Inhibition of Neprilysin by Infusion of Thiorphan into the Hippocampus Causes an Accumulation of Amyloid β and Impairment of Learning and Memory

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ABSTRACT

An imbalance between anabolism and catabolism causes an accumulation of amyloid β-peptide (Aβ), which is a proposed trigger of the onset of Alzheimer’s disease. Neprilysin is a rate-limiting peptidase that participates in the catabolism of Aβ in the brain. We examined whether rats continuously infused with thiorphan, a specific neprilysin inhibitor, into the hippocampus develop cognitive impairments through accumulation of Aβ. Thiorphan infusion elevated hippocampal Aβ40 and Aβ42 levels in the insoluble but not the soluble fraction. Thiorphan-infused rats displayed cognitive impairments in the ability to discriminate in the object recognition test, associative learning in the conditioned fear learning test, and spatial memory in the water maze test, tasks that depend on the hippocampus. These cognitive abilities in the battery of behavioral tasks inversely correlated with insoluble Aβ contents in the hippocampus. The nicotine-stimulated release of acetylcholine in the hippocampus of thiorphan-infused rats was significantly lower than that in vehicle-infused rats. These results indicate that continuous infusion of thiorphan into the hippocampus causes cognitive dysfunction and reduces cholinergic activity by raising the level of Aβ in the hippocampus and suggest that a reduction of neprilysin activity contributes to the deposition of Aβ and development of Alzheimer’s disease.

Alzheimer’s disease is a progressive neurodegenerative disorder characterized by a global cognitive decline involving memory, orientation, judgment, and reasoning that affects 20 to 30 million people worldwide (Selkoe and Schenk, 2003). The accumulation of amyloid β-peptide (Aβ) is a proposed trigger of the decades-long pathological cascade leading to the development of Alzheimer’s disease (Hardy and Selkoe, 2002). Changes in the metabolic balance of Aβ are closely associated with the accumulation (Saido, 2003; Saido and Nakahara, 2003). Several enzymes play a critical role in balancing the metabolism of Aβ. In the anabolism of Aβ, formation of Aβ from amyloid precursor protein (APP) requires the activities of β- and γ-secretases (Selkoe and Schenk, 2003). β-Site APP-cleaving enzyme 1 exhibits the functional properties of β-secretase and catalyzes the initial process of formation of Aβ (Vassar et al., 1999). Following β-site cleavage, a second cut at the C terminus of the β-stab of APP by γ-secretase produces Aβ40 or Aβ42 (Suh and Checler, 2002). Presenilin is required for γ-secretase-dependent cleavage to produce Aβ (Zhang et al., 2000). Gene mutations of APP and presenilin in early-onset familial Alzheimer’s disease patients enhance the processing of APP to form Aβ, resulting in an accelerated accumulation of Aβ (Hardy and Selkoe, 2002). However, because the promotion of anabolism in the brains of sporadic Alzheimer’s disease patients

ABBREVIATIONS: Aβ, amyloid β-peptide; APP, amyloid precursor protein; ACh, acetylcholine; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; Veh/SAL, vehicle + saline; Thio/SAL, thiorphan + saline; Thio/NAL, thiorphan + naloxone.
seems to be rare, a reduction in catabolic activity involving Aβ-degrading enzyme(s) has been considered the most likely cause of the accumulation. Reverse genetic studies have so far identified neprilysin (Iwata et al., 2000), insulin-degrading enzyme (Farris et al., 2003), and endothelin-converting enzymes 1 and 2 (Eckman et al., 2003) as Aβ-degrading enzymes.

Several lines of evidence indicate that Aβ generated from axonally transported APP is released from presynaptic sites and contributes to extracellular amyloid deposition (Lazarov et al., 2002; Sheng et al., 2002). Neprilysin is also distributed to presynaptic and axonal regions in the cerebral cortex and the limbic region, including the hippocampus (Fukami et al., 2002; Iwata et al., 2004). The brain Aβ-elevating effect of a deficiency of neprilysin is greater than that of a deficiency of any other known Aβ-degrading enzyme (Iwata et al., 2001; Eckman et al., 2003; Farris et al., 2003). These findings suggest that, among all potential Aβ-degrading enzymes, neprilysin plays a major role in the degradation of Aβ in the brain (Saito et al., 2003; Saito and Nakahara, 2003; Iwata et al., 2005). To our knowledge, neprilysin is the only peptidase capable of degrading oligomeric forms as well as the monomeric form of Aβ (Kanemitsu et al., 2003; Iwata et al., 2005). Neprilysin gene-deficient mice have shown a gene dosage-dependent elevation of endogenous Aβ levels in the brain (Iwata et al., 2001). The expression levels of neprilysin in specific regions, such as the hippocampus and cerebral cortex, have been demonstrated to be selectively reduced not only in aged rodents (Iwata et al., 2002) but also during the early stages in sporadic cases of Alzheimer’s disease (Yasojima et al., 2001).

In pharmacological experiments, continuous infusion of a specific inhibitor for neprilysin, thiorphan, into the hippocampus of rats is known to raise the hippocampal Aβ levels and form the Aβ plaque (Iwata et al., 2000). The effect of these biochemical changes induced by continuous thiorphan infusion into the hippocampus on cognitive function has not been investigated. Thus, the present study was designed to investigate the effects of infusing thiorphan into the hippocampus, on cognitive function has not been investigated. Thus, the present study was designed to investigate the effects of infusing thiorphan into the hippocampus, and the change in endogenous Aβ levels in the hippocampus of rats.

Materials and Methods

Animals

Male 8-week-old Wistar rats (Oriental BioService, Kyoto, Japan), weighing 260 to 300 g at the beginning of the experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum, and were maintained on a 12-h light/12-h dark cycle (lights on at 9:00 AM and off at 9:00 PM). All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985).

Drugs

Thiorphan (N-(RS)-2-benzyl-3-mercaptopropanoyl-glycine) and naloxone were purchased from Sigma Chemical Co. (St. Louis, MO). Thiorphan (0.5 mg/ml) was dissolved in saline containing 1 mM ascorbic acid (adjusted to pH 6.8 with NaOH). Infusion (2.5 μl/h) of thiorphan into the hippocampus was continued for 4 weeks by attaching an infusion cannula to a miniosmotic pump (Alzet model 2ML4; DURECT Corp., Cupertino, CA). Neprilysin is also known as an enkephalinase because of its ability to cleave enkephalins and terminate peptidic neurotransmission (Turner and Tzanzawa, 1997). To exclude a possible involvement of enkephalins, the metabolism of which may be affected by the inhibition of neprilysin, in behavioral changes in the rat, naloxone (1 mg/kg i.p.) or saline (1 ml/kg i.p.) was administered 30 min before each experiment.

Surgery

The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and fixed on a stereotactic apparatus (Narishige, Tokyo, Japan). The cannula for infusing thiorphan was implanted into the bilateral hippocampus (coordinates: anteroposterior, −3.0 mm; mediolateral, ±2.5 mm from the bregma; dorsoventral, 3.4 mm from the skull), according to the atlas of Paxinos and Watson (1986). As a control, rats were infused with the vehicle alone (saline containing 1 mM ascorbic acid; adjusted to pH 6.8 with NaOH). We have confirmed that the vehicle itself failed to induce any behavioral and neurochemical changes at this flow rate (data not shown).

Experimental Design

Continuous hippocampal infusion of thiorphan for 3 days increases Aβ contents (Iwata et al., 2000). Thus, the tests started on day 7 after the start of thiorphan infusion and were carried out sequentially according to the experimental schedule shown in Fig. 1.

Measurement of Spontaneous Locomotor Activity

The measurement of locomotor activity in a novel environment was carried out on day 7 after the start of thiorphan infusion. Spontaneous locomotor activity was measured as previously reported with a minor modification (Yamada et al., 1999). Rats were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (45 × 26 × 40 cm) for 15 min, and locomotor activity was measured each 5 min using digital counters with infrared sensors (Scanet SV-10; Melquest Pty Ltd., Toyama, Japan). The system was equipped with photosensor frames in the side walls. Locomotor activity was defined as the total number of beam cuts due to horizontal movement measured by photosensors. The acrylic cage was wiped with paper towel between animals and kept clean.

Novel-Object Recognition Test

The experiments were carried out on days 9 to 14 after the start of thiorphan infusion and according to the method of Ennaceur et al. (1996) with a minor modification. Namely, the apparatus consisted of an open box (80 × 80 × 20 cm) made of wood, the inside of which was painted gray. Triplicate copies were made of the objects to be discriminated out of glass, plastic, or metal. The weight of the objects

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Fig. 1. Experimental schedule.
ensured that they could not be displaced by the rats. The apparatus was placed in a sound-isolated room. A light bulb fastened in the upper part of the room provided a constant illumination of approximately 40 lux at the level of the task apparatus.

Rats were individually habituated to the open box (with no object for 3 min) for 2 days (on days 9 and 10). All rats were given two habituation sessions on days 9 and 10 during which they were allowed 3 min to explore the apparatus (with no object present). On day 11, the rats were first tested on the standard version of the task. Three days after the standard version (on day 14), they were tested on the configural version.

**Standard Version.** The task consisted of a sample phase and a choice phase. At the start of sample phase two identical objects (A1 and A2) were placed in the back corner of the box, 10 cm from the side wall. A rat was then placed in the middle front of the box, and the total time the rat spent exploring the two objects was recorded by the experimenter with two stopwatches. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose. The rat was put back in its home cage after 3 min had elapsed. One hour after the sample phase, the rats were reintroduced into the open field for 3 min (choice phase). In the standard version of the object recognition task, the open field contained a third identical copy of the familiar object (A) and a new object (B).

**Configurational Version.** The conditions in the configural version were the same as those used in the standard version of the task, except that new a third identical copy of the sample stimulus was reconfigured for the choice phase. A reconfigured stimulus (A*) consisted of a different spatial arrangement of the elements of the original sample (A). This meant that although each constituent part of the sample was familiar, the overall appearance was novel.

**Analyses of Variance.** Analyses of variance were performed on the discrimination ratio, which is the difference in exploration time divided by the total time spent exploring the two objects in the choice phase (e.g., \( B - A/B + A \) in the standard condition and \( A^* - A/A^* + A \) in the configural condition).

**Contextual Fear Conditioning Tests**

The experiments were carried out on days 15 to 16 after the start of thiorphan infusion and are summarized as follows. On the first day, for measuring basal levels of the freezing response (preconditioning phase), the rats were individually placed in a conditioning cage equipped with a metal wire floor, and the freezing time was determined for 2 min. For training (conditioning phase), rats were placed in the conditioning cage and allowed to explore the cage freely for 60 s. At the end, rats received a foot shock of 0.5 mA for 0.5 s as an unconditioned stimulus through a shock generator (Neuroscience Idea Co., Ltd., Osaka, Japan). The contextual task was carried out 1 day after the fear conditioning. The freezing response was measured in the conditioning cage for 2 min in the absence of the conditioned stimulus. The freezing response was defined as the rat keeping all paws still and stooping down with fear, which was measured with a stopwatch.

**Morris Water Maze Test**

The experiments were carried out on days 18 to 22 after the start of thiorphan infusion. The Morris water maze task was performed as previously reported with a minor modification (Nitta et al., 1994; Yamada et al., 1999). The circular water tank (140 cm in diameter and 45 cm high) had four equally spaced quadrants (north, south, east, and west). A transparent platform was set at the east quadrant of the tank, 40 cm from the wall (10 cm in diameter, surface 2 cm below the surface of the water) in the reference memory task. The pool was located in a large room, in which there were some cues external to the maze. The positions of these cues were left unchanged throughout the task.

**Reference Memory Task.** The task was conducted twice a day for 5 consecutive days, with one session consisting of two trials (2 trials × 5 days; intertrial interval: 3 h). In each trial, the rat was placed in the water at one of five starting positions (that were spaced equally around the rim of the tank), with the sequence of the positions being randomly selected. The latency to escape onto the platform was measured. If the rat found the platform, it was allowed to remain there for 15 s and was then returned to its home cage. If the rat could not find the platform within 90 s, the trial was terminated, and the animal was put on the platform for 15 s. Escape latency was assigned using the Target2 system (Neuroscience Idea Co., Ltd.).

**Probe Task.** Three hours after the 10th training trial of the reference memory task on day 22, the platform was removed from the pool, and all animals underwent a 90-s spatial probe trial. The time spent in the quadrant where the platform had been located during training was measured using the Target2 system.

**Determination of Extracellular ACh Release**

On day 26 after the start of the infusion of thiorphan, the cannula was removed, and a dialysis probe was implanted to measure the release of ACh (Tran et al., 2003). In brief, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and fixed in a stereotaxic apparatus (Narisihige). A guide cannula (EICOM, Kyoto, Japan) was implanted into the hippocampus (anteroposterior, −3.8 mm; mediolateral, 2.2 mm from the bregma; dorsalventral, 2.0 mm from the skull). On day 27 (24 h after the implantation of the guide cannula), the dialysis probe (A-I-8-03, membrane length 3 mm; EICOM) was implanted into the hippocampus, and Ringer’s solution (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl2) containing 10−4 M eserine was perfused at a flow rate of 2.0 μl/min. The dialysate was collected every 15 min, and the amount of ACh in the dialysate was determined using a high-performance liquid chromatography system with electrochemical detection. After the basal release of ACh had reached a stable level, nicotine (free base, 3 mM) was infused for 30 min. The animal in which the dialysis probe was inserted incorrectly in the hippocampus was excluded from the statistic analysis.

**Determination of Aβ40 and Aβ42**

The amounts of Aβ40 and Aβ42 in the soluble and insoluble fractions were determined by a sandwich ELISA using a combination of the monoclonal antibodies BNT77/BA27 and BNT77/BC05 (Iwata et al., 2000). On the day 27 after the start of the infusion of thiorphan, rats were sacrificed by decapitation, and brains were quickly removed and placed on an ice-cold glass plate. The hippocampus was rapidly dissected out, frozen, and stored in a deep freezer at −80°C until assayed. The four or five frozen tissues were randomly chosen from each group and 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 200,000g for 20 min at 4°C. The supernatant was used as the soluble fraction. The pellet was solubilized by sonication in buffer A containing 6 M guanidine-HCl. The solubilized pellet was then centrifuged at 200,000g for 20 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. The amounts of Aβ40 and Aβ42 in each fraction were determined by sandwich ELISA.

**Statistical Analysis**

Statistical analysis was performed using the one-way or two-way analysis of variance (ANOVA) followed by Fisher’s test. Pearson’s correlation coefficient test was used to examine the relationship between cognitive function and Aβ contents in the hippocampus. A value of \( p < 0.05 \) was considered statistically significant. Data are expressed as means ± S.E.M.
Results

Endogenous Levels of Aβ40 and Aβ42 in the Hippocampus of the Thiorphan-Infused Rats. Endogenous concentrations of Aβ40 and Aβ42 in the hippocampus were measured after a battery of behavioral tests and the measurement of ACh release (Table 1). The hippocampal infusion of thiorphan led to a significant elevation of Aβ40 and Aβ42 levels in the insoluble fraction of the hippocampus. Naloxone pretreatment did not affect the elevation in levels of Aβ40 and Aβ42 in the thiorphan-infused rats. However, there was no significant difference in the Aβ content of the soluble fraction among the three groups.

Spontaneous Locomotor Activity in the Thiorphan-Infused Rats. The counts of spontaneous locomotor activity in the vehicle + saline (Veh/SAL)-, thiorphan + saline (Thio/SAL)-, and thiorphan + naloxone (Thio/NAL)-treated rats were measured on day 7 after the start of thiorphan infusion. There were no significant differences in the time course of locomotor activity (Fig. 2A) and total locomotor activity (Fig. 2B) among the groups. It seemed that there was no difference in emotional reactivity and habituation to a novel environment among the all of the groups.

Novel-Object Recognition Task in the Thiorphan-Infused Rats. In the measure of discrimination (d2) between new and familiar objects in the standard and configural conditions, Veh/SAL- and thiorphan-treated rats showed no significant difference. A significant inverse discrimination in the thiorphan-infused rats (Fig. 3, A and B). Naloxone administered before each sample phase did not change the measures of discrimination between the objects (Fig. 3, A and B). In the standard and configural conditions, there was no difference in the total amount of time spent exploring two objects in the sample or choice phase of all groups (Fig. 3, E and F). These results suggested there was no difference in motivation among the three groups. A significant inverse relationship between cognitive ability and insoluble Aβ contents in hippocampus was observed in the both versions of the novel-object recognition task (Fig. 3, C and D).

Figure 2. Effect of continuous infusion of thiorphan into the hippocampus on the locomotor activity in rats. The experiments were carried out on day 7 after the start of thiorphan infusion. Values indicate the means ± S.E.M. for six to eight animals. A, time course of locomotor activity. Results with the two-way ANOVA were not significantly different among the groups: F(2,51) = 0.93, p = 0.39. B, total locomotor activity. Results with the one-way ANOVA were not significantly different among the groups: F(2,17) = 0.80, p = 0.46.

Contextual Fear Conditioning Tasks in the Thiorphan-Infused Rats. In the preconditioning phase, the Veh/SAL-, Thio/SAL-, and Thio/NAL-treated rats hardly showed any freezing response. There were no differences in the basal levels of the freezing response among the three groups (data not shown).

In contextual fear conditioning, animals learned the context associated with the foot shock. The Thio/SAL-treated rats, compared with the Veh/SAL-treated rats, exhibited less of a freezing response in the contextual tasks 24 h after fear conditioning (Fig. 4A). Naloxone administered before fear conditioning did not change the freezing response in the thiorphan-infused rats (Fig. 4A). There was no difference in nociceptive response (flinching/running, jumping, or vocalization) for this foot shock in all of the groups. Therefore, it is unlikely that the impairment of the contextual learning in the conditioning phase of conditioned fear learning task in the thiorphan-infused rats is due to changes in nociceptive response. A significant inverse relationship between cognitive ability and insoluble Aβ contents in hippocampus was observed in the contextual fear conditioning task (Fig. 4B).

Water Maze Task in the Thiorphan-Infused Rats. Changes of escape latency, the time taken to find the hidden platform, in training trials in each group of rats are shown in Fig. 4A. The Thio/SAL- and Thio/NAL-treated rats exhibited a significantly prolonged escape latency compared with the Veh/SAL-treated rats (Fig. 5A). A 90-s spatial probe trial was carried out following the 10th training trial (Fig. 5B). The Thio/SAL-treated rats, compared with the Veh/SAL-treated rats, showed a significant decrease in the time spent in the quadrant in which the platform had been located during training. Naloxone administered before the probe trial did not change the amount of time spent in the trained quadrant in the thiorphan-infused rats (Veh/SAL: 35.28 ± 3.78%, Thio/SAL: 24.53 ± 3.59%, and Thio/NAL: 21.38 ± 2.17%). This impaired ability of the thiorphan-infused rats in the Morris water maze test did not reflect poor swimming ability, because the rats in all groups showed similar speed of movement (data not shown). A significant inverse relationship between cognitive ability and insoluble Aβ contents in hippocampus was observed in the probe trial of the Morris water maze task (Fig. 5C).

Hippocampal Extracellular ACh Release Stimulated by Nicotine in the Thiorphan-Infused Rats. Because continuous infusion of Aβ40 impaired the nicotine-evoked ACh release in the hippocampus (Tran et al., 2003), we ex-

Table 1

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<thead>
<tr>
<th></th>
<th>Veh/SAL</th>
<th>Thio/SAL</th>
<th>Thio/NAL</th>
<th>Insoluble Aβ</th>
<th>Total Aβ</th>
</tr>
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<tbody>
<tr>
<td>Soluble Aβ</td>
<td>0.095 ± 0.002</td>
<td>0.087 ± 0.004</td>
<td>0.089 ± 0.003</td>
<td>0.409 ± 0.018</td>
<td>0.504 ± 0.017</td>
</tr>
<tr>
<td>Veh/SAL</td>
<td>0.093 ± 0.008</td>
<td>0.079 ± 0.002</td>
<td>0.077 ± 0.002</td>
<td>0.321 ± 0.036</td>
<td>0.414 ± 0.035</td>
</tr>
<tr>
<td>Thio/SAL</td>
<td>0.746 ± 0.035**</td>
<td>0.466 ± 0.020**</td>
<td>0.455 ± 0.036*</td>
<td>0.541 ± 0.035**</td>
<td>0.531 ± 0.035**</td>
</tr>
<tr>
<td>Thio/NAL</td>
<td>0.688 ± 0.061**</td>
<td>0.466 ± 0.020**</td>
<td>0.545 ± 0.019**</td>
<td>0.541 ± 0.035**</td>
<td>0.531 ± 0.035**</td>
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*p < 0.05; **p < 0.01 compared with Veh/SAL-treated rats.
Fig. 3. Effect of continuous infusion of thiorphan into the hippocampus on the performance of rats in a spontaneous object recognition test under standard (A) and configural (B) conditions. The experiments were carried out on days 9 to 14 after the start of thiorphan infusion. Values indicate the means ± S.E.M for six to eight animals. Results with the one-way ANOVA were: training trial, \( F(2,17) = 8.91, p < 0.01 \); configural version, \( F(2,17) = 3.62, p < 0.05 \). \(*, p < 0.05; **, p < 0.01 \) compared with Veh/SAL rats. Relationship between the discrimination index of novel object and insoluble total Aβ contents in a spontaneous object recognition test under standard (C) and configural (D) conditions. Results with the Pearson’s correlation coefficient test: standard version, \( r(14) = -0.753, p < 0.05 \); configural version, \( r(14) = -0.608, p < 0.05 \). The solid line represents the regression line, which was estimated by plotting the change in the discrimination index of each version against insoluble total Aβ contents. The total time spent exploring two objects in the sample or choice phase of standard (E) and configural (F) conditions. Results with the one-way ANOVA were: standard version, \( F(2,17) = 0.18, p = 0.85 \) (sample phase) and \( F(2,17) = 1.05, p = 0.37 \) (choice phase); configural version, \( F(2,17) = 0.71, p = 0.51 \) (sample phase) and \( F(2,17) = 1.77, p = 0.19 \) (choice phase).

Fig. 4. A, effect of continuous infusion of thiorphan into the hippocampus on the performance of rats in a context fear-conditioned learning test. The experiments were carried out on days 15 to 17 after the start of thiorphan infusion. Values indicate the means ± S.E.M for six to eight animals. Results with the one-way ANOVA were \( F(2,17) = 11.72, p < 0.01 \). \*, \( p < 0.01 \) compared with Veh/SAL rats. B, relationship between percentage of freezing time and insoluble total Aβ contents in a context fear-conditioned learning test. Results with Pearson’s correlation coefficient test: \( r(14) = -0.756, p < 0.05 \). The solid line represents the regression line, which was estimated by plotting the change in percentage of freezing against insoluble total Aβ contents.

Fig. 5. Effect of continuous infusion of thiorphan into the hippocampus on the performance of rats in training (A) and probe (B) trials of the water maze task. The experiments were carried out on days 18 to 22 after the start of thiorphan infusion. Values indicate the means ± S.E.M for six to eight animals. Results with the two-way ANOVA were: training trial, \( F(2,170) = 6.78, p < 0.01 \). Results with the one-way ANOVA were: probe trial, \( F(2,17) = 4.10, p < 0.05 \). \*, \( p < 0.05 \) compared with Veh/SAL rats. C, relationship between spending time on the platform quadrant and insoluble total Aβ contents in probe trials of the water maze task. Results with Pearson’s correlation coefficient test were \( r(14) = -0.557, p < 0.05 \). The solid line represents the regression line, which was estimated by plotting the change in spending time on the platform quadrant against insoluble total Aβ contents.

Discussion

Although reduced levels of neprilysin, which could cause accumulation of Aβ, have been observed in the brains of sporadic Alzheimer’s disease patients (Yasojima et al., 2001) and of aged laboratory mice (Iwata et al., 2002), there have been few studies with animal models of the diminution in catabolic activity, as achieved by continuous infusion of thiorphan (Iwata et al., 2000; Dolev and Michaelson, 2004). Therefore, we investigated the effects of the direct infusion of thiorphan into the hippocampus, which is important for the formation of memory and is a region vulnerable to Alzheimer’s disease pathology, to elucidate the relationship be-

aminated the effect of thiorphan infusion on the nicotine-mediated ACh release in the hippocampus. In vehicle-infused rats, the extracellular level of ACh in the hippocampus was elevated approximately 2-fold by perfusion of nicotine-Ringer’s solution for 30 min and returned to the basal level within 90 min (Fig. 6). However, in the thiorphan-infused rats, the nicotine-stimulated release of ACh was significantly lower than that in vehicle-infused rats (Fig. 6). There was no difference in the basal levels of ACh in the hippocampus between the vehicle- and thiorphan-infused rats (1.095 ± 0.139 versus 1.326 ± 0.202 pmol/30 μl/15 min).
between the increase in hippocampal Aβ levels induced by inhibiting catabolic activity and cognitive dysfunction.

We found that the continuous infusion of thiorphan into the rat hippocampus caused the accumulation of endogenous Aβ40 and Aβ42 in the insoluble fraction of the hippocampus and induced impairments of novelty discrimination ability in the object recognition test, associative learning in the conditioned fear learning test, and spatial memory in the water maze test. It is unlikely that the impairment in the performance of the thiorphan-infused rats in learning and memory tasks is due to changes in motivation or sensorimotor function because the motivation for each of these behavioral tasks is different and different skills are required for good performance on each task. Actually, there was no difference in locomotor activity, total time spent exploring objects in the novel object task, and swimming speed in the Morris water maze task between the vehicle- and thiorphan-infused rats, indicating no changes in motor function and exploratory activity.

Neprilysin plays an important role in the degradation of Aβ in the brain (Iwata et al., 2000, 2001). In the present experiments, continuous administration of thiorphan for 27 days, an inhibitor of neprilysin, increased hippocampal Aβ40 and Aβ42 levels in the insoluble but not soluble fraction. Previous reports have shown that thiorphan treatment for 3 days increases the total Aβ42/Aβ40 ratio and Aβ42 but not Aβ40 levels in the insoluble fraction, although it increases Aβ40 but not Aβ42 levels in the soluble fraction (Iwata et al., 2000). Because of the additional two carboxyterminal residues with Aβ42, it is more aggregation-prone than Aβ40 (Jarrett et al., 1993). In addition, Aβ42 can polymerize into the insoluble fraction at a much faster rate than Aβ40 in the hippocampus only 3 days after thiorphan treatment, and Aβ40 forms later in the process of amyloid genesis by 27 days after thiorphan hippocampal infusion. In the previous immunohistochemical experiment, continuous thiorphan infusion into hippocampus for 30 days produced Aβ plaques, as seen in Alzheimer’s disease brains, and seemed to mimic the initial stage of amyloid deposition (Iwata et al., 2000). Aβ in an aqueous solution undergoes self-assembly, leading to the transient appearance of soluble oligomers or protofibrils and ultimately to insoluble fibrils (Ramírez-Alvarado et al., 2000). Thus, the impairment of memory functions may be related to the hippocampal accumulation of insoluble Aβ induced by thiorphan. On the other hand, neprilysin hydrolyzes and inactivates several neuropeptides, such as enkephalin, somatostatin, substance P, atrial natriuretic peptide, and cholecystokinin in vitro (Iwata et al., 2005). Neprilysin, previously termed an enkephalinase, is also a potent enkephalin-degrading enzyme and may be involved in peptidic neurotransmission (Turner and Tanzawa, 1997). Enkephalin affects cognitive function, e.g., [Leu]enkephalin impaired the acquisition of a one-way step-through active avoidance response (Janak and Martinez, 1990). To exclude the possible involvement of enkephalin, metabolism of which may be affected by the inhibition of neprilysin, in memory and learning tasks in the rat, we administered naloxone (1 mg/kg i.p.) to thiorphan-infused rats before each experiment. However, naloxone treatment had no effect on cognitive performance in the thiorphan-infused rats. Because naloxone (2 mg/kg) facilitates spatial memory (Gallagher et al., 1983), it seems that naloxone itself does not impair cognitive function in thiorphan-infused rats. The genetic approach supports our finding, because a deficiency of neprilysin does not significantly elevate enkephalin levels in the brain (Saria et al., 1997). In addition, neprilysin deficiency does not seem to alter the levels of somatostatin, cholecystokinin, and substance P in the hippocampal formation and cerebral cortex (Iwata et al., 2005), ruling out the presence of a redundant mechanism or pathways to metabolize these neuropeptides. Thus, the cognitive impairment in the thiorphan-infused rats may not be caused by the presence of redundant neuropeptides.

Previous reports have shown that continuous infusion of Aβ40 (Nitta et al., 1994) and Aβ42 (Yamada et al., 1999) into the rat cerebral ventricle impairs memory in several learning and memory tasks. These animals are impaired in terms of the nicotine-stimulated extracellular release of ACh (Tran et al., 2003) and long-term potentiation (Itoh et al., 1999). In this experiment, continuous infusion of thiorphan into the hippocampus resulted in an accumulation of Aβ, an impairment of the hippocampal nicotine-stimulated release of ACh, and various cognitive dysfunctions in the rats. The inverse relationship between Aβ accumulation in the hippocampus and cognitive ability was observed in the battery of behavioral tasks. Hippocampal damage disrupts object recognition memory (Ainge et al., 2005), spatial memory of the Morris water maze (Morris et al., 1982), and contextual fear conditioning (Phillips and LeDoux, 1992). Taken together, our results suggest that these hippocampus-dependent behavioral tasks were impaired by thiorphan-induced Aβ accumulation and disruption of ACh release. Although hippocampal Aβ measurements were performed 24 h after the thiorphan treatment was stopped, there were sufficient amounts of Aβ to evaluate the effect of thiorphan on the accumulation of Aβ. Because thiorphan-induced Aβ accumulation is found even 3 days after treatment (Iwata et al., 2000), the correlation between insoluble Aβ contents and cognitive deficits in behavioral test can be estimated. To evaluate more reliable relationships, we need to investigate the measurement of Aβ levels for each behavior test as a future experiment.

It was demonstrated in experiments in vivo that the overexpression of neprilysin in the brains of APP transgenic mice decreased the deposition of Aβ (Leissring et al., 2003; Iwata et al., 2004). In addition, the exposure of APP transgenic mice to an enriched environment results in a pronounced...
reduction in cerebral Aβ levels and amyloid deposits with an elevation of neprilysin activity (Lazarov et al., 2005). Recently, Saito et al. (2005) have reported that somatostatin, a neuropeptide, decreases Aβ levels in the mouse brain through up-regulation of neprilysin. These findings together with our results suggest that a reduction in neprilysin activity contributes to the development of Alzheimer’s disease by promoting deposition of Aβ and that an increase in neprilysin activity may be a therapeutic target in the treatment of Alzheimer’s disease.

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References


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