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Intranasal Administration as a Route for Drug Delivery to the Brain: Evidence for a Unique Pathway for Albumin

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ABSTRACT

A variety of compounds will distribute into the brain when placed at the cribriform plate by intranasal (i.n.) administration. In this study, we investigated the ability of albumin, a protein that can act as a drug carrier but is excluded from brain by the blood-brain barrier, to distribute into the brain after i.n. administration. We labeled bovine serum albumin with [^{125}I] ([^{125}I]Alb) and studied its uptake into 11 brain regions and its entry into the blood from 5 minutes to 6 hours after i.n. administration. [^{125}I]Alb was present throughout the brain at 5 minutes. Several regions showed distinct peaks in uptake that ranged from 5 minutes (parietal cortex) to 60 minutes (midbrain). About 2–4% of the i.n. [^{125}I]Alb entered the bloodstream. The highest levels occurred in the olfactory bulb and striatum. Distribution was dose-dependent, with less taken up by whole brain, cortex, and blood at the higher dose of albumin. Uptake was selectively increased into the olfactory bulb and cortex by the fluid-phase stimulator PMA (phorbol 12-myristate 13-acetate), but inhibitors to receptor-mediated transcytosis, caveolae, and phosphoinositide 3-kinase were without effect. Albumin altered the distribution of radioactive leptin given by i.n. administration, decreasing uptake into the blood and by the cerebellum and increasing uptake by the hypothalamus. We conclude that [^{125}I]Alb administered i.n. reaches all parts of the brain through a dose-dependent mechanism that may involve fluid-phase transcytosis and, as illustrated by leptin, can affect the delivery of other substances to the brain after their i.n. administration.

Introduction

Drug delivery to the brain for the treatment of a wide variety of diseases is hampered by the blood-brain barrier (BBB). In recent years, intranasal (i.n.) administration has come to light as an effective and noninvasive means to circumvent the BBB for a variety of substances, including polypeptides, liposomes, virus vectors for gene therapy, and stem cells (Dhuria et al., 2010; Lochhead and Thorne, 2012a). These substances not only reach the brain tissue, but bioactive compounds such as insulin (Reger et al., 2008; Benedict et al., 2011; Craft et al., 2012), oxytocin (Veening and Olivier, 2013), and leptin (Schulz et al., 2012) do so in levels sufficient to cause significant behavioral changes in both patients and animal models of disease.

Albumin, the pre-eminent transport protein in the blood, is emerging as a versatile transport molecule for targeted drug delivery (Kratz, 2008), and albumin has recently gained attention in the field of pharmaceutical development for its ability to prolong the half-life and stability of bioactive compounds (Elsadek and Kratz, 2012). Whereas albumin binding proteins, such as gp60 (albondin), facilitate transcytosis of albumin in peripheral capillaries, there is low expression in brain endothelial cells (Schnitzer, 1992), and albumin has been shown not to cross the BBB in vivo or in vitro (Pardridge et al., 1985); however, a study has suggested that ovalbumin may be taken up by brain after i.n. administration (Migliore et al., 2010).

Here, we assessed whether i.n. administration of radioactively labeled albumin in CD1 mice is an effective method of delivery to the brain. We also examined the regional distribution and time course of administered albumin, explored the cellular mechanisms of brain uptake, and assessed i.n. albumin's effect on distribution of another potential central nervous system (CNS) therapeutic, leptin.

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ABBREVIATIONS: BBB, blood-brain barrier; BSA, bovine serum albumin; CPM, counts per minute; CNS, central nervous system; CSF, cerebrospinal fluid; DMSO, dimethylsulfoxide; GR138950, 1-[[3-bromo-2-[2-[[(trifluoromethyl)sulphonyl]amino]phenyl]-5- benzofuranyl]methyl]-4-cyclopropyl-2-ethyl-1*H*-imidazole-5-carboxamide; i.n., intranasal; LR, lactated Ringer's solution; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; MeOH, methanol; PB, phosphate buffer; PMA, phorbol 12-myristate 13-acetate.

Materials and Methods

Animals. Male CD1 mice, 8 weeks of age, purchased from Charles River Laboratories (Wilmington, MA), were used throughout these experiments. Mice had free access to food and water and were on a 12-hour light/dark cycle. All studies were performed under approved Institutional Animal Care and Use Committee protocols and by an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility.

Iodination of Albumin and Leptin. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and murine leptin (R&D Systems Inc., Minneapolis, MN) were radioactively labeled with [^{125}I] by the chloramine-T method (Greenwood et al., 1963). Briefly, 10 μg of albumin [1 μg/μl in 0.25 M phosphate buffer (PB)] or 5 μg of leptin (1 μg/μl in dIH_2O) was labeled with 0.5 mCi of [^{125}I] (PerkinElmer, Waltham, MA). The BSA was purified on a column of Sephadex G-10 (GE Healthcare, Piscataway, NJ) and the leptin on a Nap-5 column Sephadex G-25 DNA grade (GE Healthcare). The BSA fractions were collected at 1-minute intervals into 100 μl lactated Ringer's solution (LR; Baxter International, Deerfield, IL) in glass tubes treated with Sigmacote siliconizing reagent (Sigma-Aldrich); the leptin fractions were collected in plastic tubes at 1-minute intervals into 100 μl of LR solution with or without 1% BSA. An acid precipitation using 15% trichloroacetic acid was then performed to assess purity. The percentage of radioactivity precipitated was calculated by the following formula (eq. 1):

$$100 \times \left[\left(\mathrm{CPM_{pellet}}\right)/\left(\mathrm{CPM_{pellet}} + \mathrm{CPM_{supernatant}}\right)\right] \quad (1)$$

Only fractions that showed >90% activity in the precipitate were used in the experiment.

Intranasal Administration. Mice were anesthetized by i.p. injection of urethane, 40% solution (Sigma-Aldrich); 1 μl containing 400,000 counts per minute (CPM) of [^{125}I]Alb in PB was administered i.n. via a micropipette tip inserted 4 mm into the left nostril of each mouse. Leptin labeled with [^{125}I] ([^{125}I]Lep) was injected in PB with or without 1% BSA (1 g/100 ml) added to the PB saline vehicle.

Tissue Collections. Male CD1 mice were anesthetized and administered i.n. [^{125}I]Alb or [^{125}I]Lep as described. Blood was collected from the carotid artery, and the whole brain was removed at a predetermined time point after i.n. administration. Time points were 5, 10, and 30 minutes, and 1, 2, 4, and 6 hours. The brain was dissected using the method of Glowinski and Iversen (1966) on ice into 11 regions of the olfactory bulb, occipital cortex, striatum, frontal cortex, hypothalamus, thalamus, hippocampus, parietal cortex, cerebellum, midbrain, and pons medulla, and each region was weighed. The collected whole blood was allowed to clot at room temperature, centrifuged at 5400*g* for 10 minutes at 4°C, and 50 μl of resulting serum was removed for use. The level of radioactivity in each of the 11 regions and the serum sample was determined in a Wizard2 Automatic Gamma Counter (PerkinElmer) by counting for 30 minutes. Values for the whole brain were calculated by summing the regional weights and the regional levels of radioactivity of all tissue samples except the olfactory bulb. The percentage of the injected dose present in a milliliter of serum (%Inj/ml) was calculated with the following formula (eq. 2):

$$\%\mathrm{Inj/ml} = [100 \times (\mathrm{CPM/ml})]/\mathrm{Inj}], \quad (2)$$

where Inj is the CPM administered and CPM/ml is the level of radioactivity in a milliliter of serum. The percentage of the injected dose taken up per gram of brain region (%Inj/g) was calculated at each time with the following formula (eq. 3):

$$\%\mathrm{Inj/g} = [100 \times (\mathrm{CPM/R})]/\mathrm{Inj(W)}], \quad (3)$$

where W is the weight of the given brain region in grams, and CPM/R is the level of radioactivity in the brain region. The rate at which i.n. [^{125}I]Alb would have to enter into the blood to reach the levels measured in blood was calculated by the following formula (eq. 4):

$$T = \mathrm{PKVd}/\left(1 - \mathrm{e}^{-\mathrm{Kt}}\right), \quad (4)$$

where T is the entry rate into blood in %Inj/min, P is the level of [^{125}I]Alb measured in serum in %Inj/ml at time t, Vd is volume of distribution in ml, t is sampling time in minutes, and $K = (0.693/t_{1/2})$, where $t_{1/2}$ is the half-time clearance of [^{125}I]Alb from blood after i.v. injection. The values used for $t_{1/2}$ and Vd for [^{125}I]Alb were 316 minutes and 1.76 ml as previously measured (Banks and Broadwell, 1994).

Acid Precipitations. Male CD1 mice were anesthetized and administered i.n. [^{125}I]Alb as described. Blood was collected from the external jugular vein, and the whole brain was removed at 10 minutes or at 60 minutes after the i.n. administration of [^{125}I]Alb (n = 7 mice/group). Olfactory bulb, cortex, and cerebellum were dissected from the remainder of the brain on ice and collected in 1 ml of 1% BSA in LR. Tissue samples were homogenized in a Mini-BeadBeater (BioSpec Products, Inc., Bartlesville, OK) twice (30 seconds each pulse; 4800 rpm), then centrifuged at 10,000*g* for 20 minutes at 4°C. Whole blood samples were allowed to clot at room temperature and then centrifuged at 5400*g* for 10 minutes and also at 4°C. A 50-μl portion of the supernatant from each sample was used to perform an acid precipitation using 15% trichloroacetic acid. To determine the amount of degradation that occurred with processing, 400,000 CPM was added in vitro to a sample of serum or a brain region obtained from a mouse that had not received i.n. [^{125}I]Alb (n = 2). Samples were then processed as described. After the addition of trichloroacetic acid, the serum, brain, and processing controls were centrifuged at 5000*g* for 10 minutes at 4°C, and the supernatant was separated from the pellet. The radioactivity of both pellet and supernatant was determined in a Wizard2 Automatic Gamma Counter (PerkinElmer) by counting for 30 minutes, and the percentage of radioactivity precipitated (AP %) was calculated by the following formula (eq. 5):

$$\mathrm{AP\%} = 100 \times \left[\left(\mathrm{CPM_{pellet}}\right)/\left(\mathrm{CPM_{pellet}} + \mathrm{CPM_{supernatant}}\right)\right] \quad (5)$$

The percentage that was precipitated from samples was divided by that which precipitated in the processing controls and multiplied by 100 to give an index of intact [^{125}I]Alb.

[^{125}I]Alb versus [^{125}I]Alb + Unlabeled Albumin. Male CD1 mice were anesthetized as described above and administered i.n. [^{125}I]Alb with or without 10 μg (2 μg/μl in PB) of unlabeled albumin (n = 29/group). Blood was collected from the external jugular vein, and the whole brain was removed 30 minutes after the i.n. administration. Olfactory bulb, cortex, and cerebellum were dissected from the remainder of the brain on ice; each region was weighed; and the level of radioactivity was determined in a Wizard2 Automatic Gamma Counter by counting for 30 minutes. Whole brain mass and radioactivity were determined by combining the mass and radioactivity of all regions except the olfactory bulb. Whole blood was allowed to clot at room temperature and centrifuged at 5400*g* for 15 minutes at 4°C, and the radioactivity of 50 μl of the resulting serum was counted for 30 minutes.

Modifiers of Cellular Mechanisms of Uptake. Male CD1 mice were anesthetized as described herein and administered i.n. a 1-μl solution containing various substances known to alter physiologic pathways of transport. The concentrations chosen for use in this study were based on concentrations available in the literature for use of these substances in vivo. For substances for which in vivo data are not available, the in vitro concentrations were used since i.n. administration is considered an in situ model. These substances include phenylarsine oxide [100 μM in dimethylsulfoxide (DMSO); n = 14], filipin (5 μg/ml in DMSO; n = 15), LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride] (50 μM in DMSO; n = 15), PMA (phorbol 12-myristate 13-acetate) (100 ng/ml in DMSO; n = 15), histamine [100 ng/ml in methanol (MeOH); n = 10], monensin (50 μM in MeOH; n = 10), verapamil (10 μM in MeOH; n = 10), and lidocaine (1% solution in MeOH; n = 10) (all purchased from Sigma-Aldrich). For controls (n = 20), 1 μl of either DMSO or MeOH, the vehicle used to deliver the drugs, was administered. Each drug group

was done concurrently with a control group, and 30 minutes after injection of an inhibitor or control (DMSO or MeOH), 1 μl of 0.25 M PB containing 400,000 CPM of [^{125}I]Alb was administered i.n. 4 mm into the right nostril. Blood was collected from the external jugular vein, and the whole brain was removed 15 minutes after i.n. administration of [^{125}I]Alb. The olfactory bulb, cortex, and cerebellum were dissected from the remainder of the brain on ice, each region was weighed, and the level of radioactivity determined in a Wizard2 Automatic Gamma Counter by counting for 30 minutes. Whole brain mass and radioactivity were determined by combining the mass and radioactivity of all regions except the olfactory bulb. Whole blood was allowed to clot at room temperature and centrifuged at 5400*g* for 15 minutes at 4°C, and the radioactivity was counted in 50 μl of the resulting serum for 30 minutes. The percentage of the injected dose present in 1 ml of serum (%Inj/ml) and the percentage of injected dose taken up per gram of brain region (%Inj/g) were calculated according to the equations herein.

Statistical Analysis. Means are reported with their standard error and number. Means were compared by two-tailed *t* test. Statistical significance was taken as $P < 0.05$. The Prism 5.0 statistical software program (GraphPad Software Inc., San Diego, CA) was used in statistical analysis.

Results

Our first goals were to determine whether i.n.-administered albumin reaches the brain, where in the brain it distributes, and the time course of delivery and clearance. We therefore harvested the brain over a 6-hour period, dividing it into the 10 regions of Glowinski and Iversen (1966) plus the olfactory bulb. Figure 1 shows the percentage of i.n.-administered [^{125}I]Alb taken up by each brain region (%Inj/g) at the various time points and the varying regional patterns of [^{125}I]Alb uptake and clearance.

All brain regions showed uptake of [^{125}I]Alb at 5 minutes, the first time point examined (Fig. 1). The highest peaks occurred in the olfactory bulb (1.06%Inj/g $\pm$ 0.35, $n = 15$) and striatum (0.45%Inj/g $\pm$ 0.21, $n = 10$). In the frontal cortex, parietal cortex, occipital, thalamus, cerebellum, and pons medulla, the concentration of [^{125}I]Alb was highest at 5 minutes, after which it decreased gradually. Among the three cortical regions, the frontal cortex and occipital cortex remained steady at 0.10–0.15%Inj/g through the first 2 hours, whereas the parietal cortex decreased earlier, having undetectable levels by 2 hours. Several regions demonstrated distinct peaks in %Inj/g, including the olfactory bulb at 10 minutes, the striatum at 30 minutes, and the midbrain and hypothalamus at 60 minutes. The cerebellum had the lowest peak level and was the first to be cleared with undetectable levels 60 minutes after i.n. administration of [^{125}I]Alb. [^{125}I]Alb is essentially cleared from all regions by 4 hours.

The %Inj/ml of serum (Fig. 2) remained fairly steady at 1% Inj/ml, trending upward at the 6-hour time point. The rate at which [^{125}I]Alb would enter the bloodstream to produce these

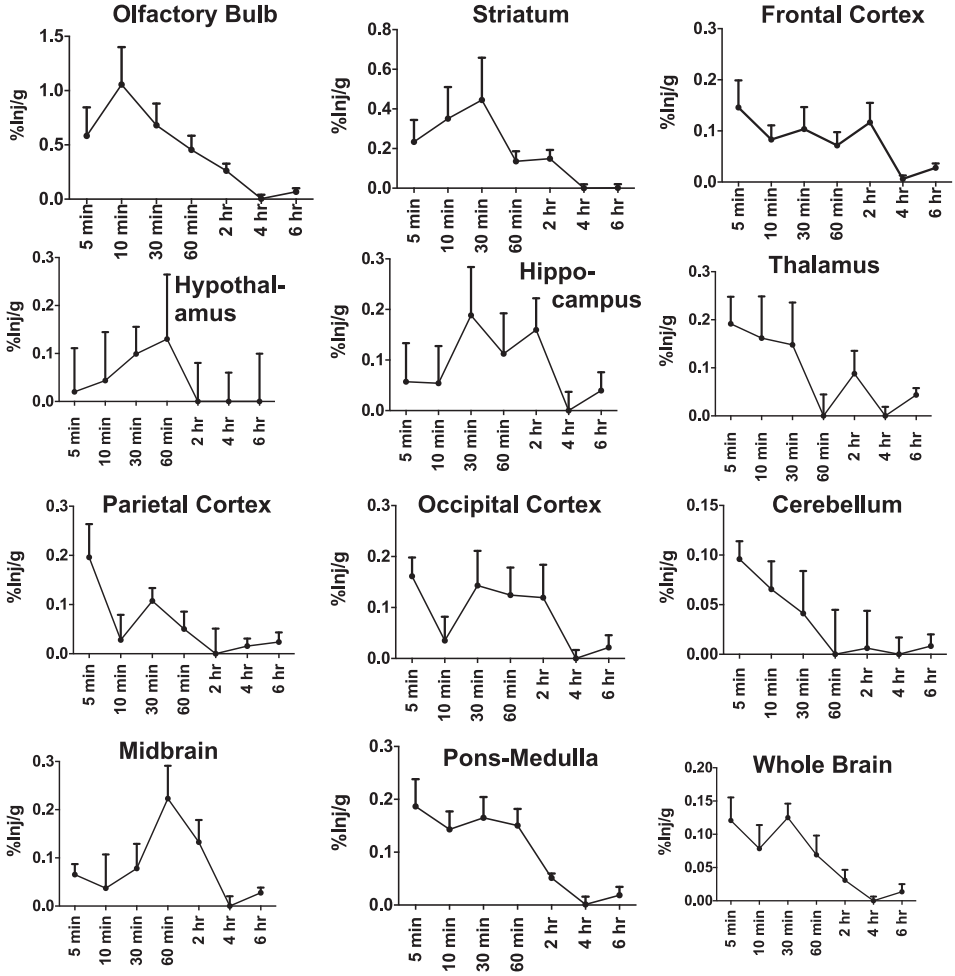

Fig. 1. Distribution in brain after i.n. administration of [^{125}I]Alb. Appearance of [^{125}I]Alb in the olfactory bulb, striatum, frontal cortex, hypothalamus, hippocampus, thalamus, parietal cortex, occipital cortex, cerebellum, midbrain, pons medulla, and whole brain at 5 minutes ($n = 15$), 10 minutes ($n = 15$), 30 minutes ($n = 10$), 60 minutes ($n = 15$), 2 hours ($n = 20$), 4 hours ($n = 15$), and 6 hours ($n = 17$) post–i.n. administration of [^{125}I]Alb. The peak value was at 5 minutes in the frontal cortex, parietal cortex, occipital cortex, thalamus, cerebellum, pons medulla and whole brain; 10 minutes in olfactory bulb; 30 minutes in striatum and hippocampus; and 60 minutes in the midbrain and hypothalamus.

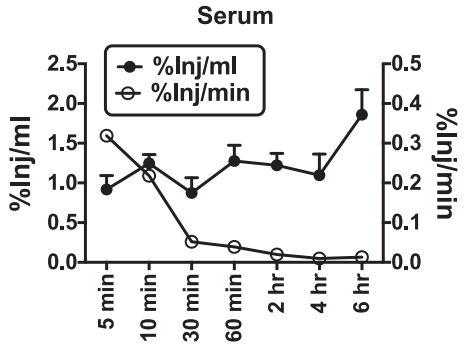

Fig. 2. Level and rate of appearance of [^{125}I]Alb in serum after i.n. administration. Appearance of [^{125}I]Alb in serum at 5 minutes (n = 15), 10 minutes (n = 15), 30 minutes (n = 10), 1 hour (n = 15), 2 hours (n = 20), 4 hours (n = 15), and 6 hours (n = 17) after i.n. administration of [^{125}I]Alb (left y-axis). Values were steady at about 1%Inj/ml, peak value at 6 hours. Rates of entry into serum during same time course (right y-axis).

levels ranged from about 0.32%Inj/min at 5 minutes to a low of 0.012%Inj/min at 360 minutes. Multiplying time by %Inj/min showed that about 1.5–4% of the i.n. dose of [^{125}I]Alb entered the blood stream during the course of the study.

To determine the extent to which the radioactivity observed in the brain after i.n. administration of [^{125}I]Alb accurately represents the presence of the administered protein, we performed an acid precipitation of the tissue. Because most of the regions showed the strongest signal within the first hour, we chose to examine time points 10 and 60 minutes after i.n. administration. Four regions were examined, and the radioactivity of each sample after precipitation is reported as a percentage of a matched processing control (Table 1). When measurements were pooled from all regions and both time points, the mean adjusted percentage was 61.36% (± 9.40%), representing the portion of radioactivity measured throughout these experiments that reliably signifies intact [^{125}I]Alb. No statistical differences were seen in any regions between the two time points ($P > 0.05$ by two-way analysis of variance).

To investigate the dose dependence of albumin uptake, we administered 400,000 CPM of [^{125}I]Alb with and without additional unlabeled albumin, the results of which are shown in Fig. 3. Inclusion of unlabeled albumin decreased [^{125}I]Alb uptake by the cortex (0.074 ± 0.008 with unlabeled albumin versus 0.146 ± 0.030 without, $P < 0.05$), whole brain (0.085 ± 0.009 with unlabeled albumin versus 0.132 ± 0.017 without, $P < 0.05$), and serum (2.290 ± 0.1759 with unlabeled albumin versus 4.645 ± 1.007 without, $P < 0.05$). Changes in olfactory bulb and cerebellum were not significant.

Cellular mechanisms for [^{125}I]Alb uptake into the brain and blood were studied by administering agents 30 minutes before [^{125}I]Alb i.n. administration and harvesting brains 15 minutes after i.n. administration. The agents were phenylarsine oxide, filipin, LY294002, and PMA, lidocaine, histamine, monensin, and verapamil. DMSO or MeOH was used as control, depending on the solubility of the inhibitor. Only PMA had a statistically significant effect (Fig. 4), increasing uptake at the olfactory bulb and cortex, but with no effect on cerebellum, or whole brain. PMA had no effect on serum levels, and the other agents were without effects on brain or serum levels (data not shown).

The ability of albumin to influence brain distribution of substances administered by the i.n. route was tested by examining the distribution of [^{125}I]Lep given with or without albumin included in the i.n. injection. Figure 5 shows that coadministration with albumin decreased [^{125}I]Lep entry into the blood and uptake by the cerebellum, but it increased hypothalamic uptake. There were also statistical trends for decreasing uptakes by the olfactory bulb and whole brain but without an influence on hippocampus.

TABLE 1

Acid precipitation of radioactivity extracted from brain regions after intranasal administration of albumin labeled with radioactive iodine

Percentage of acid precipitation for whole brain and brain regions 10 and 60 minutes after intranasal administration of albumin labeled with radioactive iodine. Values are corrected for degradation that occurred during processing. Results are means ± S.E.M. (n).

Region	%Control	
	10 Minutes	60 Minutes
Olfactory bulb	108 ± 30 (7)	75 ± 18 (6)
Cortex	62 ± 19 (5)	60 ± 18 (6)
Cerebellum	87 ± 29 (6)	81 ± 32 (5)
Whole brain	74 ± 18 (6)	74 ± 13 (4)

Discussion

Intranasal administration is a promising approach for the delivery of biologicals to the brain (Frey, 2002; Lochhead and Thorne, 2012b); however, it is unknown which substances can or cannot use this route, nor is it known what cellular mechanisms underlie this uptake. Here, we investigated albumin uptake after i.n. administration. If albumin were to be taken up after i.n. administration, it might act as a binder and carrier, helping to direct substances to specific brain regions, similar to the role albumin plays in the circulation. Albumin is by far the most versatile transport protein in the blood circulation and has been used in a variety of pharmaceutical applications, as has been recently reviewed (Elsadek and Kratz, 2012). Several strategies have been developed to link drugs, prodrugs, and bioactive polypeptides to albumin, including various forms of physical and covalent binding as well as drug encapsulation in albumin nanoparticles (Kratz, 2008; Elsadek and Kratz, 2012). Levemir and Victoza, developed by

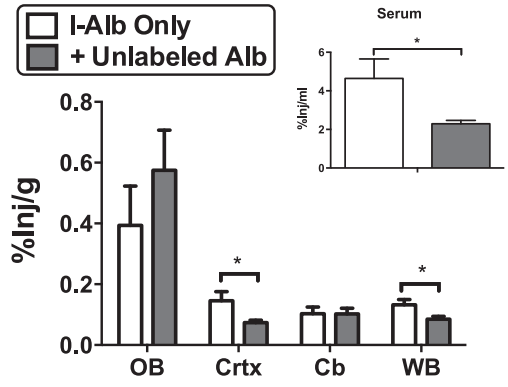

Fig. 3. Dose-dependent distribution of [^{125}I]Alb. %Inj/g of [^{125}I]Alb ± unlabeled albumin in the olfactory bulb (OB), cortex (Crtx), cerebellum (Cb), and whole brain (WB) 30 minutes after i.n. administration of [^{125}I]Alb. Inset shows %Inj/ml for serum at 30 minutes after i.n. administration. Whole brain was calculated by combining the cerebellum, cortex, and remainder but excluding olfactory bulb. Unlabeled albumin produced significant decreases (*$P < 0.05$; n = 29) in the cortex, whole brain, and serum as assessed by t test.

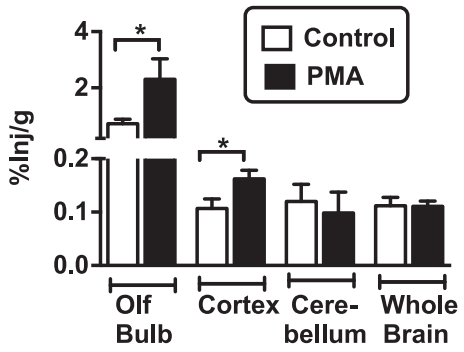

Fig. 4. Cellular mechanisms of uptake of i.n. administered [^{125}I]Alb. Influence of the fluid-phase stimulator PMA on the percentage of [^{125}I]Alb taken up per gram of tissue (%Inj/g) by the olfactory bulbs (Olf Bulb), cortex, cerebellum, and whole brain. PMA was given 30 minutes before i.n. administration of [^{125}I]Alb, and brains were harvested 15 minutes after [^{125}I]Alb administration. Filipin, LY294002, histamine, verapamil, lidocaine, monensin, and phenylarsine oxide were without effect on [^{125}I]Alb distribution, and none of the drugs affected the levels of [^{125}I]Alb in serum (data not shown). Significance ($P > 0.05$) indicated by asterisk.

Novo Nordisk (Bagsvaerd, Denmark) for the treatment of diabetes, are long-lasting derivatives of human insulin and glucagon-like peptide-1 agonist, respectively. Through the attachment of myristic acid, a saturated fatty acid that binds albumin with high affinity, the serum half-life of these compounds is extended from minutes to hours (Elsadek and Kratz, 2012; Kratz and Elsadek, 2012). Albumin-binding moieties, such as myristic acid, maleimide, and a growing number of synthetic peptides, offer a simple and effective means of linking drugs to exogenous and endogenous serum albumin. Developing albumin to play similar roles after i.n. administration could be a major advance in this approach for the delivery of biologicals to the brain.

Recent reviews of i.n. administration (Dhuria et al., 2010) make a good case for the involvement of the olfactory nerves, noting the high turnover of olfactory neurons and the presence of perineural channels created by olfactory ensheathing cells. Our results are consistent with this model: whereas [^{125}I]Alb was seen in all regions of the brain, the highest levels by far were seen in the olfactory bulbs. Numerous studies have noted the rapidity with which i.n. applied substances reach tissues distant to the nose. Most have observed the highest CNS concentrations within the first hour, many almost immediately after administration (Dhuria et al., 2010). Here, we also

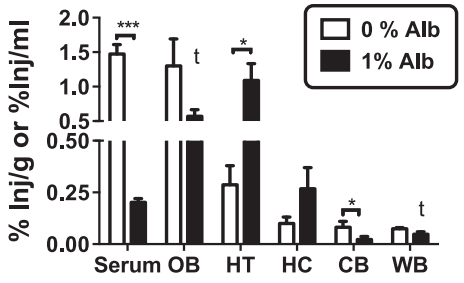

Fig. 5. Effect of albumin (Alb) on the brain distribution of i.n. [^{125}I]Lep. Influence of albumin on the distribution among brain regions and to the blood of i.n. administered [^{125}I]Lep. Coadministration of [^{125}I]Lep with 1% albumin (1g/dl) resulted in lower levels of [^{125}I]Lep appearing in the serum and cerebellum (CB) and a higher level appearing in hypothalamus (HT). Olfactory bulb (OB) and whole brain (WB), but not hippocampus (HC), showed trends (*$P < 0.05$; ***$P < 0.001$; $t = 0.05 < P < 0.10$) toward changes.

observed very rapid distribution of [^{125}I]Alb, with measurable amounts in all regions at 5 minutes. Recent work using both confocal imaging (Iliff et al., 2012) and contrast-enhanced MRI (Iliff et al., 2013) indicates that a large portion of cerebrospinal fluid (CSF) moves into the brain by bulk flow through arterial perivascular spaces, traverses the parenchyma, and is cleared along venous perivascular spaces. These perivascular spaces additionally provide a means of rapid transport of CSF within the wider subarachnoid space. The pattern of brain [^{125}I]Alb uptake described here could be explained by transport from the nasal epithelium along the olfactory nerves into the CSF, diffusion into arterial perivascular spaces, and rapid distribution throughout the brain. This explanation is supported by findings that elevated CSF levels of i.n. administered substances precede elevations in tissue levels (Charlton et al., 2008).

The results of the acid precipitation study suggest that the radioactivity of [^{125}I] in these experiments, although not an exact measure of albumin integrity, does provide a reliable proxy for its presence. Within 5 minutes of i.n. administration, [^{125}I]Alb was seen to reach all regions of the brain. After this, unique patterns of uptake and clearance emerged in different brain regions. As for other compounds (Frey, 2002; Thorne et al., 2004), we observed highest uptake of albumin in the olfactory bulbs with much lower uptake in all other regions. Many regions showed highest levels of uptake at the first time point, after which [^{125}I]Alb was gradually cleared. The rate of clearance showed regional differences, but [^{125}I]Alb was effectively cleared from all regions by the 4-hour time point. The striatum and midbrain exhibited distinct peaks in concentration, consistent with active uptake or transient sequestration of [^{125}I]Alb in these regions. These patterns differ from those seen after i.n. administration of other peptides, including pituitary adenylate cyclase–activating polypeptide (Nonaka et al., 2012), the angiotensin antagonist GR138950 (1-[[3-bromo-2-[2-[[(trifluoromethyl)sulphonyl]amino]phenyl]-5-benzofuranyl] methyl]-4-cyclopropyl-2-ethyl-1*H*-imidazole-5-carboxamide) (Charlton et al., 2008), and testosterone (Banks et al., 2009). This variability could suggest that different molecules may undergo unique mechanisms of uptake.

We found little [^{125}I]Alb entering the bloodstream when assessed either as the percentage of the injected dose that accumulated in blood or when calculated as a transport rate. The transport rate suggests that most entry into blood occurs early and that less than 4% of the injected dose reaches the bloodstream. This rate is even less than other biologicals given by the i.n. route (Banks et al., 2004) and suggests that coadministration with albumin may act to keep the coadministered substance within the CNS.

In these experiments, we sought to characterize the mechanism of albumin uptake. The decreases in [^{125}I]Alb uptake by whole brain and cortex seen when coadministered with unlabeled albumin may indicate competition for a saturable mechanism. The decrease in the [^{125}I]Alb level in serum seen with coadministration of unlabeled albumin supports the idea of dose-dependent uptake. However, levels in the olfactory bulb were not decreased, suggesting that the dose dependence may have more to do with distribution within the CNS after initial entry than with entry itself.

We saw that pretreatment with PMA increased the uptake of [^{125}I]Alb in the olfactory bulb and cortex, but not the cerebellum or whole brain, suggesting a role for PKC signaling in the active nose-to-brain transport of [^{125}I]Alb (Wu-Zhang and

Newton, 2013). More specifically, PMA can stimulate fluid phase transcytosis, the same pathway that albumin uses to cross endothelial cells (Guillot and Audus, 1990). Phenylarsine oxide, which has effects on N-type Ca^{2+} channel currents (Searl and Silinsky, 2000), protein tyrosine phosphatases (Lohmann et al., 2004), and phosphatidylinositol 4-kinase (Wiedemann et al., 1998), an important step in the 4,5-biphosphate signal pathway (Toker, 1998) that blocks receptor-mediated transcytosis (Knutson et al., 1983), was without effect on i.n. [^{125}I]Alb uptake. The same was true of verapamil, the L- and T-type Ca^{+} channel blocker (Bergson et al., 2011), which can also inhibit P-glycoprotein, an ATP-dependent drug transport protein (Schinkel, 1999). Albumin-binding proteins, such as endothelial membrane–associated gp60 (albondin) (Schnitzer, 1992), use caveolae-dependent mechanisms to transport albumin from peripheral blood vessel lumen to interstitium and are inhibited by filipin (Schnitzer et al., 1994; Tiruppathi et al., 1997), a cholesterol-binding agent that inhibits caveolae-dependent transcytosis (Schnitzer et al., 1994). Pretreatment with filipin did not affect [^{125}I]Alb uptake here, indicating that i.n. transport of albumin is not dependent on caveolae. The phosphoinositide 3-kinase inhibitor LY294002 was also without effect. Monensin, a compound that inhibits acidification of intracellular organelles to prevent endocytosis and delivery of macromolecules to lysosomes, had no effect on albumin transport (Hastings et al., 1994). Lidocaine, which acts on axons of sensory neurons to block the conduction of action potentials by closing voltage-dependent Na^{+} channels, has an attenuating effect on transport of materials in axons but had no effect on albumin (Lavoie et al., 1989; Hiruma et al., 2008). Also, vasogenic agents, such as histamine, did not increase transport of albumin, despite increasing BBB permeability (Stamatovic et al., 2008).

The consideration that albumin can be used to aid in the uptake or targeting of other biologicals was confirmed in our study with [^{125}I]Lep. Leptin is a 16-kDa protein that has effects on both feeding and cognition (Pelleymounter et al., 1995; Farr et al., 2006). As such, it has been considered for development both as an anorectic and as a treatment of cognitive impairments. However, the anorectic effects, although desirable in the treatment of obesity, would be a serious untoward effect in the treatment of dementia. Here, albumin had several beneficial effects on i.n. administered [^{125}I]Lep uptake by the brain. It decreased serum levels by supposedly retarding its clearance from brain, it increased its uptake to the hypothalamus, which would supposedly enhance its potency as an anorectic, and it decreased its uptake to nontarget sites, such as the cerebellum and possibly the olfactory bulb and whole brain.

In conclusion, we found that [^{125}I]Alb was taken up by and distributed throughout brain after i.n. administration, with little [^{125}I]Alb entering blood. [^{125}I]Alb uptake was dose-dependent and stimulated by PMA. Albumin influenced the distribution of [^{125}I]Lep, suggesting that i.n. albumin may be used to deliver substances to the CNS. Our observation that albumin readily reaches the brain after i.n. administration could indicate new applications for albumin in designing CNS-targeting drugs for the treatment of neurologic disease.

Authorship Contributions

Participated in research design: Banks, Salameh, Falcone, Kabanov, Yi.

Conducted experiments: Salameh, Falcone, Yi, Cordy, Mortell.

Performed data analysis: Banks, Yi, Falcone, Salameh.

Wrote or contributed to the writing of the manuscript: Falcone, Banks, Salameh, Yi, Kabanov.

References

Banks WA and Broadwell RD (1994) Blood to brain and brain to blood passage of native horseradish peroxidase, wheat germ agglutinin, and albumin: pharmacokinetic and morphological assessments. *J Neurochem* **62**:2404–2419.

Banks WA, During MJ, and Niehoff ML (2004) Brain uptake of the glucagon-like peptide-1 antagonist exendin(9-39) after intranasal administration. *J Pharmacol Exp Ther* **309**:469–475.

Banks WA, Morley JE, Niehoff ML, and Mattern C (2009) Delivery of testosterone to the brain by intranasal administration: comparison to intravenous testosterone. *J Drug Target* **17**:91–97.

Benedict C, Frey WH, 2nd, Schiöth HB, Schultes B, Born J, and Hallschmid M (2011) Intranasal insulin as a therapeutic option in the treatment of cognitive impairments. *Exp Gerontol* **46**:112–115.

Bergson P, Lipkind G, Lee SP, Duban M-E, and Hanck DA (2011) Verapamil block of T-type calcium channels. *Mol Pharmacol* **79**:411–419.

Charlton ST, Whetstone J, Fayinka ST, Read KD, Illum L, and Davis SS (2008) Evaluation of direct transport pathways of glycine receptor antagonists and an angiotensin antagonist from the nasal cavity to the central nervous system in the rat model. *Pharm Res* **25**:1531–1543.

Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, Arbuckle M, Callaghan M, Tsai E, and Plymate SR, et al. (2012) Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. *Arch Neurol* **69**:29–38.

Dhuria SV, Hanson LR, and Frey WH, 2nd (2010) Intranasal delivery to the central nervous system: mechanisms and experimental considerations. *J Pharm Sci* **99**: 1654–1673.

Elsadek B and Kratz F (2012) Impact of albumin on drug delivery: new applications on the horizon. *J Control Release* **157**:4–28.

Farr SA, Banks WA, and Morley JE (2006) Effects of leptin on memory processing. *Peptides* **27**:1420–1425.

Frey WH, II (2002) Bypassing the blood-brain barrier to deliver therapeutic agents to the brain and spinal cord. *J Drug Deliv Sci Technol* **2**:46–49.

Glowinski J and Iversen LL (1966) Regional studies of catecholamines in the rat brain. I. The disposition of [3H]norepinephrine, [3H]dopamine and [3H]dopa in various regions of the brain. *J Neurochem* **13**:655–669.

Greenwood FC, Hunter WM, and Glover JS (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem J* **89**:114–123.

Guillot FL and Audus KL (1990) Angiotensin peptide regulation of fluid-phase endocytosis in brain microvessel endothelial cell monolayers. *J Cereb Blood Flow Metab* **10**:827–834.

Hastings RH, Wright JR, Albertine KH, Ciriales R, and Matthay MA (1994) Effect of endocytosis inhibitors on alveolar clearance of albumin, immunoglobulin G, and SP-A in rabbits. *Am J Physiol* **266**:L544–L552.

Hiruma H, Shimizu K, Takenami T, Sugie H, and Kawakami T (2008) Effects of clonidine on lidocaine-induced inhibition of axonal transport in cultured mouse dorsal root ganglion neurones. *Br J Anaesth* **101**:659–665.

Iliff JJ, Lee H, Yu M, Feng T, Logan J, Nedergaard M, and Benveniste H (2013) Brain-wide pathway for waste clearance captured by contrast-enhanced MRI. *J Clin Invest* **123**:1299–1309.

Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SA, et al. (2012) A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med* **4**:147ra111.

Knutson VP, Ronnett GV, and Lane MD (1983) Rapid, reversible internalization of cell surface insulin receptors: correlation with insulin-induced down-regulation. *J Biol Chem* **258**:12139–12142.

Kratz F (2008) Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J Control Release* **132**:171–183.

Kratz F and Elsadek B (2012) Clinical impact of serum proteins on drug delivery. *J Control Release* **161**:429–445.

Lavoie PA, Khazen T, and Filion PR (1989) Mechanisms of the inhibition of fast axonal transport by local anesthetics. *Neuropharmacology* **28**:175–181.

Lochhead JJ and Thorne RG (2012a) Intranasal delivery of biologics to the central nervous system. *Adv Drug Deliv Rev* **64**:614–628.

Lohmann C, Krischke M, Wegener J, and Galla HJ (2004) Tyrosine phosphatase inhibition induces loss of blood-brain barrier integrity by matrix metalloproteinase-dependent and -independent pathways. *Brain Res* **995**:184–196.

Migliore MM, Vyas TK, Campbell RB, Amiji MM, and Waszczak BL (2010) Brain delivery of proteins by the intranasal route of administration: a comparison of cationic liposomes versus aqueous solution formulations. *J Pharm Sci* **99**: 1745–1761.

Nonaka N, Farr SA, Nakamachi T, Morley JE, Nakamura M, Shioda S, and Banks WA (2012) Intranasal administration of PACAP: uptake by brain and regional brain targeting with cyclodextrins. *Peptides* **36**:168–175.

Pardridge WM, Eisenberg J, and Cefalu WT (1985) Absence of albumin receptor on brain capillaries in vivo or in vitro. *Am J Physiol* **249**:E264–E267.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, and Collins F (1995) Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* **269**:540–543.

Reger MA, Watson GS, Green PS, Baker LD, Cholerton B, Fishel MA, Plymate SR, Cherrier MM, Schellenberg GD, and Frey WH, 2nd, et al. (2008) Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults. *J Alzheimers Dis* **13**:323–331.

Schinkel AH (1999) P-glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv Rev* **36**:179–194.
Schnitzer JE (1992) gp60 is an albumin-binding glycoprotein expressed by continuous endothelium involved in albumin transcytosis. *Am J Physiol* **262**: H246–H254.
Schnitzer JE, Oh P, Pinney E, and Allard J (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* **127**:1217–1232.
Schulz C, Paulus K, Jöhren O, and Lehnert H (2012) Intranasal leptin reduces appetite and induces weight loss in rats with diet-induced obesity (DIO). *Endocrinology* **153**: 143–153.
Searl TJ and Silinsky EM (2000) The phosphatidylinositol 4-kinase inhibitor phenylarsine oxide blocks evoked neurotransmitter release by reducing calcium entry through N-type calcium channels. *Br J Pharmacol* **130**:418–424.
Stamatovic SM, Keep RF, and Andjelkovic AV (2008) Brain endothelial cell-cell junctions: how to "open" the blood brain barrier. *Curr Neuropharmacol* **6**:179–192.
Thorne RG, Pronk GJ, Padmanabhan V, and Frey WH, 2nd (2004) Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience* **127**: 481–496.
Tiruppathi C, Song W, Bergenfeldt M, Sass P, and Malik AB (1997) Gp60 activation mediates albumin transcytosis in endothelial cells by tyrosine kinase-dependent pathway. *J Biol Chem* **272**:25968–25975.
Toker A (1998) The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr Opin Cell Biol* **10**:254–261.
Veening JG and Olivier B (2013) Intranasal administration of oxytocin: behavioral and clinical effects, a review. *Neurosci Biobehav Rev* **37**:1445–1465.
Wiedemann C, Schafer T, Burger MM and Sihra TS (1998) An essential role for a small synaptic vesicle-associated phosphatidylinositol 4-kinase in neurotransmitter release. *J Neurosci* **18**:5594–5602.
Wu-Zhang AX and Newton AC (2013) Protein kinase C pharmacology: refining the toolbox. *Biochem J* **452**:195–209.

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