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RESEARCH PAPER

Delivery of Brain-Derived Neurotrophic Factor via Nose-to-Brain Pathway

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ABSTRACT

Purpose To investigate the plausibility of delivering brain-derived neurotrophic factor (BDNF) to brain via nose-to-brain pathway using chitosan as barrier-modulating agent.

Methods Effect of different viscosity grades chitosan at different concentrations on permeation of fluorescein isothio-cyanate dextran (FD 40 K) across bovine olfactory mucosa was studied using Franz diffusion cells. Medium viscosity chitosan was used to carry out permeation studies of BDNF. Pharmacokinetic and pharmacodynamic studies were carried out in Sprague dawley rats upon intranasal/i.v administration of different formulations.

Results Medium viscosity chitosan more efficiently enhanced permeation of FD 40 K across olfactory mucosa compared to other grades. In case of BDNF, medium viscosity chitosan (0.25% w/v) enhanced permeation ~14-fold over control (18.78 ± 16.69 ng/cm^2). Brain bioavailability of rats administered intranasally with BDNF solution containing chitosan was significantly enhanced ~13-fold compared to rats administered with same concentration of BDNF solution without chitosan. In rats subjected to immobilization stress, BDNF solution containing chitosan significantly decreased immobility time.

Conclusions Intranasal formulations containing chitosan as barrier-modulating agent significantly enhanced brain bioavailability of BDNF. Delivery of BDNF was found to counteract stress-induced depression in rats.

KEY WORDS BDNF· chitosan · nose-brain · pharmacodynamics · pharmacokinetics

INTRODUCTION

Degenerative disorders of the nervous system are caused due to progressive loss of structure and function of neurons. One of the reasons leading to degeneration of neurons is insufficient supply of neurotrophic factors. Hence, the endogenous levels of neurotrophins (NTs) deplete in the brain in case of neurodegenerative disorders (1). Depletion of NTs is known to cause several other central nervous system (CNS) and systemic problems as well (2). The potential treatment approach for CNS disorders caused due to depletion of neurotrophins is to restore the normal neurotrophin levels. Currently followed invasive methods of delivering neurotrophins to the brain do not allow frequent administration (3,4).

Brain-derived neurotrophic factor (BDNF) is one of the most important neurotrophic factors which promotes survival of all major neuronal types affected in neurodegenerative disorders and also responsible for generation of cholinergic neurons in the basal forebrain and hippocampus region (5–7). BDNF is relatively more capable of

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protecting neurons from neurotoxic amyloid peptides in contrast to other members of neurotrophin family (8). BDNF and its receptors are abundantly expressed in the central nervous system, especially in the hippocampus and basal forebrain region (5,9–11). Presence of normal level of BDNF in the brain is very important because of its ability to inhibit the cell death cascades, modulate the plasticity and to increase the cell survival proteins (12–14). Therefore, it seems necessary to target BDNF to hippocampus and basal forebrain region, as prominent neurodegeneration is observed in these regions in case of Alzheimer's, Parkinson's and Huntington's disease.

BDNF depletion has also been found to be responsible for psychiatric disorders particularly chronic depression. Decreased BDNF levels have been reported in various animal models subjected to stress. The regulation of BDNF expression by antidepressant drugs has been demonstrated by many groups (15–18). However, the treatment of chronic depression condition using antidepressant drugs is associated with a long delayed onset of action and prolonged treatment duration (19,20). In addition, the systemic side effects associated with such prolonged antidepressant drug therapy is considerably high. Therefore, delivery of exogenous BDNF directly to the brain would be one of the ways to overcome the limitations of antidepressant drugs while treating the BDNF depletion related disorders.

Various strategies employed for the delivery of BDNF into the brain includes injection or use of minipumps or gene therapy (21,22). Unfortunately, all these methods lack practical clinical relevance due to the invasive nature of administration (23,24). Burke *et al.*, observed that intracerebroventricular injection of BDNF resulted in relatively poor diffusion of BDNF into the brain parenchyma (25). Moreover, BDNF when delivered systemically via parenteral route is not bioavailable to the brain due to metabolism of the peptide. The hydrophilic and macromolecular nature (~28 kDa dimeric protein of non-covalently linked 14 kDa subunits) of the therapeutic agent limits its permeation across biological barriers (27). In addition, the bioavailability is also limited by the blood brain barrier (BBB) and blood cerebrospinal fluid (BCSF) barrier as well (26). Therefore, there is need for an alternative, safe, non invasive route of delivery which bypasses the BBB and delivers the drug to the affected regions of the brain.

Nose-to-brain pathway has been explored by many research groups as a potential alternative route for targeting drugs to brain via the olfactory pathway (28–30). Moreover, this route is more patient compliant than the current modes of administration and allows frequent administration of BDNF, as it has a short elimination half life in brain. However, the delivery of macromolecules via this pathway has been less investigated as this pathway is associated with a major limiting factor, the olfactory epithelial barrier. One of the barrier modulating agents, chitosan was found to improve the delivery of macromolecules by safe and transient permeabilization of the olfactory mucosa (30). In this study we investigated the potential of chitosan in enhancing the brain uptake of BNDF when delivered via intranasal route. These studies also provide new information regarding the pharmacokinetics of exogenously administered BDNF to the brain.

MATERIALS AND METHODS

Materials

BDNF E_{max} ImmunoAssay system and rhBDNF were purchased from Promega Corporation, Madison, WI. Krebs-Ringer bicarbonate buffer (KRB, premixed powder), fluorescein isothio-cyanate dextran of molecular weight 40 kDa (FD40K), chitosan of different grades, low viscosity (20–200 cP), medium viscosity (200–800 cP) and high viscosity (800–2000 cP) with varying percentage of deacetylation content were procured from Sigma chemicals (St. Louis, MO). Freshly excised bovine olfactory epithelium was purchased from Pel-Freez Biologicals (Rogers, AR).

Preparation of Chitosan and FD40K/BDNF Solution

Different grades of chitosan solutions were prepared by dissolving required quantity of chitosan in a fraction of 1% glacial acetic acid solution prepared in Kreb's ringer buffer (pH 5.5). The second fraction of the Kreb's ringer buffer was used to dissolve the FD40K/BDNF. The FD40K/BDNF and chitosan solutions were mixed by vortexing. The solutions prepared without incorporation of chitosan served as control.

In Vitro Permeation Studies

Permeation studies were carried out across bovine olfactory mucosa sandwiched between donor and receiver compartments of vertical Franz diffusion cell (Logan instruments, Somerset, NJ). The tissue was mounted such that the olfactory mucosa side was in contact with the upper donor compartment and the ventral side with the receiver compartment. The active diffusion area was 0.64 cm^2.The temperature of the chamber was regulated at $37 \pm 1°C$ by water circulation. In both the donor and receiver compartments Ag/AgCl electrode wires of 0.5 mm in diameter (obtained from Alfa Aesar, Ward Hill, MA) made in the form of circular rings were placed 2 mm away from the olfactory mucosa. The trans-olfactory epithelial electrical resistance was monitored in presence and absence of

chitosan using an electrical set up consisting of a wave form generator and multimeter (Agilent Technologies, Santa Clara, CA) in order to determine the effect of chitosan on permeability status of the olfactory mucosa. Initially the donor and the receiver compartments were filled with 500 µL and 5 mL KRB, respectively, for electrical resistance measurements.

In order to investigate the effect of different viscosity grades chitosan (low viscosity, medium viscosity and high viscosity) on the permeation of FD 40 K, the donor solution was replaced after equilibration for 30 min with a solution of 100 µL of FD40K (1 mg/mL) with different concentrations of chitosan (0.1% or 0.25% or 0.5% w/v). The receiver compartment was filled with 5 mL KRB buffer. Samples were collected at the end of 2 h from the receiver compartment and the amount of FD 40 K transported across bovine olfactory mucosa was determined by measuring the fluorescence emission intensity at 520 nm by excitation at 494 nm using Perkin Elmer fluorimeter. In control set of experiments chitosan was not incorporated in the donor solution.

In another set of studies the donor compartment was filled with 100 µL of BDNF solution (50 µg/mL prepared in KRB) with medium viscosity grade chitosan of different concentrations (0.1% or 0.25% or 0.5% w/v) and the receiver compartment with 5 mL KRB buffer. Control set of experiments were run without incorporation of chitosan in the donor solution. The amount of BDNF transported across the bovine olfactory mucosa was determined by assaying the samples collected from the receiver compartment at the end of 2 h by ELISA using "BDNF E_{max} ImmunoAssay system" following the protocol supplied by the manufacturer. The sensitivity of the BDNF assay was about 7.8 pg/mL.

Stability of BDNF in Tissue Homogenate

Freshly procured bovine olfactory mucosa was placed in a glass vial and homogenized in KRB buffer. A known concentration of BDNF was spiked into tissue homogenate and incubated at 37°C for 12 h. Before the start of experiment, zero time point sample was taken to know the initial activity of BDNF. Samples were withdrawn at definite intervals of time, diluted accordingly and quantified by ELISA.

In Vivo Brain Microdialysis

The *in vitro* calibration of microdialysis probes (CMA 12, CMA Microdialysis, Sweden) was carried out by the method described by Vaka and Co-workers (30). Brain microdialysis was carried out in male, Sprague dawley rats (250–300 g, Harlan Company, Indianapolis, IN, U.S.A) under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia (i.p. injection). The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi (Protocol # 10–017). The rats were divided into 4 groups ($n=3$). The first 3 groups of rats were laid on their back after anesthetizing and formulations were administered intranasally using a microsyringe connected with a soft polymer capillary directly into the posterior segment of the nose. Chitosan solution (0.25% w/v prepared in KRB buffer was administered intranasally (vehicle control group) to group 1. BDNF solution prepared in KRB (100 µL of 50 µg/mL) without incorporation of chitosan was administered intranasally to group 2, whereas, BDNF solution (100 µL of 50 µg/mL) with 0.25% w/v chitosan prepared in KRB was intranasally administered to group 3. To rats belonging to group 4, BDNF solution (100 µL of 50 µg/mL) with 0.25% w/v chitosan prepared in KRB was administered via i.v route. After administration of formulations to different groups the rats were secured on a stereotaxic frame (Harvard Instruments, Holliston, MA) and brain microdialysis was carried out by inserting the probe into the hippocampal region (anterior-posterior=5.6 mm, medio-lateral=5 mm, dorso-ventral=7 mm, from bregma) (30). The microdialysis probes (CMA 12) were equilibrated by perfusing KRB at the rate of 2 µL/min using a microinjection pump for a period of 1 h. The rats were given maintenance doses of anesthesia [ketamine (40 mg/kg) and xylazine (10 mg/kg)] after 3 h from the start of the study. The microdialysis samples were collected at hourly intervals for 6 h and quantified by ELISA. The pharmacokinetic parameters were calculated using a noncompartmental pharmacokinetic model. The area under the curve was calculated by trapezoidal rule.

Pharmacodynamic Studies

These studies were approved by the institutional animal ethical committee (Protocol # VCP/IAEC/AB23-2010). The rats were divided into 5 groups ($n=6$). The first four groups of rats were subjected to immobilization stress before carrying out the forced swim test by taping the four limbs of rats to a wooden board with two metal loops around the neck for 2 h a day for seven consecutive days. The formulations were administered 2 h prior to study on 8th day as described in previous section (*In Vivo* Brain Microdialysis) by anesthetizing the rats using isoflurane (5%). One more vehicle control group of rats which were not subjected to immobilization stress (Group 5) was included in these studies in order to determine the immobility time in the absence of stress. After the rats were completely recovered from anesthesia they were placed individually in a glass container with water at a depth of 30 cm (23–25°C) for 10 min. The immobility time

of five groups of rats was recorded. Fresh water was used each time and the experiments were performed during the same time of the day. The rats which were floating and making only the necessary movements to keep its nostrils above the water surface were considered immobile.

Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). The unpaired *t*-test/Analysis of Variance with Tukey test was selected as the test of significance and p value less than 0.05 was considered as level of significance. The data points provided in graphs are averages of three trials with error bars representing standard error of mean (SEM).

RESULTS AND DISCUSSION

In Vitro Permeation Studies Across Bovine Olfactory Mucosa

Bovine olfactory mucosa has been reported to be the most appropriate model for investigation of nose-to-brain delivery of drugs (31). To assess the ability of different chitosan grades in enhancing the transport of macromolecules, preliminary *in vitro* permeation studies were carried out using FD40K as a model permeant. FD40K was chosen as a marker as it is a hydrophilic macromolecule and its molecular weight is somewhat comparable to that of BDNF.

Chitosan is a polycationic linear polysaccharide available in different viscosity and degree of acetylation (DA) grades. It is a biodegradable, biocompatible polymer and possesses bioadhesive property. Due to its bioadhesive property the formulations retain at the site of application thereby facilitating enhanced absorption. Chitosan has been shown to render the olfactory mucosa more permeable to macromolecules. The mechanism of enhancing membrane permeability has been attributed to opening of tight junctions in the mucosa which paves convective transport channels across the barrier (32–34).

Invariably, all the chitosan grades (low viscosity, medium viscosity and high viscosity) at three different concentrations (0.1% or 0.25% or 0.5% w/v) used in this study were able to enhance the transport of FD40K across the olfactory mucosa at the end of 2 h (Fig. 1). However, the extent of enhancement differed between different grades and concentrations. In general, the enhancement factor did not increase further at 0.5% w/v chitosan concentration over 0.25% w/v in all the cases which is likely due to increased viscosity of the donor solution. The medium viscosity grade chitosan was more efficient compared to other two grades

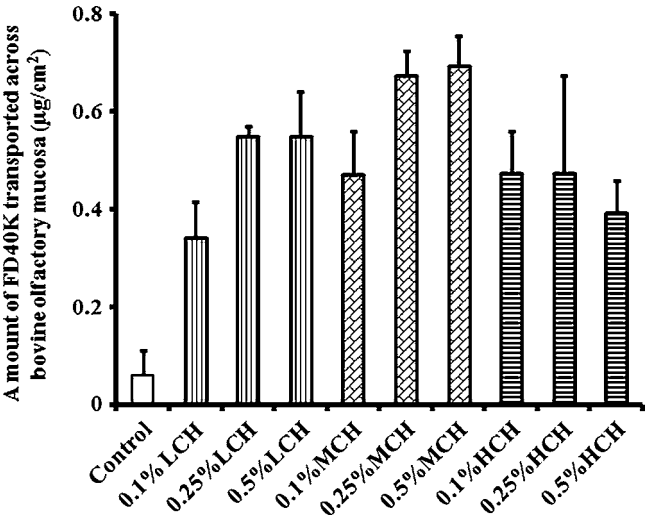

Fig. 1 Effect of different grades of chitosan (low viscosity (LCH), medium viscosity (MCH) and high viscosity (HCH)) at different concentrations (0.1%, 0.25% and 0.5% w/v) on *in vitro* transport of FD40K across bovine olfactory mucosa. The data points provided are an average of six trails with SEM as *error bars*.

of chitosan as an enhancer. The medium viscosity grade chitosan enhanced the transport of FD40K by ~ 7 fold, ~ 11 fold and ~11 fold at 0.1%, 0.25% and 0.5% w/v concentration respectively, over control (0.06 ± 0.04 µg/cm^2). Therefore, this grade of chitosan was used to investigate the effect on transport of BDNF in *in vitro* studies. Medium viscosity grade chitosan was found to enhance the BDNF transport across the bovine olfactory mucosa by ~7 fold, ~ 14 fold and ~ 5 fold over control (18.78 ± 16.69 ng/cm^2) at 0.1%, 0.25% and 0.5% w/v concentration respectively, (Fig. 2). Hence, 0.25% w/v medium viscosity grade chitosan was used for *in vivo* studies.

The amount of BDNF transported across the olfactory mucosa was enhanced by chitosan due to a combination of several mechanisms. Vaka and co-workers reported earlier that chitosan lead to a significant drop in the electrical resistivity of the mucosa indicating the possible interaction of the polymer with the mucosal membrane. The electrical resistivity was also found to recover with time suggesting that the permeabilization of the membrane by the chitosan is reversible. It is likely that the drop in electrical resistivity is due to opening of the tight junctions present in the membrane (30). However, the role of ionic charge interaction between the polymer and BDNF, hydrokinetic transport and other potential mechanisms have plausibly contributed significantly.

Stability of BDNF in Tissue Homogenate

The olfactory mucosa has a milieu of enzymes in it (35). The enzymes serve a protective function to prevent accidental entry of toxins via nose-to-brain pathway. BDNF

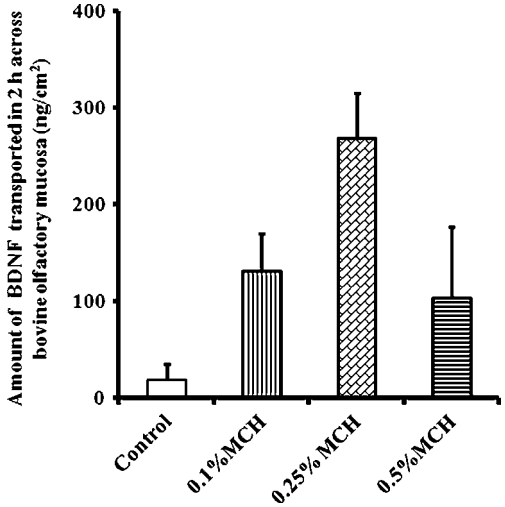

Fig. 2 Effect of different concentrations of medium viscosity grade chitosan (MCH) on *in vitro* transport of BDNF across the bovine olfactory mucosa. The data points provided are an average of six trails with SEM as *error bars*.

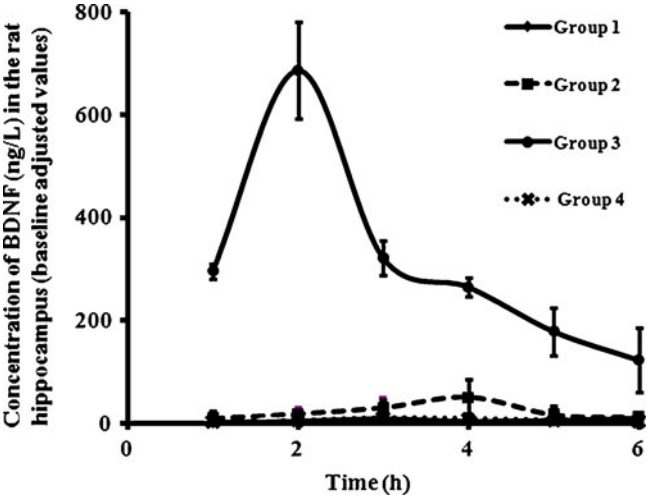

Fig. 3 Concentration-time profile of BDNF in rat hippocampus following intranasal/i.v administration of formulations to four different groups of rats. The data points represent baseline adjusted values and are averages of three animals with SEM as *error bars*.

being a peptide in nature is very amenable to enzymatic degradation. It has been reported that there are several proteolytic enzymes on the nasal mucosa surface, which could result in degradation of peptide and protein drugs (36–38). Therefore, the stability of BDNF in the homogenate of the mucosal tissue was investigated as it contains both inter and intracellular enzymes. The BDNF degradation followed an apparent first order mechanism. The degradation rate constant calculated from the semi logarithmic plot of percentage BDNF remaining as a function of time was found to be 0.39 ± 0.05 h^{-1}. These studies indicate that the poor transport of BDNF across the olfactory mucosa is also likely due to significant degradation of BDNF in the membrane in addition to poor membrane permeability.

Pharmacokinetic Studies: Brain Microdialysis

Generally, in pharmacokinetic studies, the total BDNF (bound and unbound) levels in the whole brain homogenates are estimated (19,39). Microdialysis of the brain is useful in continuous sampling the unbound agent of interest. The time course of unbound agents in the brain may be of more pharmacological relevance than the total amount in general. The sampling could be obtained from specific regions of the brain as well. Moreover, microdialysis sampling reduces the number of animals required for pharmacokinetic experiments and results in relatively less variability than studies involving one animal per time point. As the objective was to compare the extent of delivery of BDNF from different formulations, microdialysis technique was used to sample BDNF in the brain from the hippocampus region in the present study. The recovery of microdialysis probes was determined *in vitro* and found to be $2.98 \pm 0.2\%$. The BDNF levels in group 1 served as baseline endogenous levels (206.91 ± 56.94 ng/L). The time course of exogenous BDNF in the hippocampus (the difference between the total amount of BDNF and baseline endogenous level) was plotted and the bioavailability parameters were calculated. The bioavailability in group 3 rats was significantly enhanced by ~13 fold compared to group 2 (Fig. 3). This increased bioavailability of BDNF could be attributed to chitosan which acts as a barrier modulating agent by transient permeabilization of the olfactory epithelium. In addition, significant amounts of BDNF might be transported by receptor mediated endocytosis due to the presence of the

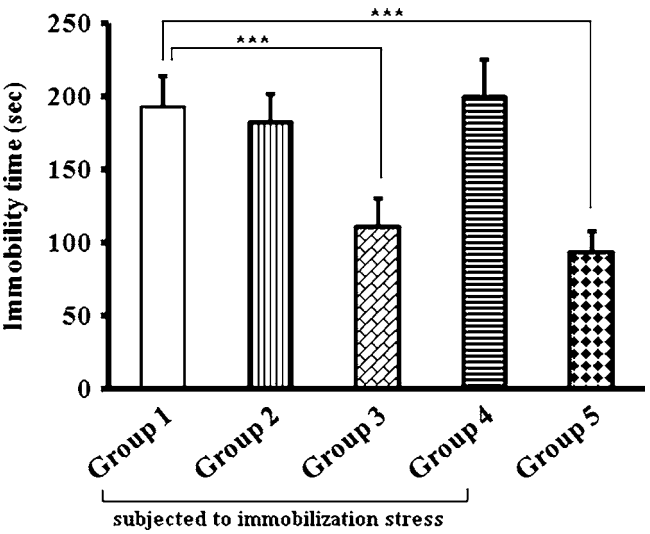

Fig. 4 The effect of BDNF on duration of immobility in the forced swim test upon intranasal/i.v administration of formulations to five different groups of rats. The data points represent baseline adjusted values and are an average of six animals with SEM as *error bars* (***$p < 0.001$).

physiologic receptor for BDNF *(trkB)* on mature olfactory neurons and by intracellular transport (40). There was no significant enhancement of BDNF levels in case of group 4 over group 1 (vehicle control). The C_{max} achieved in group 3 at 2 h was 685.63±94.37 ng/L which was significantly higher than the C_{max} achieved (50.62± 35.85 ng/L) in group 2 at 4 h. However, the concentrations achieved in rat hippocampus upon intranasal administration at 60 min in our studies were less than that reported by Alcala-Barraza and co-workers (1700±600 ng/L) upon intranasal administration of [^{125}I]-BDNF (70 μg) (39). This difference in concentrations is likely, as these authors have estimated the total amount of BDNF (bound and unbound) in homogenate of whole brain.

Pharmacodynamic Studies

The forced swim test is one of the most commonly used paradigms to assess the effect of stress on immobility time in rodent models (18,41,42). In this test the rats subjected to acute stress are forced to swim in a restricted space from which they cannot escape. When rats are previously subjected to stress leading to depression, the immobility time was found to increase (16,18).

The immobility time of group 5 rats, which were not subjected to immobilization stress is ~2 fold less than that of group 1 rats (Fig. 4). Smith and coworkers have shown that repeated immobilization of rats reduces BDNF levels in the rat brain, especially in the hippocampus (16). Intranasal administration of formulation containing BDNF solution (100 μL of 50 μg/mL) with 0.25% w/v chitosan prepared in KRB to group 3 significantly decreased the immobility time compared to that of group 1 (vehicle control group). These results clearly indicate the potential of nose-to-brain delivery of BDNF formulations containing chitosan in replenishing the depleted BDNF levels. Siuciak *et al.*, and Shirayama *et al.*, studied the effect of infusion of BDNF into the rat brain on the immobility time and reported such similar observations (17,18). The antidepressant effect of BDNF is believed to be the result of interference of hippocampal synaptic transmissions by BDNF with stress (18,43–45). In case of rats belonging to groups 2 and 4 the immobility time was not significant statistically ($p > 0.05$) compared to that of group 1 (vehicle control group). This is likely due to the poor brain bioavailability of BDNF as seen in pharmacokinetics studies.

These studies showed that significant amounts of BDNF could be delivered to brain via nose-to-brain pathway with the aid of barrier modulating agent like chitosan. Continuous stress in rats leads to depression like condition, likely due to depletion of BDNF. Repletion of BDNF using intranasal formulations can counteract the stress induced depression. Therefore, it appears that intranasal delivery of BDNF with chitosan could potentially treat CNS disorders associated with BDNF depletion.

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