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Intranasal Phosphoramidon Increases Beta-Amyloid Levels in Wild-Type and NEP/NEP2-Deficient Mice

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Abstract Intranasal administration is emerging as a reliable and non-invasive method to bypass the blood–brain barrier and deliver drugs to the brain. This approach has been primarily used to explore therapeutic avenues for neurological diseases. However, intranasal administration could also be used to create animal models of brain disease. Beta-amyloid peptide (Aβ) accumulation is a key feature of Alzheimer's disease (AD), and the most common models of AD are transgenic mice expressing mutant human genes linked to familial AD. An alternative model of amyloidosis utilizes intracerebroventricular infusion of thiorphan or phosphoramidon to block the activity of key Aβ degrading enzymes (NEP, NEP2) resulting in accumulation of Aβ. Here, we demonstrate that intranasal administration of phosphoramidon produces significantly elevated cerebral Aβ levels in wild-type mice. Furthermore, intranasal phosphoramidon administration in double knockout mice lacking NEP and NEP2 also showed increased levels of $A\beta_{40}$. These data show that intranasal delivery of drugs can be used to model AD and suggest that other phosphoramidon-sensitive peptidases are degrading Aβ in NEP/NEP2-deficient mice.

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Introduction

Intranasal administration has been shown to bypass the blood–brain barrier and deliver a wide range of agents to the brain including small molecules, growth factors, viral vectors, and even stem cells (Hanson et al. 2009; Dhuria et al. 2010; Hanson and Frey 2008; Danielyan et al. 2009). This approach has the advantage of reduced invasiveness of the delivery procedure while limiting systemic exposure. The delivery of drugs (including phosphoramidon) that inhibit neprilysin to the brain by direct infusion using mini-osmotic pumps has been shown to induce dramatic elevations in beta-amyloid peptide (Aβ) levels in mice, rats, and rabbits (reviewed in Marr and Spencer 2010). This approach has also been used to model Alzheimer's disease (AD) (Dolev and Michaelson 2004). Furthermore, the observation that NEP inhibitors can induce amyloid pathology in rodents has implicated this Aβ-degrading enzyme as a primary mediator of Aβ catabolism (Iwata et al. 2000; Mouri et al. 2006; Nisemblat et al. 2007). However, the observation that NEP knockout mice do not develop dramatic elevations in Aβ has raised the possibility that other "NEP-like" enzymes that exist are blocked by NEP inhibitors (Iwata et al. 2001). Previously, it has been shown that a close homolog of NEP, termed neprilysin-2 (NEP2), can degrade Aβ and is sensitive to NEP inhibitors (Huang et al. 2008; Ikeda et al. 1999). In this study, we explore the use of intranasal administration of phosphoramidon as a method to elevate brain Aβ levels in mice. We also use this approach to show that NEP/NEP2 double knockout mice retain phosphoramidon-sensitive Aβ-degrading activity.

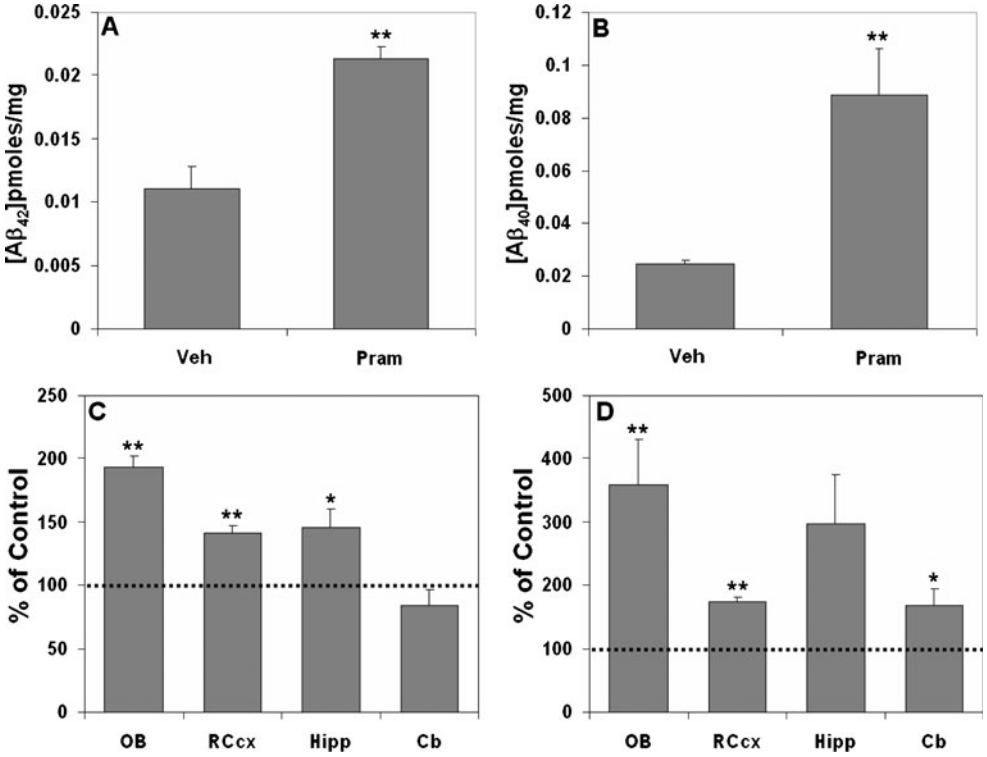

Fig. 1 Increased total Aβ after intranasal administration of phosphoramidon in wild-type mice. Levels of total $A\beta_{42}$ (**a**) and $A\beta_{40}$ (**b**) in the olfactory bulb (*OB*) of mice treated intranasally with 24 μl per day for 5 days of phosphoramidon (*Pram*, 30 mM) or vehicle (*Veh*). Relative levels of $A\beta_{42}$ (**c**) and $A\beta_{40}$ (**d**) presented as the percent of average control (vehicle) Aβ levels show increases in several brain regions including the rostral cerebral cortex (*RCcx*), hippocampus (*Hipp*), and cerebellum (*Cb*). Values are means±S.E.M, $n=6$. *$p<0.05$; **$p<0.01$

Methods

Intranasal Administration of Phosphoramidon

Phosphoramidon was dissolved in phosphate-buffered saline (PBS+1 mM ascorbic acid) at a concentration of 30 mM. Mice were treated intranasally as previously described (Hanson et al. 2004) except that isoflurane anesthesia was used. Briefly, anesthetized mice were placed on their backs and eight 3-μl drops of phosphoramidon solution were administered to alternating nares every 2 min. This was done once per day for 5 days. Mice were

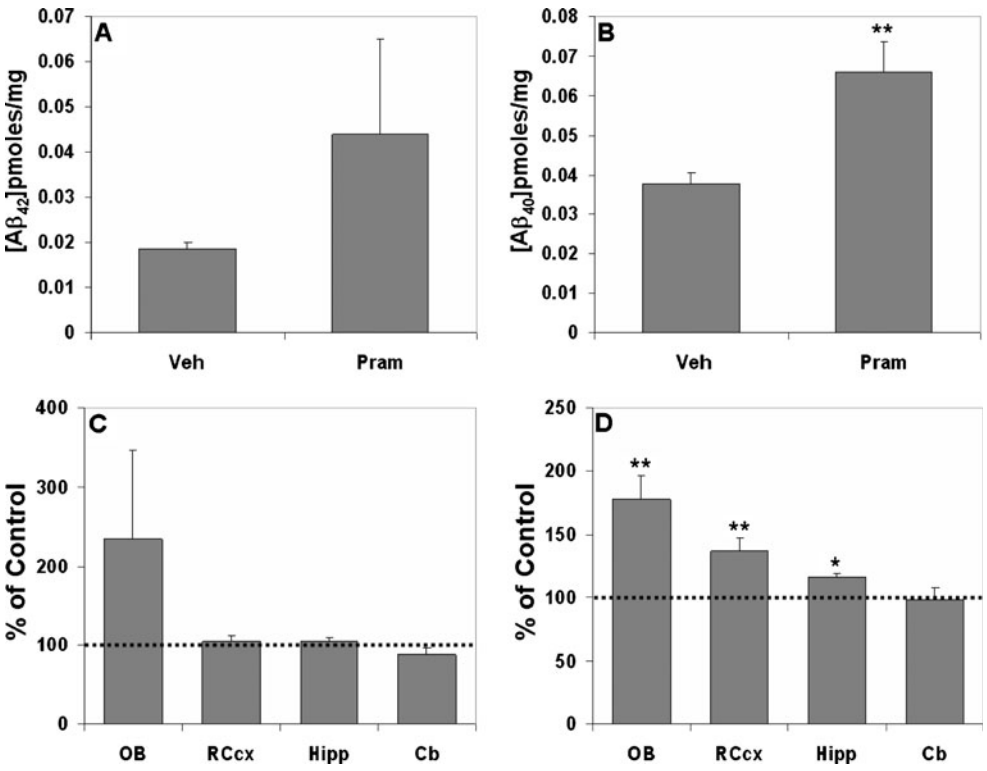

Fig. 2 Increased total Aβ after intranasal administration of phosphoramidon in NEP/NEP2 DKO mice. Levels of total $A\beta_{42}$ (**a**) and $A\beta_{40}$ (**b**) in the olfactory bulb (*OB*) of mice treated intranasally with 24 μl per day for 5 days of phosphoramidon (*Pram*, 30 mM) or vehicle (*Veh*). Relative levels of $A\beta_{42}$ (**c**) and $A\beta_{40}$ (**d**) presented as the percent of average control (vehicle) Aβ levels show increased $A\beta_{1-40}$ in several brain regions including the rostral cerebral cortex (*RCcx*) and hippocampus (*Hipp*), but not in cerebellum (*Cb*). Values are means±S.E.M, $n=10$. *$p<0.05$; **$p<0.01$

euthanized under anesthesia for tissue collection 2 h post phosphoramidon administration on day 5. Control mice were treated with intranasal PBS vehicle solution alone. Brains were removed and dissected into the desired brain regions before being homogenized in 5 M guanidine HCl to extract total Aβ. After centrifugation (16,000×*g*), the supernatants were diluted tenfold and Aβ (1–42 and 1–40) was quantified by specific ELISA (Wako Chemicals).

Results and Discussion

Intranasal Phosphoramidon in Wild-Type Mice

Figure 1 shows that treatment with phosphoramidon produced significantly elevated levels of total $A\beta_{1-42}$ and $A\beta_{1-40}$ in the olfactory bulb (OB) (Fig. 1a, b). This increased Aβ was found in other brain regions including the rostral cerebral cortex (RCcx) and hippocampus (Hipp) as shown by their levels relative to the average values from vehicle treated mice (Fig 1c, d). There was a general trend towards decreased elevations in Aβ as the distance from the nose increased, with little to no effect seen in the cerebellum (Cb). The hippocampus and cerebral cortex are both susceptible to AD pathology and important in AD.

Intranasal Phosphoramidon in NEP/NEP2 Double Knockout Mice

We generated double knockout mice by crossing mice genetically deficient for the NEP and NEP2 genes (Carpentier et al. 2004; Lu et al. 1995). These mice were also intranasally treated with phosphoramidon. Since NEP and NEP2 are known Aβ-degrading enzymes targeted by phosphoramidon, it was hypothesized that these mice would no longer be responsive to the drug with increased Aβ levels. However, Fig. 2 shows that Aβ levels were significantly elevated by phosphoramidon. The levels of total $A\beta_{1-42}$ showed a trend towards increased expression in the OB (Fig. 2a), while a significant increase was found for total $A\beta_{1-40}$ (Fig. 2b). Elevations in $A\beta_{1-40}$ were observed in multiple brain regions, with decreasing effects seen in samples taken at greater distance from the OB (Fig. 2d). These data suggest that as yet undetermined phosphoramidon sensitive enzymes are still present in NEP/NEP2 double knockout mice. However, the effect appears to be selective for the $A\beta_{1-40}$ isoform, suggesting that the remaining phosphoramidon sensitive enzyme(s) is/are selective for $A\beta_{1-40}$.

These data show that intranasal administration of drugs that affect aspects of AD pathology could be used to create new animal models of AD. The usefulness of this approach would be dependent on several factors, including the activity and stability of the drug and the duration of administration needed to affect pathology. In our case, 5 days was sufficient to observe significant effects on Aβ. Equally as important, behavioral studies must be done to determine if the changes in brain pathology are reflected in changes in key behavioral measures of memory and other AD-related behaviors. Finally, the results shown in Fig. 2 suggest that in addition to NEP and NEP2, there are more phosphoramidon-sensitive enzymes controlling Aβ levels in the mouse brain. While other members of the M13 family (including ECE, PHEX, and DINE) are clear candidates, additional enzymes outside this family that show some sensitivity of phosphoramidon may also be contributing. Inhibition (by thiorphan) or knockout of NEP has been shown to increase both $A\beta_{1-42}$ and $A\beta_{1-40}$(Mouri et al. 2006; Iwata et al. 2001). Also, we are finding that genetic ablation of NEP2 has a clear effect on both $A\beta_{1-42}$ and $A\beta_{1-40}$ levels (Hafez et al. 2010, *American Journal of Pathology*, in press). However, in the absence of NEP and NEP2, phosphoramidon clearly elevates $A\beta_{1-40}$ levels relative to $A\beta_{1-42}$ levels. This would suggest that NEP and NEP2 may be the major "NEP-like" $A\beta_{1-42}$-degrading (phosphoramidon-sensitive) enzymes in the rodent brain.

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