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RESEARCH PAPER

Cellular Uptake Mechanism and Therapeutic Utility of a Novel Peptide in Targeted-Delivery of Proteins into Neuronal Cells

Ailing Fu • Zizhen Zhao • Feiyan Gao • Miaomiao Zhang

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ABSTRACT

Purpose The peptide-based delivery system constitutes a potent approach to overcome the limitations of drug delivery *in vitro* and *in vivo*. We recently proposed a novel peptide RDP, which enables brain-targeting delivery of proteins into neuronal cells. Here we investigate the possible internalization mechanism of RDP, and identify the therapeutic effects of functional proteins when linked with RDP in brain disease.

Methods The RDP fusion proteins are produced through recombinant DNA technology, and cell culture is used to investigate the uptake mechanism of RDP and its fusion protein. Experimental Parkinson's disease (PD) model is prepared in mice by intra-striatal injection of 6-hydroxydopamine, and is tested by apomorphine- and amphetamine-induced rotation.

Results The results suggest that the possible route for RDP cellular uptake might involve GABA receptor-dependent, clathrin-mediated endocytosis pathway. Additionally, the conjugate of RDP and glial cell-derived neurotrophic factor (GDNF) exhibits the neuroprotective effect in experimental PD animals, including reduction of apomorphine- and amphetamine-induced rotation following toxin administration.

Conclusions RDP may become an effective tool for the targeted delivery of proteins into brain for disease treatment.

KEY WORDS Brain-targeting delivery · Internalization mechanism · Parkinson's disease · RDP

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INTRODUCTION

The poor permeability of the blood-brain barrier (BBB) of mammalian to drugs is a major barrier for the development of therapeutic molecules for brain diseases (1). BBB is composed of specialized endothelial cells with tight junctions, and prevents the passage of more than 98% of small-molecular weight drugs and almost 100% of all macromolecular neurotherapeutic drugs into brain parenchyma (2,3). To overcome this barrier, considerable efforts have been made in the past years to design new approaches and technologies.

The use of biological active proteins as therapeutic agents constitutes a very promising approach for the treatment of brain diseases. However, the transvascular delivery of these large molecules into brain remains a challenge since they can not cross BBB. Though different types of lipid- and polymer-based vectors have been used for protein delivery, including liposomes, microparticles and nanoparticles, most of them have relatively poor efficiency (4,5).

Peptide-based delivery system is an alternative for protein delivery, partly because of the advantages of peptide, including biodegradability, biocompatibility, low toxicity and ease of synthesis (6). For example, cell-permeable peptides (CPPs) have been shown to mediate numbers of proteins into various tissues, including brain (7,8). CPPs provide a means for delivery of exogenous proteins, but they can not deliver them with cell specificity since CPPs are effective in a very large number of different cell types (9,10). This limitation contributes to one of the major drawbacks of CPPs, and hampers the most promising applications of CPPs.

We recently proposed a novel peptide, RDP, consisting of 39 amino acid residues (KSVRTWNEIIPSK GCLRVGGRCHPHVNGGGRRRRRRRRR), for efficient and targeted delivery of proteins into brain cells following systemic administration (11). RDP is a peptide

derived from rabies virus glycoprotein (RVG), the only protein component that interacts specifically to enable viral entry into neuronal cells (12). In this study, we demonstrated the possible mechanism of cellular uptake of RDP by neuronal cells, and confirmed the targeted delivery and pharmacological activity of biological active proteins as conjugated with RDP. The results suggested that the mechanism of selective cellular uptake of RDP might be mediated by GABA receptor-dependent, clathrin-mediated endocytosis, and the fusion protein of RDP and glial cell-derived neurotrophic factor (GDNF) exhibited significant therapeutic effect in experimental Parkinson's disease (PD) animals following intravenous injection.

MATERIALS AND METHODS

Construction of Recombinant Plasmid and Expression of Fusion Protein

The plasmid pET28b (+) (invitrogen) was used as the expression vector. The nucleotide acids of RDP and linker (GGGSGGGS) were inserted into the pET28b to construct the plasmid pET/RDP. Subsequently, enhanced green fluorescent protein (EGFP) gene or human GDNF gene was cloned into the pET/RDP to construct the recombinant plasmid pET-RDP-EGFP or pET-RDP-GDNF. As controls, we also constructed the pET-EGFP and pET-GDNF plasmid. The nucleotide acid sequences of the plasmids were analyzed for their accuracy in Invitrogen Biotech. Co. LTD. (Shanghai, China). The plasmids were transformed into the *E. Coli.* BL21 strain, then induced by 1 mmol/L IPTG to express the fusion proteins. After the cells were harvested and sonicated, the proteins were purified by Ni-NTA resin column (Amersham) and analyzed by SDS-PAGE.

Cell Culture

Chinese hamster ovary (CHO) cells, Hela (Human cervic carcinoma) cells, and human neuroblastoma SH-SY5Y cells were obtained from Beijing Dingguo Changsheng Biotech. Co. Ltd., China. These cells were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml Streptomycin. All media were obtained from Gibco. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2.

Fluorescence Analysis

N-terminal rhodamine B labeled RDP and its scrambled control peptide (RSP; GRGGRNVRHPRHCRGGVRLC GRKSRPIRIENRWTRVRSK) were synthesized by Chinapeptides Co. Ltd. using standard solid-phase FMOC method, and the peptides were purified to >99% by high-performance liquid chromatography. The RDP, RSP and RDP-EGFP were dissolved in sterile saline as concentrations of 80 μM. In the presence of 400 μl DMEM and 10% FCS, the RDP, RSP and RDP-EGFP (10 μl) were added respectively to CHO, Hela and SH-SY5Y cells of 60% confluency in a 24-well plate. After incubated for 30 min, the cells were visualized under a fluorescent microscope (Olympus, Japan), and the fluorescences of EGFP and rhodamine were photographed with an attached camera.

To examine the mechanism of cellular uptake of RDP and RDP-EGFP, colchicine, acetylcholine (Ach), γ-aminobutyric acid (GABA) or chlorpromazine (Sigma) of different concentrations were added to the SH-SY5Y cells. After 15 min, RDP or RDP-EGFP (10 μl) was added into the DMEM for 30 min, and then the fluorescence images were obtained with excitation wavelength of 470 nm for EGFP (emission wavelength 510 nm), and 550 nm for rhodamine (emission wavelength 610 nm) under the fluorescent microscope.

Animals

Healthy male mice, C57BL/6 J species, weighing 25–30 g, were used in the animal model of PD. Healthy male null mice (weighing 20–25 g) were used for *in vivo* imaging analysis. Both species were purchased from the Department of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, Beijing. Animals were maintained under standard housing conditions with *ad libitum* access to standard laboratory mouse chow and water. All animal experiments were carried out in accordance with guidelines evaluated and approved by the Animal Committee of Southwest University, China.

In Vivo Imaging Analysis

Rhodamine labeled RDP, RDP-EGFP and their respective control, rhodamine and EGFP, were injected respectively into the tail veins of nude mice at the dose of 1.0 mg/kg body weight. The mice were anesthetized by isofluorane 30 min following injection. After the mice were placed in the cassette of *In-Vivo* Imaging System FX Pro (Carestream, USA), images were captured in X-ray and fluorescence, and then overlaid according to the protocol of the manufacturer (Carestream, USA). The fluorescence images were taken at excitation wavelength of 470 nm for EGFP (emission wavelength 535 nm), and 530 nm for rhodamine (emission wavelength 610 nm).

Western Blot Analysis

RDP-GDNF in saline (1.0 mg/kg body weight) was injected into mouse tail veins. The mice were euthanized at 0.5, 1, 3,

5 h after injection. The livers, kidneys and cerebra of mice were removed and frozen immediately in liquid nitrogen. The western blot was performed according to previous reports (13,14). Briefly, the tissues of mice were homogenized in lysis buffer, then the supernatant fluids were collected, and samples were subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in blocking solution for 2 h at room temperature, then immersed in goat anti-GDNF monoclonal antibody (Santa Cruz) overnight at 4°C. Membranes were washed in wash buffer and incubated for 2 h with HRP-conjugated secondary antibody, rabbit anti-goat IgG (1:5000; Dingguo Changsheng Biotech, Co. Ltd., Beijing, China). After washing twice for 15 min each in wash buffer, the signal was detected by the ECL system (Pierce). A western blot of GADPH was performed in the same way, using a monoclonal GADPH antibody (Kangcheng Biotech. Co. Shanghai, China.) as the first antibody, and a goat anti-mouse antibody as the second antibody.

Immunofluorescence Staining of GDNF

The method of immunofluorescence staining was described previously (15). The mice were intravenously injected RDP (synthesized by Chinapeptides Co. Ltd., purity >99%), GDNF and RDP-GDNF (1.0 mg/kg), respectively. At 30 min after injection, the mice were euthanized, and the brains were dissectioned. The coronal sections of brains were cut on a cryostat microtome. The slices were incubated in 10% normal goat serum, subsequently in human GDNF monoclonal antibody (1:1000; Santa Cruz) and FITC-labeled goat anti-human IgG (Beijing Boaoshen Biotech. Co., China). The PBS was used to wash the slices before each addition. All immunostaining sections were observed with the fluorescence microscope and were photographed.

Preparation of PD Model and Treatment

The mice were anesthetized with pentobarbital (30 mg/kg), and received a unilateral intra-cerebral injection of a total of 12 μg of 6-hydroxydopamine · HBr (6-OHDA, Sigma) dissolved in 0.02% ascorbic acid in 0.9% saline. The 6-hydroxydopamine (6 μg in 2 μL) was microinjected into the right striatum at two locations as described previously (16,17). Mice were treated intravenously with saline, RDP, GDNF or RDP-GDNF (1.0 mg/kg) at the day 2 after toxin injection, followed by once daily for 2 weeks. Ten mice were used in each group.

Behavioral Tests

Beginning 1 week after the 6-OHDA injection, mice were tested weekly for apomorphine- and amphetamine-induced rotation, which was performed on separate days. For the apomorphine testing, mice were subcutaneously injected apomorphine (0.6 mg/kg). Full (360°) and contralateral rotations only were counted over 20 min, starting 5 min after administration. For the amphetamine testing, mice were intraperitoneally injected amphetamine (2.5 mg/kg). Full (360°) and ipsilateral rotations only were counted over 20 min, starting 5 min after administration.

A vibrissae-elicited forelimb placing trial was carried out as described previously for mice (17,18). Each session included 120 trials (60 left side and 60 right side), in which a forelimb motor response to ipsilateral facial whisker stimulation was scored. In trials scored as a "3," paw pads made full contact with table top. In trials scored as a "2," paw pads do not make contact with the table. In trials scored as a "1" the limb moves forward only. In trials scored as a "0," the limb does not move.

Tyrosine Hydroxylase Activity Measurement

Tyrosine hydroxylase (TH) activity in mouse brain frontal cortex and in striatum (left and right side) was measured with [3,5-^{3}H]-L-tyrosine as substrate; the labelled [^{3}H]-water and [^{3}H]-L-DOPA were assayed with a charcoal separation technique, as described previously (17,18). Briefly, left and right striatum, and frontal cortex were removed and homogenized in phosphate buffer (pH = 6.2). The homogenates were centrifuged and the supernatant fluids were stored at −20°C before use. The buffer composition of TH activity assay included NADPH, (6R)-5,6,7,8-tetrahydrobiopterin, bovine liver catalase, $Fe(NH_4)_2(SO_4)_2$, L-tyrosine (Sigma), and ^{3}H-L-tyrosine (Perkin Elmer) in phosphate buffer. TH enzyme activity was expressed as pmol/h/mg protein.

Statistical Analysis

All data were given as mean ± S.E.M. For comparison between three groups a one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test was performed. A p value of <0.05 was considered to be statistically significant.

RESULTS

RDP and RDP-EGFP Specifically Entered Neuronal Cells

RDP-EGFP was produced in pET expression vector *in vitro*. SDS-PAGE analysis showed that the expression band of RDP-EGFP was about 30 kDa (Fig. 1a), which was consistent with its theoretical molecular weight (32.7 kDa).

To identify the cell specificity of RDP delivery, three different cell lines, CHO, Hela and SH-SY5Y cells, were used in the study. The results showed that strong fluorescence was only

observed in SH-SY5Y cells following rhodamine labeled RDP or RDP-EGFP treatment, suggesting that RDP and RDP-EGFP specifically entered neuronal cells (Fig. 1b). However, no fluorescence was detected after rhodamine labeled RSP was added in all tested cells, indicating that amino acid sequence of RDP was essential for cell transport. Moreover, cellular distributions of RDP and RDP-EGFP in SH-SY5Y cells were examined, and the results indicated that rhodamine labeled RDP was located in the whole cells, while RDP-EGFP was only distributed in the cytoplasm.

The time course of RDP-EGFP in SH-SY5Y cells showed that RDP-EGFP rapidly entered the cells within 10 min, and lasted for at least 2 days (Fig. 1c). Additionally, all cells in the vision field showed green fluorescence following RDP-EGFP treatment, implying that RDP might deliver EGFP into 100% cells.

We also examined the ability of RDP injected intravenously to cross the BBB and enter brain cells. Mice were injected with fluorescence labeled RDP or RDP-EGFP and, 30 min later, fluorescence intensities were examined by *in vivo* imaging system. The results showed that the fluorescence obviously accumulated in the brains of the mice treated with RDP and RDP-EGFP (Fig. 1d), while there was no significant fluorescence in the brains of the rhodamine and EGFP treated mice, indicating that the RDP peptide could cross the BBB and enter the brain cells. In addition, since fluorescence accumulation in the lung was detected in peptide/protein (RDP, RDP-EGFP and EGFP) treated mice, we assume that the exogenous peptides/proteins transport across alveolar epithelium might opt for nonspecific fluid phase endocytosis or other paracellular routes of lung, such as passive diffusion (19).

RDP Entered Neuronal Cells by an Energy-Dependent Mechanism

Cell energy status plays an essential role in efficient internalization of some peptides, including CPPs (20–22). Thus, we tested whether RDP was internalized in an energy-dependent manner. To address this question, the impact of temperature was evaluated on RDP transport efficiency. SH-SY5Y cells were incubated with RDP or RDP-EGFP at 37°C or 4°C, for 30 min, the transport efficiency was observed using fluorescence microscopy. The results showed that the fluorescence intensities in RDP and RDP-EGFP treated cells were increased in a concentration-dependent manner at 37°C, but only weak fluorescence appeared at 4°C when the concentration of RDP or RDP-EGFP arrived at 2 μM (Fig. 2), suggesting that RDP could efficiently enter metabolically active cells.

RDP Entered Cells Dependent on Endocytosis

Endocytosis serves as an important energy-dependent mechanism by which cells internalize macromolecules (23).

Fig. 1 RDP specifically enter neuronal cells *in vitro* and *in vivo*. (**a**) SDS-PAGE of the expressed and purified RDP-EGFP fusion protein. Lane 1, protein molecular weight markers; lanes 2, cell lysate of induced *E. coli* harboring pRDP-EGFP; lane 3, purified RDP-EGFP; lane 4, uninduced *E. coli*.; lane 5, uninduced *E. coli* harboring pRDP-EGFP. (**b**) SH-SY5Y, CHO and HeLa cells were respectively incubated with rhodamine labeled RDP and RSP, or RDP-EGFP (2 μM). Scale bar 20 μm. (**c**) The time course of RDP-EGFP in SH-SY5Y cells. RDP-EGFP were transported into 100% of cells of a given cell culture population. (**d**) Specific brain-targeting of the RDP and RDP-EGFP *in vivo*. Images were taken at 30 min after intravenous injection.

Here we used a common endocytosis inhibitor, colchicine, to examine whether RDP enter neuronal cells through endocytosis pathway. SH-SY5Y cells were incubated with 2 μM fluorescently labeled RDP or RDP-EGFP, in the presence of the high and low concentrations of colchicine. The results showed that colchicine dramatically prevented the internalization of both RDP and RDP-EGFP (Fig. 3), and the cells treated with high concentration of colchicine (40 μM) exhibited weaker fluorescence intensity than that of low concentration (10 μM), indicating that the cellular internalization of RDP and its conjugate RDP-EGFP may be dependent on endocytosis pathway.

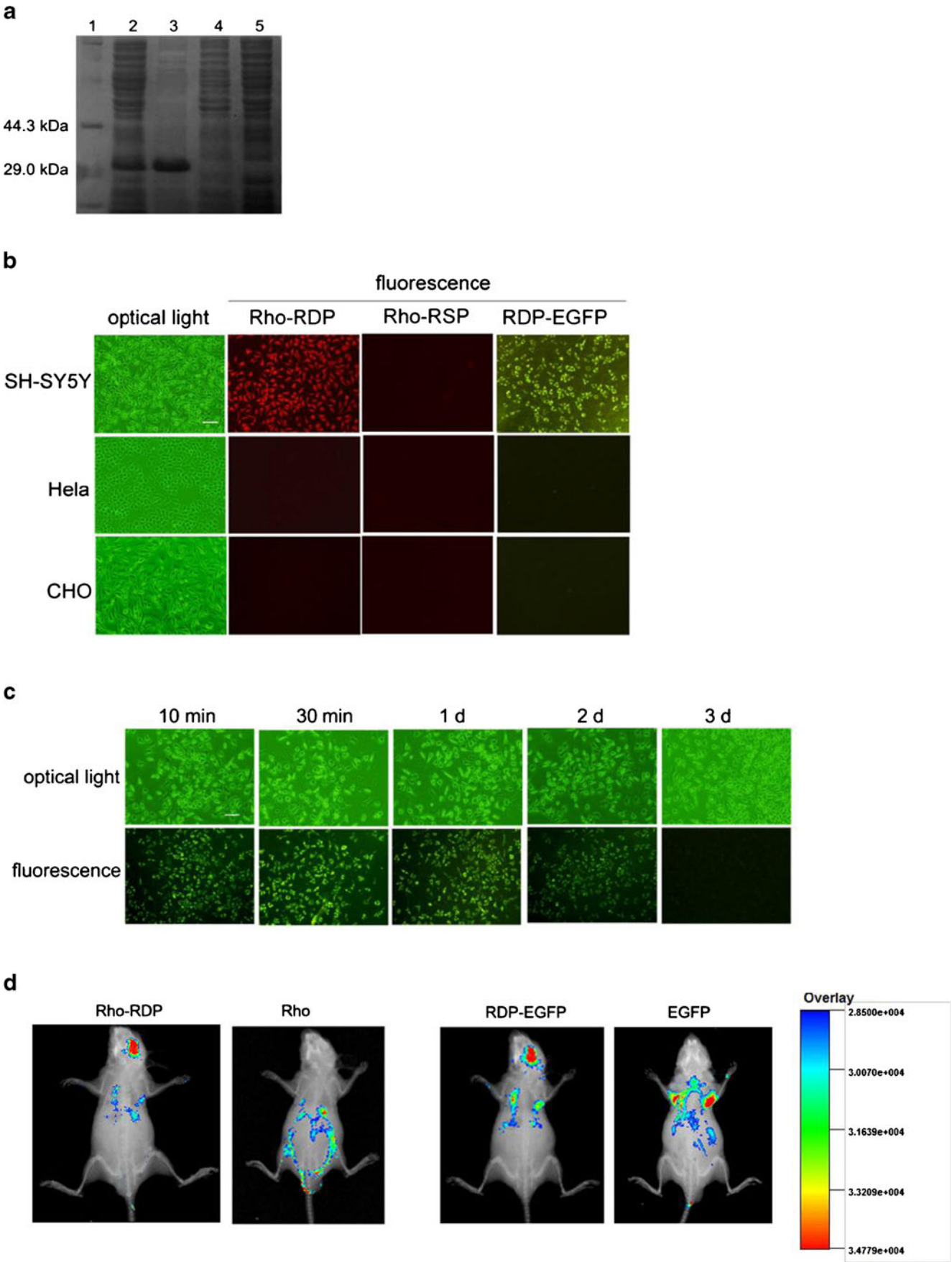
RDP-EGFP Prevented Cellular Uptake of RDP

To test the interaction between RDP and RDP-EGFP during internalization process, fluorescently labeled RDP and RDP-EGFP were co-incubated in SH-SY5Y cells. The results showed that green fluorescence intensities of RDP-EGFP had no significant change when the cells were co-treated with fluorescence labeled RDP, while red fluorescence of rhodamine labeled RDP became weak as co-incubation with RDP-EGFP (Figs. 4 and 2), suggesting that RDP-EGFP might competitively inhibit the cellular uptake of RDP.

GABA Prevented Internalization of RDP and RDP-EGFP

Because RDP peptide was derived from RVG, the only protein component by which virus interacts specifically with the nicotinic acetylcholine receptor (AchR) of neuronal cells (24). We therefore tested whether AchR mediated the internalization of RDP and RDP-EGFP. Ach, the non-selective AchR agonist, was added and co-incubated with RDP or RDP-EGFP. As shown in Fig. 5a, the presence of either high or low concentration of Ach (100 μM or 400 μM), during the treatment of SH-SY5Y cells with RDP or RDP-EGFP, had no significant effect on the fluorescence intensity. The results indicated that the internalization of RDP and RDP-EGFP might be irrelevant to AchR.

Because GABA is a major neurotransmitter which is widely distributed throughout the central nervous system (CNS), and its receptor or transporter is also widely expressed in brain and


a
1 2 3 4 5
44.3 kDa
29.0 kDa
b
fluorescence
optical light
Rho-RDP
Rho-RSP
RDP-EGFP
SH-SY5Y
Hela
CHO
c
10 min
30 min
1 d
2 d
3 d
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d
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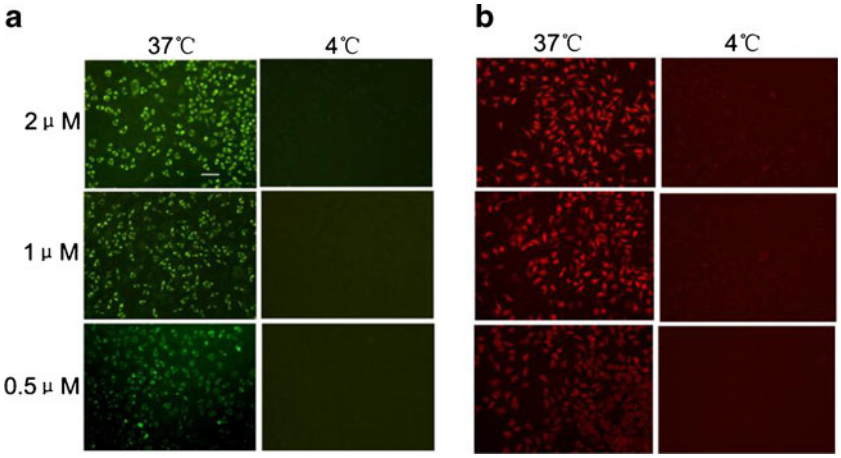

Fig. 2 RDP enters neuronal cells by an energy-dependent mechanism. SH-SY5Y cells were incubated with 10 μM of RDP or RDP-EGFP at 37°C or 4°C, for 30 min, and the transport efficiency was observed using fluorescence microscopy. Scale bar 20 μm.

endothelial cells of brain capillaries (25), we examined whether GABA could affect the internalization of RDP. The results showed that the cells co-treated with GABA resulted in dramatic decreases of fluorescent intensity (Fig. 5b), and high concentration of GABA completely inhibited the cellular uptake of RDP and RDP-EGFP, indicating that the GABA receptor might mediate RDP and its conjugate in cell entry.

Since GABA enters cells by clathrin-mediated endocytotic pathway, we further tested whether the clathrin-mediated endocytotic inhibitor, chlorpromazine, affected the internalization of RDP and RDP-EGFP. The results showed that strong reductions of cellular uptake of RDP and RDP-EGFP were observed after the cells were treated with chlorpromazine (Fig. 5c), suggesting that RDP entered cells probably through GABA receptor-dependent, clathrin-mediated endocytotic pathway.

RDP Peptide Delivers GDNF into Brain

To confirm the ability of brain-targeting delivery of RDP, we tested whether RDP could deliver a potential therapeutic protein, GDNF, into brain. SDS-PAGE showed that the expression band of RDP-GDNF was about 20 kDa (Fig. 6a), which is similar to its theoretical molecular weight (20.8 kDa).

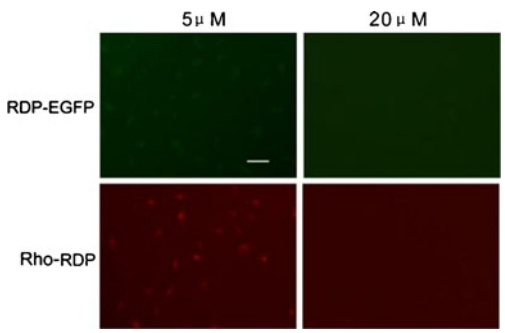

Fig. 3 Cellular uptake of RDP and RDP-EGFP in the presence of endocytosis inhibitor. SH-SY5Y cells were treated with different concentrations of colchicine (10 μM and 40 μM). Scale bar 20 μm.

Western blot analysis and immunofluorescence staining were used to examine the biodistribution and level of GDNF *in vivo*. The results of western blot showed that GDNF levels in the mouse brains were significantly increased at 0.5 h after RDP-GDNF administration, and then gradually decreased to the base level (the endogenous level of mouse brain) at about 3 h after injection (Fig. 6b). The slight signals were obtained in the livers at 0.5 and 1 h, whereas no signal was detected in the kidneys.

Additionally, the result of immunofluorescence staining confirmed the ability of RDP in brain delivery. A number of immunopositive cells were detected in the brains of RDP-GDNF treated mice, and the fluorescence appeared mainly in the cytoplasm of the GDNF-positive cells, whereas there were few fluorescence positive cells in the control groups (Fig. 6c). These results indicated that RDP enabled the intravenous delivery of protein into neuronal cells following systemic administration.

The Therapeutic Effect of RDP-GDNF in Experimental PD Mice

Experimental PD model was induced in mice by intra-striatal injection of 6-OHDA. Mice were tested for apomorphine-

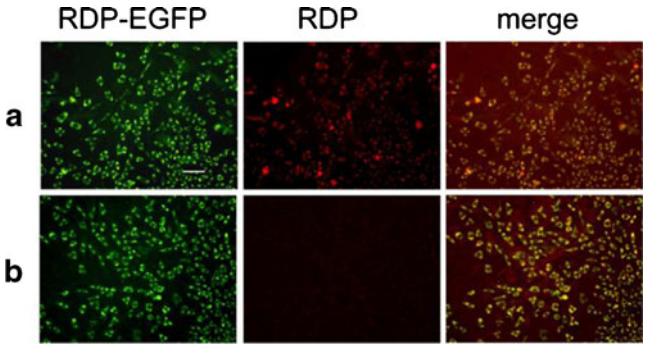

Fig. 4 RDP prevents the cellular uptake of RDP-EGFP. SH-SY5Y cells were co-treated with both RDP-EGFP (2 μM) and (**a**) RDP (1 μM), or (**b**) RDP (0.5 μM). Scale bar 20 μm.

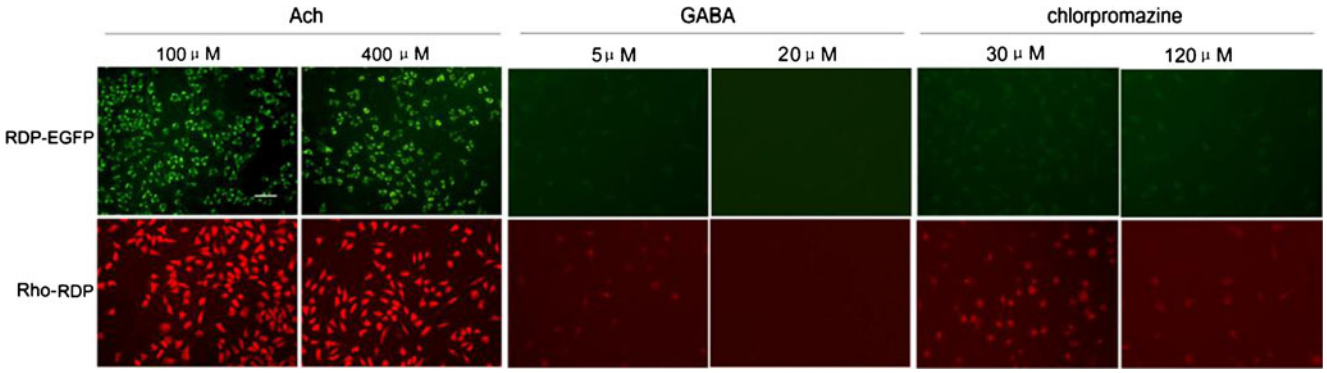

Fig. 5 Cellular uptake of RDP and RDP-EGFP in the presence of different concentrations of Ach, GABA or chlorpromazine. Scale bar 20 μm.

and amphetamine-induced rotation at 1, 2, and 3 weeks after toxin administration. The results were shown in Fig. 7. The mice treated with RDP and GDNF exhibited an increase in apomorphine- and amphetamine-induced rotation at 1–3 weeks after toxin injection (Fig. 7a and b). However, the mice treated with the RDP-GDNF showed significant decreases in both apomorphine- and amphetamine-induced rotation compared to the RDP or GDNF group, suggesting that the mice in the RDP-GDNF group had a marked improvement in induced rotation behaviors.

At the end of the study, the mice were evaluated with the vibrissae-elicited forelimb placing test to identity the pharmacologic effect of the RDP-GDNF therapy. The unilateral 6-OHDA lesioned mouse failed to place the forelimb contralateral to the lesion. The results showed that all of mice in the different groups had maximal placing scores on the right side, which is ipsilateral to the side of toxin injection (Fig. 7c). The mice treated with RDP and GDNF showed a reduction in placing score on the lesioned side, while the mice treated with RDP-GDNF showed a significant increase in placing score, relative to the saline treated mice, on the lesioned side (Fig. 7c).

TH is a marker enzyme of dopaminergic neuron. Our results indicated that the TH activity in the cortex or left (non-lesioned) striatum of RDP-GDNF treated mice was not significantly different from that of saline treated mice (Fig. 7d), whereas the RDP-GDNF treatment caused about 4-fold increase in the TH activity of the right (lesioned) striatum compared to that of saline treated mice, suggesting that the function of dopaminergic neuron was recovered in PD model mice after RDP-GDNF administration.

DISCUSSION

Targeted delivery of drug molecules to brain is one of the most challenging research areas in pharmaceutical sciences (26,27). In the study, we identified the brain-targeting effect and possible internalization mechanism of RDP. The results also suggested that RDP had the capability of penetration within 100% of cells of a given cell culture population, and proved the therapeutic effect of biological active proteins as conjugated with RDP in brain diseases following systemic administration. The study provides an effective approach for targeted delivery of proteins into neuronal cells *in vitro* and *in vivo*.

Understanding cellular uptake of molecules remains an important task in order to improve their potency or promote their *in vivo* application. In the present study, we firstly examined the cell specificity of RDP delivery, then investigated the internalization mechanism of RDP using fluorescently labeled-RDP and RDP-EGFP in live cells. The concentration of RDP was selected according to previous studies of CPPs (28,29). The uptake process showed a temperature-depending manner as the fluorescence intensity decreased significantly at 4°C, the cellular transport of RDP was blocked by endocytosis inhibitor, as well as the conjugate of RDP and EGFP clearly influences the cellular uptake of RDP, suggesting that the energy-depending mechanism involved active endocytosis and competitive inhibition character. Also, the studies revealed that though RDP enabled proteins into the neuronal cells, the cellular location seemed dependent on the protein delivered. RDP was distributed in the whole cells, while RDP-EGFP and RDP-GDNF were located in the cytoplasm.

Receptor-mediated endocytosis is one of the major mechanisms by which macromolecules cross biological membranes. In previous studies, a 29 amino-acid peptide, derived from the 189–214 amino acid sequences of RVG (RVG29-d9R, YTIWMPENPRPGTPCDIFTNSRGKRASNGGGG(d)R RRRRR RRR), enables siRNA and cationic-polymers specifically to enter neuronal cells, probably through nicotinic AchR-mediated transcytosis (30–32). Besides 189–214 amino acid sequences, the analysis reveals that the 330–357 amino acid sequences is another important nerve binding region of RVG (33,34). Since part of sequences of RDP was originated from the 330–358 amino acid sequences of RVG, we assumed that AchR might mediate the internalization of RDP. However, the results showed that the cellular internalization of RDP was not affected

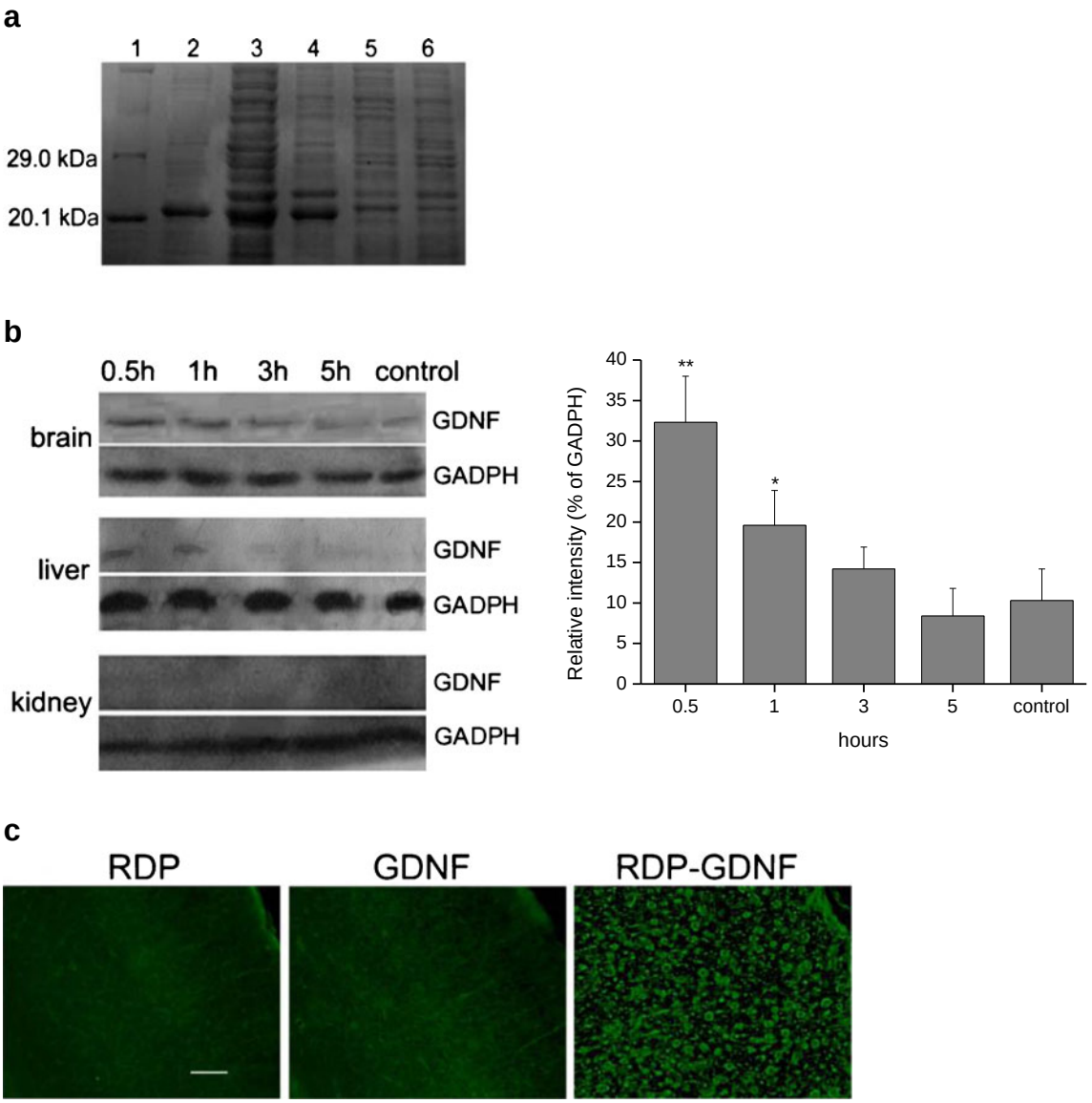

Fig. 6 Targeted delivery of RDP-GDNF into mouse brain after intravenous injection. (**a**) SDS-PAGE for RDP-GDNF fusion protein. Lane 1, protein molecular weight markers; lanes 2, purified RDP-GDNF; lane 3, cell lysate of induced *E. coli* harboring pRDP-GDNF; lane 4, supernatant of cell lysate of induced *E. coli* harboring RDP-GDNF; lane 5, uninduced *E. coli* harboring pRDP-GDNF; lane 6, uninduced *E. coli*. (**b**) Western blot analysis of the GDNF in brain, kidney and liver of mice. GDNF levels were detected on 0.5 h. 1 h, 3 h, 5 h after injection. Data are mean ± SEM ($n = 3$ mice/group). *$p < 0.05$, **$p < 0.01$ compared to the control. (**c**) Representative photographs of GDNF immunostraining in cerebral cortex. Scale bar 100 μm. The mice were respectively injected RDP, GDNF and RDP-GDNF (three mice in per group) and were euthanized at 0.5 h after injection. The results clearly showed that RDP mediated the GDNF selectively into the brain, while the control protein GDNF did not cross the BBB when delivered in the same manner.

by different concentration of Ach, implying that the binding site of Ach might be different from that of RDP.

GABA is a common neurotransmitter whose receptors are expressed in all major brain structures and brain capillary endothelial cells (25,35). Both GABA type A (GABAA) receptors and nicotinic AchR belong to the superfamily of ligand-gated ion channel, and clathrin-dependent endocytosis is their common internalization mechanism when ligands enter cells (36,37). In the study, the specific cellular uptake of RDP was inhibited by GABA and chlorpromazine (clathrin-mediated endocytotic inhibitor), suggesting that RDP, derived from RVG, might interact with GABA receptor, then enter cells by clathrin-mediated endocytosis pathway.

GDNF, a member of the transforming growth factor-β superfamily, is a potent neurotrophic factor that promotes the survival and morphological differentiation of dopaminergic neurons and motor neurons (38,39). However, GDNF molecule is a protein that does not readily pass BBB. Although the neuroprotective effects of GDNF have been established in PD animal models by intracerebral microinjection (40,41),

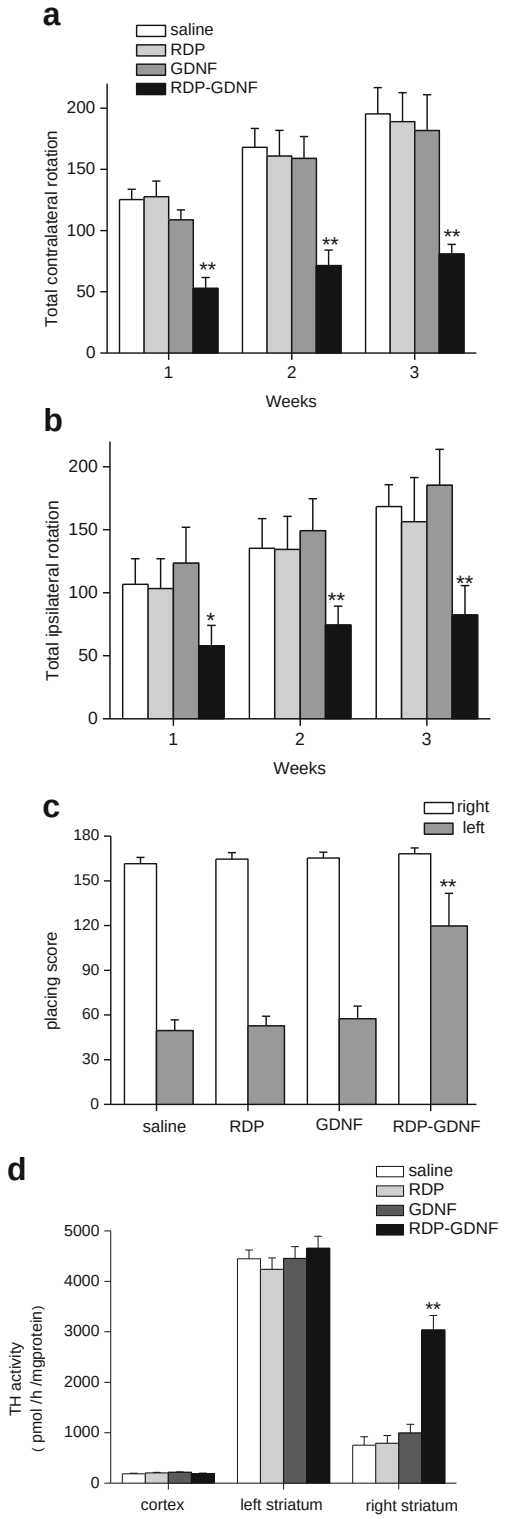

Fig. 7 Therapeutic effect of RDP-GDNF on experimental PD mice. Rotation measurements following the administration of either apomorphine (**a**) or amphetamine (**b**) for PD mice treated with saline, RDP, GDNF or RDP-GDNF. (**c**) Vibrissae-elicited forelimb placing test at 3 weeks following toxin injection. Neurologic deficit is recovered in RDP-BDNF treated mice. (**d**) TH activity in mouse cortex, left and right striatum. Brain TH activity was measured at 3 weeks after toxin administration. Data are mean ± SEM ($n = 10$ mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the saline group.

this method results only in local diffusion around the injection site, also it's invasive and unadapted for human therapy.

The use of peptide as vector may be a safe alternative for GDNF delivery. It's reported that an 11-amino-acid CPP of Tat delivers the rat GDNF mature protein across the BBB in vivo (42,43). However, Tat-GDNF did not provide neuroprotection of dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) induced subchronic PD model mice (43). The possible cause for the result might be that Tat-CPP is lack of cell specificity and can enter almost all types of cells, which induces lower concentration of protein in brain than in other tissues at even large injection dose (9,44). Contrary to CPP, RDP exhibits high cell specificity, and selectively delivers proteins into brain. RDP-GDNF fusion protein showed biological activity in the brain and produced neuroprotection in the experimental PD model. Moreover, since repeated administration of RDP-GDNF fusion protein for 14 days did not show obvious toxic reactions in mice, this approach might be a potential new treatment for PD.

CONCLUSION

The study suggests that RDP is a novel peptide that enables proteins targeted-delivery into neuronal cells both *in vitro* and *in vivo*. Also, the conjugates of RDP and neurotrophic proteins show neuroprotective function in brain diseases, including PD. The mechanism of RDP in cell specificity might be characterized as clathrin-mediated endocytosis and probably relating to the GABA receptor. Although the mechanisms of efficient delivery of RDP are not fully understood and under investigation, we believe that RDP holds great promises in noninvasive, efficient and brain-targeting molecule delivery.

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REFERENCES

1. Luissint AC, Artus C, Glacial F, Ganeshamoorthy K, Couraud PO. Tight junctions at the blood brain barrier: physiological architecture and disease-associated dysregulation. Fluids Barriers CNS. 2012;9(1):23.
2. Lossinsky AS, Shivers RR. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Histol Histopathol. 2004;19(2):535–64.
3. Pardridge WM. Biopharmaceutical drug targeting to the brain. J Drug Target. 2010;18(3):157–67.

4. Trabulo S, Cardoso AL, Mano M, de Lima MCP. Cell-penetrating Peptides—mechanisms of cellular uptake and generation of delivery systems. Pharmceuticals. 2010;3:961–93.
5. Tan ML, Choong PF, Dass CR. Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. Peptide. 2009;31(1):184–93.
6. Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol. 2009;157(2):195–206.
7. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. *In vivo* protein transduction: delivery of a biologically active protein into the mouse. Science. 1999;285(5433):1569–72.
8. Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev. 2005;57(4):637–51.
9. Martín I, Teixidó M, Giralt E. Building cell selectivity into CPP-mediated strategies. Pharmceuticals. 2010;3:1456–90.
10. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, *et al.* Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem. 2003;278(1):585–90.
11. Fu A, Wang Y, Zhan L, Zhou R. Targeted delivery of proteins into the central nervous system mediated by Rabies Virus glycoprotein-derived peptide. Pharm Res. 2012;29(6):1562–9.
12. Tuffereau C, Benejean J, Alfonso AR, Flamand A, Fishman AC. Neuronal cell surface molecules mediate specific binding to rabies virus glycoprotein expressed by a recombinant baculovirus on the surfaces of lepidopteran cells. J Virol. 1998;72:1085–91.
13. Fu AL, Fan L, Li S, Manji S. Role of extracellular signal-regulated kinase signal transduction pathway in anxiety. J Psychiatr Res. 2008;43(1):55–63.
14. Fu AL, Wu SP, Dong ZH, Sun MJ. A novel therapeutic approach to depression via supplement with tyrosine hydroxylase. Biochem Bioph Res Co. 2006;351(1):140–5.
15. Fu AL, Zhou CY, Chen X. Thyroid hormone prevents cognitive deficit in a mouse model of Alzheimer's disease. Neuropharmacology. 2010;58(4–5):722–9.
16. Alvarez-Fischer D, Henze C, Strenzke C, Westrich J, Ferger B, Höglinger GU, *et al.* Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and alpha-synuclein-deleted mice. Exp Neurol. 2008;210(1):182–93.
17. Fu AL, Zhou QH, Hui EK, Lu JZ, Boado RJ, Pardridag WM. Intravenous treatment of experimental Parkinson's disease in the mouse with an IgG-GDNF fusion protein that penetrates the blood-brain barrier. Brain Res. 2010;1352:208–13.
18. Zhang Y, Pardridge WM. Near complete rescue of experimental Parkinson's disease with intravenous, non-viral GDNF gene therapy. Pharm Res. 2009;26(5):1059–63.
19. Kim K, Malik AB. Protein transport across the lung epithelial barrier. Am J Physiol Lung Cell Mol Physiol. 2003;284(2):L247–59.
20. Rydström A, Deshayes S, Konate K, Crombez L, Padari K, Boukhaddaoui H, *et al.* Direct translocation as major cellular uptake for CADY self-assembling peptide-based nanoparticles. PLoS One. 2011;6(10):e25924.
21. Zhang X, Jin Y, Plummer MR, Pooyan S, Gunaseelan S, Sinko PJ. Endocytosis and membrane potential are required for HeLa cell uptake of R.I.-CKTat9, a retro-inverso Tat cell penetrating peptide. Mol Pharm. 2009;6(3):836–48.
22. Mussbach F, Franke M, Zoch A, Schaefer B, Reissmann S. Transduction of peptides and proteins into live cells by cell penetrating peptides. J Cell Biochem. 2011;112(12):3824–33.
23. Mellman I. Endocytosis and molecular sorting. Annu Rev Cell Dev Biol. 1996;12:575–625.
24. Tang Q, Orciari LA, Rupprechti CE, Zhao XQ, Li ZG, Yang WS. Sequencing and position analysis of the glycoprotein gene of four Chinese rabies viruses. Virol Sin. 2000;15(1):22–33.
25. Young AB, Chu D. Distribution of GABA, and GABA, receptors in mammalian brain: potential targets for drug development. Drug Develop Res. 1990;21(3):161–7.
26. Guo L, Ren J, Jiang X. Perspectives on brain-targeting drug delivery systems. Curr Pharm Biotechnol. 2012;13(12):2310–8.
27. Ambikanandan M, Ganesh S, Aliasgar S. Drug delivery to the central nervous system: a review. J Pharm Pharmaceut Sci. 2003;6(2):252–73.
28. Keller A, Mussbach F, Breitling R, Hemmerich P, Schaefer B, Lorkowski S, *et al.* Relationships between cargo, cell penetrating peptides and cell type for uptake of non-covalent complexes into live cells. Pharmaceuticals. 2013;6:184–203.
29. Ma D, Qi X. Comparison of mechanisms and cellular uptake of cell-penetrating peptide on different cell lines. Acta Pharmacol Sin. 2010;45(9):1165–9.
30. Kumar P, Wu H, McBride JL, Jung KE, Kim MH, Davidson BL, *et al.* Transvascular delivery of small interfering RNA to the central nervous system. Nature. 2007;448(7149):39–43.
31. Hwang do W, Son S, Jang J, Youn H, Lee S, Lee D, *et al.* Brain-targeted rabies virus glycoprotein-disulfide linked PEI nanocarrier for delivery of neurogenic microRNA. Biomaterials. 2011;32(21):4968–75.
32. Liu Y, Huang R, Han L, Ke W, Shao K, Ye L, *et al.* Brain-targeting gene delivery and cellular internalization mechanisms for modified rabies virus glycoprotein RVG29 nanoparticles. Biomaterials. 2009;30(25):4195–202.
33. Tuffereau C, Leblois H, Bénéjean J, Coulon P, Lafay F, Flamand A. Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. Virol. 1989;172(1):206–12.
34. Benmansour A, Leblois H, Coulon C, Tuffereau C, Gaudin Y, Flamand A, *et al.* Antigenicity of rabies virus glycoprotein. J Virol. 1991;65:4198–203.
35. Whiting PJ. GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? Drug Discov Today. 2003;8(10):445–50.
36. Smith KR, Muir J, Rao Y, Browarski M, Gruenig MC, Sheehan DF, *et al.* Stabilization of GABA(A) receptors at endocytic zones is mediated by an AP2 binding motif within the GABA(A) receptor β3 subunit. J Neurosci. 2012;32(7):2485–98.
37. Ulens C, Hogg RC, Celie PH, Bertrand D, Tsetlin V, Smit AB, *et al.* Structural determinants of selective alpha-conotoxin binding to a nicotinic acetylcholine receptor homolog AChBP. Proc Natl Acad Sci USA. 2006;103(10):3615–20.
38. Yasuda T, Mochizuki H. Use of growth factors for the treatment of Parkinson's disease. Expert Rev Neurother. 2010;10(6):915–24.
39. Mickiewicz AL, Kordower JH. GDNF family ligands: a potential future for Parkinson's disease therapy. CNS Neurol Disord Drug Targets. 2011;10(6):703–11.
40. Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, *et al.* Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons *in vivo*. Neurosci Lett. 1994;182(1):107–11.
41. Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, *et al.* Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol. 2006;59(3):459–66.
42. Kilic U, Kilic E, Dietz GP, Bähr M. Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice. Stroke. 2003;34(5):1304–10.
43. Dietz GP, Valbuena PC, Dietz B, Meuer K, Müeller P, Weishaupt JH, *et al.* Application of a blood-brain-barrier-penetrating form of GDNF in a mouse model for Parkinson's disease. Brain Res. 2006;1082(1):61–6.
44. Cai SR, Xu G, Becker-Hapak M, Ma M, Dowdy SF, McLeod HL. The kinetics and tissue distribution of protein transduction in mice. Eur J Pharm Sci. 2006;27(4):311–9.