Mapping the active site in vasoactive intestinal peptide to a core of four amino acids: Neuroprotective drug design

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ABSTRACT The understanding of the molecular mechanisms leading to peptide action entails the identification of a core active site. The major 28-aa neuropeptide, vasoactive intestinal peptide (VIP), provides neuroprotection. A lipophilic derivative with a stearyl moiety at the N-terminal and norleucine residue replacing the Met-17 was 100-fold more potent than VIP in promoting neuronal survival, acting at femtomolar–picomolar concentration. To identify the active site in VIP, over 50 related fragments containing an N-terminal stearic acid attachment and an amidated C terminus were designed, synthesized, and tested for neuroprotective properties. Stearyl-Lys-Lys-Tyr-Leu-NH₂ (derived from the C terminus of VIP and the related peptide, pituitary adenylate cyclase activating peptide) captured the neurotrophic effects offered by the entire 28-aa parent lipophilic derivative and protected against β-amyloid toxicity in vitro. Furthermore, the 4-aa lipophilic peptide recognized VIP-binding sites and enhanced choline acetyltransferase activity as well as cognitive functions in Alzheimer’s disease-related in vivo models. Bio-distribution studies following intranasal administration of radiolabeled peptide demonstrated intact peptide in the brain 30 min after administration. Thus, lipophilic peptide fragments offer bioavailability and stability, providing lead compounds for drug design against neurodegenerative diseases.

With aging population outnumbering the young population, Alzheimer’s disease will become an immense health problem in the early years of the next century. Epidemiological studies indicate that senile dementia of the Alzheimer’s type afflicts 3–5 million people in the United States alone (1, 2). The neurochemistry of Alzheimer’s disease involves degeneration of cholinergic systems as well as other neurotransmission pathways (3). Mismetabolism of the amyloid precursor protein and abnormal phosphorylation of the tau protein lead to pathological deposits in the diseased brain (4). Recent studies have identified genetic mutations in the amyloid precursor protein (presenilin-1 and -2) and the inheritance of the lipid carrier apolipoprotein E4 (ApoE) as major risk factors in Alzheimer’s disease, allowing the development of animal models (5–8). Genetic counseling and novel cognition-enhancing drugs represent future research with major clinical relevance (9).

The structurally related neuropeptides, vasoactive intestinal peptide (VIP, 28 aa) (10, 11) and pituitary adenylate cyclase activating peptide (PACAP, 27 or 38 aa) (12), are important molecules in the maintenance of neuronal survival (11, 13–15). A lipophilic VIP derivative, stearyl-norleucine17-VIP (SNV) (16–19), was previously described that was 100-fold more potent than VIP in promoting neuronal survival (19), acting at femtomolar–picomolar concentration (16, 19). Neuroprotection was observed in Alzheimer’s disease-relevant models: β-amyloid neurotoxicity in vitro, cholinergic blockade (16), and genetic impairments (ApoE knockout) in vivo (8).

It is desirable to have smaller molecules mimicking the parent peptide action while offering applicable benefit in better penetration through biological barriers and reduced possible enzymatic degradation sites. Indeed, in the case of nerve growth factor (NGF), small fragments, including antagonists (20) and agonists (21), were synthesized and exhibited certain efficacy. An original stepwise approach for the minimization of peptide hormones was employed with atrial natriuretic peptide and resulted in a size reduction from 28 residues to 15 residues, however, the final molecule was one-fifth as potent as the parent peptide (22).

In the case of VIP, structure–function analyses were carried out and indicated that for most activities, the entire sequence of the peptide is required for full biological function. Thus, only VIP(2–28) retained similar biological activity to the parent peptide (23, 24), whereas shorter fragments (amino acids 7–28, 15–28, and 14–28) exhibited 1/10 to 1/1,000 potency as measured for vasodilation and smooth muscle relaxation (25) and even lower potency for other activities (24). Interestingly, the 17-norleucine (Nle) replacement in VIP(14–28) resulted in a peptide that was as potent as the parent VIP(14–28) and had about 2% of the entire VIP potency (26). Thus, the Nle replacement does not decrease (26), but may increase, potency of VIP derivatives, with Nle17-VIP being 10-fold more potent than VIP in maintaining neuronal survival (19). As in our assays, SNV was 100-fold more potent than VIP in maintaining neuronal survival (19); it is possible that the stearyl moiety is an important addition to the VIP molecule (19). With this rationale, a series of lipophilic VIP/PACAP fragments were prepared that shared an N-terminal addition of stearyl moiety and an amidated C terminus. This study resulted in the discovery of a 4-aa lipidic derivative that surpassed the activity of the parent whole molecules.

MATERIALS AND METHODS

Peptide Syntheses. Synthesis of a battery of small peptides was achieved by automated procedure with an Abimed AMS 422 synthesizer (Abimed Analyses-Technik, Langenfeld, Germany) using the commercially available protocols via the fluorenlymethoxycarbonyl strategy. Peptide chains were assembled on a 4-[(2,4-dimethoxyphenyl)-fluorenlymethoxycarbonyl-aminomethyl]phenoxy resin (Rink amide resin, Nova

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: VIP, vasoactive intestinal peptide; SNV, steryl-Nle17-VIP; AF64A-ethylcholine aziridinium; ApoE, apolipoprotein E; NGF, nerve growth factor; PACAP, pituitary adenylate cyclase activating peptide; St-KKYL-NH₂, stearyl-Lys-Lys-Tyr-Leu-NH₂; Nle, norleucine.

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The lipophilic stearyl group was added at the termination of the reaction to the free α-amino group (16). The cleaved peptide (after treatment with 90% trifluoroacetic acid/5% water/5% triethylsilane, vol/vol/vol) was precipitated, dissolved in water, and lyophilized. Purification of the crude peptide was achieved by semipreparative HPLC on an RP-8 column (Merck 250 × 10 mm; 7 μm) employing a linear gradient established between 35% acetonitrile and 75% acetonitrile in water containing 0.1% trifluoroacetic acid. Purity was ascertained by analytical HPLC (RP-18, Merck, 250 × 4 mm; 5 μm), using the same gradient. Further measurements used amino acid analysis and electrospray ionization mass spectrometry on VG Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.). Peptide labeling was performed as described (18).

**Cell Culture.** Cultures from astrocyte-enriched (27) and mixed neuronal–glial cultures were used for preparations of cerebral cortical cultures derived from newborn rats. Astrocytes were used for binding and displacement assays as described (27). For mixed neuronal–glial cultures, used to assess neuronal survival (16), dissociated cerebral cortical tissue was seeded on to a confluent layer of astroglial cultures (27). Cells (1.5 × 10^6) were placed into the 35-mm dish in a volume of 1.5 ml. The mixed cultures were maintained in medium consisting of 5% horse serum in MEM supplemented with defined medium components and 5-fluoro-2′-deoxyuridine (15 μg/ml) plus uridine (3 μg/ml) as described (16). Cultures were treated once and assayed for neuronal survival after a 5-day incubation period. The β-amyloid peptide (α-amino acids 25–35) was added to a final concentration of 25 μM. The test peptides (1 mg) were dissolved in 10 μl of DMSO, followed by serial dilutions in saline (which was different than the method described in ref. 16). Neuronal cell counts were conducted as described (16) after fixation and staining with antibodies against neuron-specific enolase (a neuron-specific marker).

**An in Vivo Model of Cholinergic Inhibition.** Male Wistar rats (300–350 g) were subjected to two daily tests in a water maze that included a hidden platform (28). Every day, for the first test, both the platform and the animal were situated in a new location with respect to the pool, which was immobile. The experiment was performed as follows: the animal was placed on the platform for 0.5 min and then placed in the water, and the time required to reach the platform (indicative of learning and intact reference memory) was measured (first test). After 0.5 min on the platform, the animal was placed back in the water (in the previous position) for a second test to search for the hidden platform (which also remained in the previous position). The time required to reach the platform in the second trial was recorded, indicative of short-term (working) memory (7). Animals were tested for 4 consecutive days to eliminate random memory-defective animals. The best performers were injected i.c.v. on each side at a rate of 0.21 μl/min with the cholinotoxin ethylhchine aziridium (AF64A, 3 nmol/2 μl); control animals received an injection of saline (16). Animals were allowed to recover for 1 week, followed by daily exposure to intranasal administration of 40 μl of 5% Sefsol (Sigma) and 20% isopropanol (control) or containing 1 μg of peptide (experimental) (16). After 1 week of peptide treatment, the animals were subjected to two daily tests in the water maze (as above). During the test period, animals received an intranasal administration of peptide or vehicle (carrier) 1 hr before the daily test.

**ApoE-Deficient Mice.** ApoE-deficient mice were used as described (8). Subcutaneous injection of the peptide included 1-μg daily injections on the first 4 days of life, 2 μg on days 5–10, and 4 μg on days 11–14. Peptide preparation involved dissolving 1 mg of peptide in 50 μl of DMSO followed by serial dilutions in a saline solution. Three-week-old mice were tested for performance in the Morris water maze as above. Choline acetyl transferase measurements were performed on 21-day-old animals (8).

**Biodistribution After Intranasal Administration.** Labeled-peptide distribution was monitored after intranasal administration (to ether-anesthetized rats) of 2 μl of peptide in a solution containing 5% Sefsol and 20% isopropanol (16).

**Statistical Evaluation.** Comparisons were made with ANOVA followed by the Student–Newman–Kuel’s multiple comparison of means test.

**RESULTS**

A 4-aa Lipophylic Derivative of VIP Provides Neuroprotection *in Vitro.* To test VIP-related peptide analogues for neuroprotective actions, cerebral cortical cultures containing a mixed population of neurons and glial cells were treated with a fragment (α-amino acids 25–35) of the Alzheimer’s-related neurotoxin [the β-amyloid peptide (e.g., ref. 16)], resulting in 38–66% neuronal cell death, in over 100 reproducible experiments. Fig. 1 demonstrates the *in vitro* neuroprotective properties against β-amyloid neurotoxicity of the 4-aa lipophylic peptide St-KKYL-NH₂ (Stearyl-Lys-Lys-Tyr-Leu-NH₂). Neuroroprotection was observed at a wide range of concentration of St-KKYL-NH₂ (from 10⁻¹⁴ to 10⁻⁹ M, P < 0.002; estimated EC₅₀ = 0.5 × 10⁻¹⁵M) and ranged from 80–110% survival, in comparison to 52% survival with β-amyloid alone and 100% survival in the absence of both the neurotoxin and the protective peptide fragment. 110% survival probably reflects defense against naturally occurring cell death in this system (16). The identification of St-KKYL-NH₂ as an active VIP-derived fragment resulted from multiple screening experiments by using the same neuronal survival assays that analyzed and determined the activity of about 50 VIP-related derivatives. With VIP sequence HSD4AVFTDNYTRLQMAKVKYLNNSIL-NH₂ initial studies determined inactivity for stearyl-AVFTDNYT-NH₂, stearyl-AVFTTNDNY-NH₂ and steryl-AVFTDNYT-NH₂.
stearyl-DNYTRL-NH₂ peptides residing at the N terminus of the VIP molecule (italicized) that were tested at concentrations ranging from 10⁻¹³ to 10⁻⁸ M. Further studies showed neuroprotective activity for VIP fragments residing at the C terminus of the molecule, and these peptides were studied extensively. Table 1 compares 16 of the peptides, related to the C-terminal domain, tested for their relative potency and efficacy in the in vitro neuroprotection assay. A survival rate of 100% reflected the number of neurons in control cultures (not treated with the β-amyloid peptide). A peptide protecting also against naturally occurring cell death (see above), St-KKYLN-NH₂, was chosen for further studies. St-KKYLN-NH₂ contained a very short peptidergic backbone and was easily soluble, in contrast to other peptides (e.g., St-NSILN-NH₂, peptide 11 in Table 1). Structure–function studies revealed that the Leu-4 residue of St-KKYLN-NH₂ might be replaced by a hydrophobic amino acid residue (e.g., peptides 2–4, Table 1), while preserving some neuroprotective capacities. However, removal of the amide from the C-terminus resulted in loss of function (peptide 13, Table 1). Similarly, exchange of the second Lys, or the third Tyr with D-Ala resulted in inactive peptide (peptides 14, 15). Furthermore, exchange of the Lys residues with Arg residues resulted in loss of activity (peptide 16). In contrast, removal of one of the lysine residues resulted in a peptide with substantial biological activity (peptide 5).

Comparison of the protective action against β-amyloid neurotoxicity of St-KKYLN-NH₂, SNV (the parent 28-aa lipophilic peptide), and St-KKYLN-OH is shown in Fig. 1. As evidenced, St-KKYLN-NH₂ offered neuroprotection that was similar to SNV (estimated EC₅₀ = 10⁻¹⁰ M), whereas St-KKYLN-OH (a control peptide) was inactive in this system. Furthermore, tarcine (an inhibitor of acetylcholinesterase, a current drug treatment against Alzheimer’s disease symptoms, e.g., ref. 9) also was inactive in this system (data not shown).

Binding and displacement experiments were performed as described (27). Input was 125I-VIP 200,000 cpm per ml; 100% binding represented 18,700 cpm (in two independent experiments with 10⁻⁶ M VIP indicated a similar 80%–110% binding). Comparison of the protective action against β-amyloid toxicity of St-KKYLN-NH₂, SNV (the parent 28-aa lipophilic peptide), and St-KKYLN-OH is shown in Fig. 1. As evidenced, St-KKYLN-NH₂ offered neuroprotection that was similar to SNV (estimated EC₅₀ = 10⁻¹⁰ M), whereas St-KKYLN-OH (a control peptide) was inactive in this system. Furthermore, tarcine (an inhibitor of acetylcholinesterase, a current drug treatment against Alzheimer’s disease symptoms, e.g., ref. 9) also was inactive in this system (data not shown).

Binding and displacement experiments were performed as described (27). Input was 200,000 cpm ¹²⁵I-VIP per ml; 100% binding represented 18,700 cpm (in two independent experiments, each performed in triplicate). Results indicated that 10⁻⁵ M St-KKYLN-NH₂ displaced 68% ± 2% iodinated VIP from its binding sites on astroglial cells (P < 0.0001). Parallel experiments with 10⁻⁵ M VIP indicated a similar 80% ± 3% displacement (see also ref. 27).

St-KKYLN-NH₂ Provides Neuroprotection in Vivo. Previous results have shown a reduction in choline acetyltransferase activity in the cerebral cortex of ApoE-deficient animals (8). Here, ApoE-deficient animals were injected daily with St-KKYLN-NH₂ or vehicle (see Materials and Methods) for the first 14 days of life. At day 21, animals were sacrificed and cortical choline acetyltransferase activity was assessed (8). Fig. 2A shows that St-KKYLN-NH₂ treatment resulted in increased enzymatic activity in the deficient mice (P < 0.008) to levels similar to those found in control animals. Furthermore, St-KKYLN-NH₂ administration facilitated faster development of milestones of behavior (comparable to results obtained with SNV administration; ref. 8 and data not shown).

Cognitive functions were assessed in two animal model systems capturing aspects of Alzheimer’s disease: (i) ApoE-deficient mice injected daily with St-KKYLN-NH₂ for the first 2 weeks of life with memory tests performed 1 week after cessation of treatment (as above, for cholinergic measurements) and (ii) cholinergically impaired animals (treated by the cholinoxin AF64A as described in ref. 16) with St-KKYLN-NH₂ delivered intranasally before daily trials. Improvements in learning and memory functions were tested in a swim maze, including a hidden-platform test (28) performed twice daily.

Results showed improvements in both reference memory (first daily test) and short-term memory (second daily test, Fig. 2B and C) after drug treatments in the two model systems. In the ApoE-deficient animals (3-week-old mice derived from three different litters, n = 15), the improvement in reference memory already was significant on the second testing day and persisted for an additional two days (P < 0.001, data not shown). Similarly, for short-term memory evaluations (Fig. 2B), significant improvements were observed by the first day of testing and persisted for 4 additional days (P < 0.01). Animals treated with a control peptide that included the sequence VLGGSALLRSIPA (29) were not significantly different from vehicle-treated animals (data not shown). In AF64A-treated adult rats (n = 9–11 animals per test group), significant improvements in reference memory were observed in the second day of testing (P < 0.02, data not shown). A more dramatic influence was observed in the short-term memory test (Fig. 2C), with improvements persisting for the entire trial period (P < 0.02). When the platform was removed from the water maze and the time spent by the trained animals at the original location of the platform was measured, significant increases were observed with peptide-treated animals. Control rats treated with St-KKYLN-NH₂ spent 22 ± 3.7% more time at the memorized location as compared with vehicle-treated controls (P < 0.0001). In contrast, control animals treated with St-KKYLN-OH did not show an improvement after peptide

<table>
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<tr>
<th>Peptide</th>
<th>Structure</th>
<th>EC₅₀, M</th>
<th>Maximal Survival, %</th>
<th>Active concentration, M</th>
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<td>1</td>
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<tr>
<td>4</td>
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<td>76–84 (low efficacy)</td>
<td>10⁻¹⁴–10⁻¹²</td>
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<td>83 (low efficacy)</td>
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<td>84 (low efficacy)</td>
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Table 1. Peptide activity in the neuronal survival assay: Protection against β-amyloid toxicity

Neuronal survival was assessed as in Figure 1. 100% survival represented the surviving neurons in a given experiments that were not treated with neither toxin (β-amyloid) or a VIP-derived peptide. After addition of β-amyloid only 38%–66% (50.4 ± 1.7%) neurons survived. Peptide concentrations tested were as in Fig. 1, except for peptides 3 and 4 (tested at 10⁻¹⁵ M–10⁻¹¹ M) and peptide 6 tested also at 10⁻⁸ M. St = stearyl. Conc. = concentration.
treatment. These data suggest increased efficacy for St-KKYL-NH₂ in comparison to St-KKYdAla-NH₂.

Bioavailability and Stability of St-KKYL-NH₂. A time course of distribution of ¹²⁵I-radiolabeled St-KKYL-NH₂ that was applied intranasally was measured in the various organs of the rat body. Results (Fig. 3A) demonstrated high levels of radioactivity in the intestine and kidney even 150 min after administration. The incorporation into the brain (frontal cortex) represented ~3.5% of the original input 150 min after administration (Fig. 3B). For the determination of the percentage of radioactivity remaining in the intact molecule, each animal received 7.8 million counts. Thirty minutes after nasal administration, animals were sacrificed. Postmitochondrial supernatant obtained from the cerebral cortex [0.965 g of original tissue weight, containing 1,392 cpm (5.3%) of the applied radioactivity] was subjected to HPLC analysis. Results shown in Fig. 3C indicated that ~1% of this total radioactivity

**Fig. 2.** (A) ApoE-deficient mice exhibit a reduction in choline acetyltransferase (ChAT) activity: amelioration by St-KKYL-NH₂. The graph depicts incorporation of radiolabeled choline into acetylcholine in cortical extracts. Activity levels of 100% in the control age-matched mice corresponded to 669–758.4 pmol/mg protein per min. Because the experiments were repeated 8–10 times, results were standardized against the control calibrated at a 100%. (B) ApoE-deficient mice exhibit an impairment in learning and memory that is ameliorated by prophylactic St-KKYL-NH₂ treatment. Two daily water maze tests were performed on 3-week-old animals. Groups tested included control animals injected with vehicle for the first 2 weeks of life [32 animals of six different litters (5–7 animals from each litter; ○)]; ApoE-deficient animals injected with vehicle for the first 2 weeks of life [18 animals derived from three different litters (5–7 pups per litter); ●]; control animals chronically treated with St-KKYL-NH₂ for the first 2 weeks of life (15 animals derived from three different litters; □); and ApoE-deficient mice chronically treated with St-KKYL-NH₂ for the first 2 weeks of life (15 animals derived from three different litters; ■). (C) AF64A-treated rats exhibit learning and memory deficiencies—protection by St-KKYL-NH₂. AF64A was used as described (16, 42). Two daily water maze tests were performed. Groups included control animals receiving daily nasal administration of vehicle (n = 10; ○), AF64A-treated animals receiving daily nasal administration of vehicle (n = 10; ●), control animals receiving daily nasal administration of St-KKYL-NH₂ (n = 14; □), and AF64A-treated animals receiving daily nasal administration of St-KKYL-NH₂ (n = 10; ■). (B and C) Latency was measured in seconds to reach the hidden platform in the second daily trial and remaining on it for additional 0.5 min (indicative of intact working memory processes). Tests were performed over 4–5 consecutive days. ApoE, ApoE-deficient animals. There was no difference between animals treated with vehicle and untreated animals (data not shown).

**Fig. 3.** (A and B) Intranasally applied ¹²⁵I-St-KKYL-NH₂ reaches the body and the brain. Each rat (300 g) received 2 µl containing 1,723,373 ± 33,315 cpm, (i.e., 5,744 cpm/g). Animals were sacrificed at indicated times after administration, and tissue samples were weighed and assayed (in duplicate) for radioactivity in a γ-counter. (C) Intact ¹²⁵I-St-KKYL-NH₂ reached the brain after intranasal administration. Radioactive tissue samples (cerebral cortex) were homogenized and subjected to low-speed centrifugation. Supernatants were analyzed by using HPLC fractionation against St-KKYL-NH₂. Samples were monitored for radioactivity in a γ-counter.
was represented by soluble St-KKYL-NH₂. Thus, ~0.05% of the total radioactivity applied was found in intact St-KKYL-
NH₂ in the brain 30 min after nasal application.

**DISCUSSION**

An active site of 4 aa has been discovered that mimics the neuroprotective activity of the 28-aa peptide, VIP. St-KKYL-
NH₂ offers a few advantages over the parent peptide. (i) The EC₅₀ for the VIP neuroprotective effect against β-amylloid neurotoxicity was shown to be 10⁻¹² M (16), suggesting a several-fold increased potency for St-KKYL-NH₂. (ii) In contrast to Apo-E-deficient mice treated with SNV where significant improvements of short-term memory were manifested only on the second trial day (8), mice treated with St-KKYL-
NH₂ already exhibited significant improvements of short-term memory on the first trial day.

The structure of KKYL (Lys-Lys-Tyr-Leu) is conserved in both VIP and PACAP, suggesting an area of functional
importance. Both VIP and PACAP exert neuroprotection (13–16), and it is tempting to hypothesize that areas of structural similarities between the molecules are responsible for the neuroprotective properties. Though photoaffinity labeling and further analysis of the interaction of PACAP with the PACAP receptor, it was discovered that the Tyr-22 is important for receptor binding (30). This Tyr-22 corresponds to the Tyr in Lys-Lys-Tyr-Leu (KKYL). Because PACAP binds all of the cloned VIP receptors with similar or higher affinity than VIP (31), the KKYL epitope may represent an important site for receptor association. In this respect, we have recently shown (using specific antisense oligodeoxynucleotides) that the neuroprotective effects of VIP are mediated through a PACAP receptor splice variant (hop2, or PACAP4, ref. 32). The neuroprotective effects of VIP/SNV were shown previously to be independent of cAMP production in glial cells (19, 27). Furthermore, in mouse cortical neurons, VIP and PACAP potentiated the glutamate-evoked release of arachidonic acid through a PACAP receptor-associated cAMP-independent mechanism (33).

It is an interesting question whether VIP is physiologically cleaved to yield active fragments. According to Romualdi and colleagues (34), authentic VIP (22–28) can be found in the rat central nervous system, suggesting that VIP can be cleaved naturally. Furthermore, two primary cleavage sites on the VIP molecule were identified as the amide bonds between Ser-25 and Ile-26 and Thr-7 and Asp-8 (35). It is possible that exopeptidases cleave the VIP fragments to yield smaller derivatives; however, those peptide derivatives may have a very short half-life. Our previous results with stearyl-VIP (17) indicate that it has a longer half-life and increased activity as compared with VIP (half-life <1 min in the blood). The rationale of the current study was that stearyl-VIP/PACAP fragments might mimic stearyl-VIP. VIP has been previously shown to act in part, after receptor-mediated endocytosis (36). The finding of ~0.05% of the total radioactivity applied in intact St-KKYL-NH₂ in the brain 30 min after nasal application may suggest some loss of intact peptide caused by cellular internalization, similar to VIP.

The neurotrophic milieu produced by neuropeptide VIP-stimulated astroglia contains a number of proteins, the most potent of which are represented by the activity-dependent neurotrophic factors (ADNFs; refs. 37–39). It is possible that St-KKYL-NH₂ acts directly on neurons to provide neuroprotection and, indirectly, as a secretagogue for ADNF or ADNF-like molecules from glial cells.

Regardless of the mechanism of action and receptor sites involved, a lipophilic tetrapeptide peptide is suggested here for noninvasive (inhalation) Alzheimer’s treatment. The neuroprotection observed after peptide treatment in the genetic model of Alzheimer’s disease (ApoE-deficient mice, ref. 8), was reflected not only in improvements of learning and memory after cessation of peptide application (Fig. 2B) but also in faster acquisitions of developmental milestones of behavior (data not shown). Enhancement of learning and short-term memory was probably obtained, in part, by increasing cholinergic functions (Fig. 2A), as was noted before for VIP (8). St-KKYL-NH₂, like the parent VIP and PACAP may act in concert with classic neurotrophins (e.g., 40, 41) to provide cellular defense. With Alzheimer’s disease being the fourth leading cause of death in adults and with the steep rise with age that is so prevalent in advanced populations, the need for innovative, noninvasive Alzheimer’s disease therapies is obvious (4, 9).

The present study reveals a short peptide fragment, carrying a lipophilic moiety, that captures the activities of the 7-fold larger parent peptide. This peptide may provide a novel avenue for drug design for abundant neurodegenerations and a prototype approach for drug design with other major neuropeptides.

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