum. By contrast, an inhibition of oligonucleotide uptake by more than 50% occurred, as determined by fluorescence measurements in three independent experiments, when the slices had been preincubated with GN-2640 antibodies at a 1:10 dilution (Fig. 5C). Although the uptake of oligonucleotide lipoplexes was only affected by decreasing the temperature (Fig. 3E,F), but not by blocking the channel or by competing with heparan sulfate (data not shown). Hence, these data suggest that a functionally active nucleic acid channel is present on brain cells, which contributes to the translocation of naked oligonucleotides in brain slices.

L-malate, which is a substrate for cMDH, has been identified as a regulatory subunit of the nucleic acid channel in kidney, and, upon reconstitution of the channel in liposomes, L-malate was shown to block the reconstituted activity (Hanss et al., 2002). However, when L-malate was preincubated with brain slices at concentrations ranging from 20 to 200 μ g/mL prior to addition of the oligonucleotides, no significant effect on oligonucleotide uptake was apparent (Fig. 5G, H). Hence, unlike an effective inhibition by the antibody raised against the

FIG. 5. Modulation of oligonucleotide uptake in brain slices, following pretreatment with oligonucleotide channel antibodies. In A, 1 nmol FITC-oligonucleotides (1 μ M) were incubated with a brain slice for 30 minutes. In B, prior to incubation with the same amount of FITC-oligonucleotides, the brain slice was pretreated with preimmune serum or with the channel antibody GN-2640, at a dilution of antibody of 1:10 (C) or 1:100 (D). Note the strong inhibition of uptake of oligonucleotides was seen in C and D, in which the nucleic acid channel had been preblocked with the channel-specific antibody, whereas such effect was not observed in B with the preimmune serum. In E and F, the brain slices were preincubated with heparan sulfate before the addition of the same amount of FITC-oligonucleotides. Note that the inhibitory effect of heparan sulfate was dose dependent, showing no effect at 20 μ g/mL (E), while at 200 μ g/mL, an inhibition of approximately 30% was apparent (F). In G and H, the brain slices were treated with L-malate prior to the addition of oligonucleotides (1 nmol). L-Malate acid (G, at 20 μ g/mL; H, at 200 μ g/mL) did not interfere with the uptake of oligonucleotides by the brain slices (see A). Scale bar, 100 μ m, $n = 3$.

pore-forming subunit, no effect was seen when modulating the regulatory subunit, of this channel with its substrate. However, the failure of L-malate to block oligonucleotide uptake in brain slices is possibly due to the likelihood that cMDH resides inside the cell and, as such, L-malate would block the channel only from inside the cell.

Plasmid uptake does not occur via oligonucleotide channels and requires cationic-lipid mediated delivery

Because the data obtained thus far strongly suggest the presence of an oligonucleotide-conducting channel in the plasma membrane of brain cells, as detected in brain slices, we next examined whether plasmids could also be translocated via the channel protein. Thus, rhodamine-labeled plasmids encoding GFP were incubated with brain slices, either at 4°C or 37°C. However, except for some fuzzy fluorescence at the cell's surface, no significant internalization of plasmid was apparent at either condition, as illustrated in Fig. 6A for the interaction at 37°C. Consistent with this observation, neither was the expression of GFP fluorescence, acting as a reporter gene, apparent at either condition (Fig. 6C). This would imply that the nucleic acid channel is not adapted for carrying large size plasmids across the plasma membranes of brain cells. However, substantial internalization of rhodamine-labeled plasmids could be detected when complexed with cationic liposomes, and a subsequent incubation with the brain slices at 37°C (Fig. 6B). As a result, a relatively high level of GFP expression was seen at the margin of the slice, culminating in the transfection of approximately 100 cells per slice (Fig. 6D), as typically seen in three independent experiments. At present, we have no clear explanation as to why the cells at the margin of the sample displayed a relatively high expression of GFP, despite the uptake of labeled plasmids in nearly all the cells. Possibly, the difference in efficiency may reflect cell type-dependent differences in gene expression efficiency. Taken together, although relatively small oligonucleotides are readily translocated via oligonucleotide channel proteins in brain cells present in brain slices, the internalization of larger-sized plasmids requires cationic lipids for delivery at elevated temperature. This suggests, as demonstrated before for *in vitro* cultures of a variety of cells, including neuronal cell lines, that also in cells in brain slices endocytic entry, followed by permeation across endosomal membranes, represents the entry pathway for lipoplexes.

The remarkable observation that oligonucleotides, by contrast, might enter brain cells in slices via a nucleic acid channel, raised the intriguing question of whether entry *in vivo* could be accomplished by a similar mechanism. Hence to evaluate further the extent to which *in vitro* versus *in vivo* data could be extrapolated and compared, we carried out similar experiment *in vivo*.

Similar mechanisms operate for oligonucleotide and plasmid entry into brain slices and brain tissue in vivo

To determine whether oligonucleotide uptake in brain slices reflected an analogous mechanism by which oligonucleotides could be taken up *in vivo* as well, FITC-oligonucleotides were locally infused into the right nucleus accumbens in rats. After 4 hours, massive uptake of FITC-oligonucleotides in cells localized near the point of injection was observed in three rats (Fig. 7A arrow). Most interestingly, FITC-oligonucleotides were not predominantly entrapped in vesicular compartments within the cell, but rather showed a fairly diffuse appearance. This indicates that endocytosis is not the only mechanism for the uptake of oligonucleotides in rat brain. This observation is consistent with the uptake of oligonucleotides by the brain slices but contrasts with *in vitro* uptake by cell culture systems. Over a period of another 24 hours, the number of cells that showed incorporation of oligonucleotides further increased (Fig. 7B), although the region that became stained was largely restricted to that near the injection site, implying that further penetration of oligonucleotides into the brain was rather poor. In contrast to the relatively rapid translocation of free oligonucleotides into brain cells *in vivo*, plasmids did not penetrate when infused in free form (data not shown). By contrast, but in line with the observations on brain slices, pGFP complexed with cationic lipids transfected around 100 cells per brain near the injection site in three infused rats (Fig. 7C). Thus, in terms of a requirement for a vector and the efficiency of transfection, the brain explant system and *in vivo* brain tissue displayed a remarkably similar behavior.

In summary, the data presented in this work demonstrate that brain cells, both *in vivo* and in brain explants, contain nucleic acid channel proteins in their plasma membranes, which effectively carry free oligonucleotides directly into the cytosol. This direct translocation facilitates their homing into the nucleus, a prerequisite for their ability to express antisense activity. In previous work, we observed (Shi et al., 2001, 2003) that cultured cells do not express such an activity, and delivery requires a vector such as cationic lipids, which allows transfer of oligonucleotides into the cytosol after endocytic internalization and subsequent perturbation of endosomal membrane integrity. At present, it is unclear why brain cells in the slices apparently express the channel protein, like *in vivo*, whereas neuronal cells in culture, such as raphe cells, RN46, and septal neurons, SN48, do not (Shi et al., 2003). Yet for therapeutic purposes, the consequence of the presence of a channel for oligonucleotides could be of potential interest.

Although antisense oligonucleotides hardly cross the blood-brain barrier, they readily accumulate in brain tissue when directly injected into rat brain. Thus, when lo-

FIG. 6. Uptake and transfection of pGFP in brain slices. Brain slices were treated with rhodamine-labeled pGFP alone or when complexed with cationic lipids (lipoplexes), prepared as described in Materials and Methods. The transfection efficiency was examined 6 days after treatment. When pGFP alone was applied onto the brain slice, no significant uptake of rhodamine-labeled plasmid could be observed (**A**) and no GFP transfection could be detected (**C**). When pGFP lipoplexes were applied, the uptake of rhodamine-labeled pGFP could be observed (**B**), and around 100 cells per sample at the margin of the slice expressed GFP (**D**). Scale bar, 100 μm , $n = 3$.

FIG. 7. Infusion of oligonucleotides or pGFP into rat brains *in vivo*. FITC-oligonucleotides were infused into rat brains as described in Materials and Methods. The rat was sacrificed at 4 or 24 hours after infusion, and brains were sectioned. The pictures were taken from areas that were near the infusion point. Note that after 4 hours (**A**) FITC-oligonucleotides had prominently accumulated within the cells (arrow) and were also found in association with the extracellular space (arrowhead). After 24 hours, the pool of extracellular oligonucleotides (arrowhead, **B**) had decreased, while concomitantly an increase in cellular uptake of oligonucleotides (arrow) was apparent. (**C**) pGFP lipoplexes were infused into the brain of the rat as described in Materials and Methods. The GFP expression was examined after 2 days. Substantial expression, i.e., around 100 transfected cells, was seen directly adjacent to the injection site. No pGFP expression could be detected when pGFP alone was applied (data not shown). Scale bar, 100 μm , $n = 3$.

cally applied, specific antisense molecules may readily acquire access to brain cells for down-regulation of specific target proteins, the specificity being dictated by the target, rather than by the nature of the brain cells. On the other hand, if specific cellular targeting is required, the use of an appropriately targeted vector will be unavoidable. As shown in the present work, vector-mediated delivery can be readily accomplished in brain slices and in *in vivo* brain tissue, although an apparent limitation is clearly the restriction in diffusion of the injected sample from the injection site.

In this context, it is interesting to note that in terms of efficiency cationic lipid-mediated delivery of oligonucleotides is nearly identical to the uptake of free oligonucleotides in the brain slices. There are several possibilities that could account for the similarity in uptake at either condition. First, the entire complex of cationic lipid and oligonucleotides is endocytosed, followed by the release of oligonucleotides from endosomes and the nucleotide's accumulation in the nuclei (Fig. 2C), which has been well-documented for *in vitro* uptake by cell cultures (Shi et al., 2001). However, we also observed that cationic complexes were poorly penetrating, whereas oligonucleotides were seen throughout the slice (Fig. 1E). Accordingly, it is also possible that oligonucleotide complex/plasma membrane interaction may cause a destabilization of the complexes by charge neutralization and induce the release of oligonucleotides from the complexes. Dissociated oligonucleotides may diffuse into the slice and subsequently enter the cells through the nucleic acid channel. The possibility of the latter scenario is suggested by observations that the cationic lipids were mostly seen at the surface or at the periphery of the slice (Fig. 2B,C).

Evidently, these and other issues require further work. Yet, the unanticipated presence of nucleic acid channels on brain cells raises novel challenges and opportunities in the development of therapeutic strategies in the treatment of brain diseases. In this context, it will be of particular interest to develop devices that cross the blood-brain barrier so that oligonucleotide-based approaches could rely on noninvasive protocols.

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