Neuroscience Letters 480 (2010) 83–86

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Amyloid-β production in aged guinea pigs: atropine-induced enhancement is reversed by naloxone

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ARTICLE INFO

Article history:
Received 1 April 2010
Received in revised form 15 May 2010
Accepted 2 June 2010

Keywords:
Alzheimer's disease
Aging
Cholinergic antagonist
Enkephalin
β-Amyloid (1–40)
β-Amyloid (1–42)
Guinea pigs

ABSTRACT

Advanced age, cholinergic deficit, and elevated brain levels of enkephalin are associated with sporadic Alzheimer's disease. The influence of these factors on production of amyloidogenic peptides (Aβ) is uncertain. In the present experiments, the levels of 40/42 amino acid-residue Aβ were measured in the brain cortex of guinea pigs aged 15–16 weeks (young) and 25–26 months (aged). As was found, injections of atropine (21 days, 5 mg/kg/day) increase Aβ levels in aged but not young animals. This atropine-induced effect was antagonized by simultaneous injections of naloxone (3 mg/kg/day) whereas naloxone alone failed to affect Aβ accumulation. These results are discussed in the light of a possible "acetylcholine – Aβ" feedback loop and an influence of enkephalin on the loop function.

Alzheimer's disease is characterized by the progressive brain pathology, including neuronal degeneration and functional decline. A sporadic form of the disease afflicts the elderly people and represents the major cause of senile dementia [10].

In recent years, much attention has been paid to the amyloidogenic peptides (Aβ) the levels of which are elevated in the brain tissue of patients [12,13,33,34,38]. Brain accumulation and deposition of Aβ is considered a critical event in sporadic Alzheimer's disease (sAD) [31]. It is widely accepted that preventing Aβ accumulation in the brain might alter the course of this disease. Meanwhile, no effective methods for an inhibition of the Aβ accumulation have been found up to now.

The increased Aβ deposition in the brain might be explained by an enhancement of the production of these peptides. In view of this, of interest is to determine whether the brain Aβ production can be stimulated by factors associated with sAD. Among such factors, advanced age as the most salient risk factor for the disease [19,24,41] should be noted first. Other typical sign of the disease is a decrease in brain cholinergic activity, including a loss of the brain cholinergic neurons [40] and a fall in acetylcholine concentration in cerebrospinal fluid [7,18,36,37]. A notable manifestation of sAD is also an elevated level of endogenous opioid peptides, enkephalins, in frontal cortex [27] and hippocampus [22].

To our knowledge, to date there has been no examination of how cholinergic deficit or enkephalin influences Aβ levels in normal aged animals. The present study addresses this issue.

The research protocol was approved by the local Animal Care and Use Committee. Male Dunkin Hartley guinea pigs aged 15–16 weeks (young) and 25–26 months (aged) were used.

Animals, which were housed in a temperature (22 ± 1 °C) and humidity (50–70%) controlled environment with a 12:12-h light/dark cycle (lights on from 6:00 to 18:00 h), had ad libitum access to food and water.

For use in experiments, animals of each age were divided randomly into groups.

Atropine sulfate salt monohydrate (atropine) and naloxone dihydrate hydrochloride (naloxone) (Sigma–Aldrich, St. Louis, MO) were dissolved in sterile saline (0.9% sodium chloride) just before use.

sAD-associated decreased intrabrain cholinergic drive was mimicked by multiple injections of muscarinic antagonist, atropine. This agent is known to be centrally active following systemic administration.

To examine the ability of enkephalins to affect an amyloid accumulation, naloxone, an antagonist of opioid receptors [21], was used. The drug after systemic administration readily enters the brain [8] and reverses enkephalin effects in the brain tissue [5,6].

Atropine and naloxone were injected subcutaneously (s.c.) at the doses 5 and 3 mg/kg/day, respectively. These doses were selected since they were found to induce brain effects in other studies [11,20]. The injections were performed once a day for

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doi:10.1016/j.neulet.2010.06.010

Table 1
Mean concentrations of Aβ40 and Aβ42 in guinea pig cerebral cortex (pg/g wet weight).

Group	Soluble Aβ40	Insoluble Aβ40	Soluble Aβ42	Insoluble Aβ42
I, young control (saline)	1031 ± 239[a]	<300[ND]	142 ± 16[d]	<300[ND]
II, young, atropine	1144 ± 286[a]	<300[ND]	133 ± 12[d]	<300[ND]
III, young, naloxone	1026 ± 234[a]	<300[ND]	145 ± 21[d]	<300[ND]
IV, aged control (saline)	1138 ± 311[a,b]	317 ± 68[c]	153 ± 17[d,e]	336 ± 44[f]
V, aged, atropine	2123 ± 686[*]	534 ± 91[*]	292 ± 28[*]	751 ± 123[*]
VI, aged, naloxone	1047 ± 314[a,b]	<300[ND]	146 ± 17[d,e]	329 ± 45[f]
VII, aged, atropine, naloxone	1256 ± 307[a,b]	328 ± 77[c]	144 ± 14[d,e]	357 ± 49[f]

Measurements are expressed as means ± SD ($n = 14$).
Means followed by same letter are not significantly different, $p > 0.05$.
[ND] Value is not detectable since sample Aβ concentration is below the detection limit of the ELISA used.
[*] Significant difference from all other groups, $p < 0.01$.

21 consecutive days. It is the longest reported administration of naloxone in small laboratory animals [28]. In the preliminary experiments in young guinea pigs, the combined s.c. injections of atropine and naloxone at the above-mentioned doses for 21 consecutive days induced no visible signs of toxicity; there was also no changes in body weight in treated animals ($n = 5$) compared to non-medicated controls ($n = 5$). Both in the preliminary and main experiments, the drugs were administered between 8:00 and 9:00 h.

As a control, animals received injections of sterile 0.9% sodium chloride.

The following groups of guinea pigs (14 animals each) were tested: I, young, administration of saline (young control); II, young, administration of atropine; III, young, administration of naloxone; IV, aged, administration of saline (aged control); V, aged, administration of atropine; VI, aged, administration of naloxone; VII, aged, administration of atropine and naloxone.Approximately 24 h after the final drug or saline injection, animals were sacrificed by carbon dioxide. The brains were immediately removed, snap-frozen, and stored at −80 °C until assayed [26].

Soluble and insoluble amyloidogenic peptides containing 40 (Aβ40) or 42 (Aβ42) residues were determined separately since both may participate in sAD-associated amyloidogenesis [39].

The Aβ levels were measured in the cerebral cortex, an area of the most intensive Aβ deposition in humans [25]. Cortical samples for analyses were prepared as described previously [42]. The frozen tissues were homogenized in 4 volumes of buffer A containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and a protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany) with 10 strokes of a Teflon glass homogenizer and centrifuged at 200,000 × *g* for 25 min at 4 °C. The supernatant was used as the soluble fraction. The pellet was solubilized by sonication with ultrasonic homogenizer in buffer A containing 6 M guanidine-HCl. The solubilized pellet was then centrifuged at 200,000 × *g* for 25 min at 4 °C, after which the supernatant was diluted 12-fold to reduce the concentration of guanidine-HCl and used as the insoluble fraction.

Aβ concentration in each sample was measured in duplicates.

The amino acid sequence of guinea pig Aβ is identical to human [4] therefore the antibodies against human Aβ can be used for the detection of the Aβ in guinea pig brain tissue [26].

Aβ40 and Aβ42 levels in the samples were determined using ELISA kits (TKHS-Set, The Genetics Company, Switzerland) following the manufacturer's instructions. Signals from ELISA were quantified at 450 nm using a microplate reader (Victor 3 1420, Perkin Elmer).

Data are expressed as means ± SD. The Kolmogorov–Smirnov one-sample test was used to assess normality of the data distribution. To analyze the effects of drugs in young and aged groups, a two-way ANOVA on ranks was used. Within groups of aged animals, atropine–naloxone interaction was analyzed with a one-way ANOVA on ranks. ANOVAs were followed by a non-parametric Tukey's test. Differences with a p value of less than 0.05 were considered statistically significant.

We found that 21-day injections of atropine or/and naloxone do not reduce body weight both in young and in aged animals (data not shown).

As is shown in Table 1, young and aged animals displayed similar Aβ levels in cerebral cortex tissue (significant differences between Groups I and IV are not revealed).

In aged animals (Group V), administration of atropine significantly ($p < 0.01$) elevated the levels of soluble and insoluble Aβ40 and Aβ42 compared to saline-treated controls (Group IV). In contrast, atropine was ineffective in young animals (Group II).

Naloxone abolished the atropine-induced elevations of Aβ levels in aged animals but did not affect basal Aβ accumulation both in aged (Group VI) and in young (Group III) guinea pigs.

Thus, injections of a cholinergic receptor antagonist, atropine, significantly increase Aβ concentration in cortex of aged but not young animals. The atropine ability to stimulate Aβ formation is not unexpected. As was observed elsewhere, the inhibition of cholinergic drive through cortical cholinergic denervation increases Aβ concentration in rabbit cerebral cortex [2,29]. At the same time, the stimulation of cholinergic processes with a muscarinic receptor agonist, carbachol, or with an acetylcholinesterase inhibitor, physostigmine, lowers the release of amyloidogenic peptides from cells in vitro [14] and reduces Aβ levels in guinea pig brain in vivo [3].

What is less clear is the reason why atropine stimulated Aβ formation in aged animals (Group V) only. It is possibly linked with a more profound cholinergic deficit in these animals. As was reported, normal aging is associated with marked damage of brain cholinergic neurons (for ref. see [15]). In view of these findings, the Aβ accumulation in Group V can be provided by net cholinergic deficit that is induced by both a physiological age-related neurodegeneration and atropine action. Atropine administration alone may be insufficient to stimulate Aβ accumulation in the absence of age-related cholinergic deficit, i.e. in young animals. It is undoubted, however, that some other aging-associated factor(s), along with the physiological damage of cholinergic neurons, may contribute to the atropine effect in aged animals.

Whatever the reason(s) of the above-mentioned age-selectivity of atropine effect, the atropine administration obviously produces a certain cholinergic deficit. Thereby, atropine in aged animal mimics sAD wherein the brain cholinergic processes are decreased compared to age-matched controls [7,18,36,37,40]. Now it was found for the first time that such an additional cholinergic deficit being produced in normal aged organism, can stimulate Aβ accumulation. Thus, the present data suggest a potential role of the excessive cholinergic deficit, which differentiates patients and age-matched controls, in sAD-associated overaccumulation of amyloid.

As was shown in the present experiments, the atropine-induced Aβ formation is inhibited by an opioid antagonist, naloxone. In