Behavioural Brain Research 219 (2011) 63–67

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Memory impairment in rats by hippocampal administration of the serine protease subtilisin

Edgar Kornisiuk[a], Marina Snitcofsky[a], Carlos Blanco[a], Alan L. Harvey[b], Trevor W. Stone[c,*,1], Diana Jerusalinsky[a,d,1]

[a] Instituto de Biología Celular & Neurociencias "Prof Eduardo De Robertis", Facultad de Medicina, University of Buenos Aires and CONICET, Buenos Aires, Argentina
[b] Institute of Pharmacy and Biological Sciences, University of Strathclyde, Glasgow G4 0NR, UK
[c] Institute of Neuroscience and Psychology, University of Glasgow, West Medical Building, Glasgow G12 8QQ, UK
[d] CBC, Universidad de Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form
13 December 2010
Accepted 16 December 2010

Keywords:
Avoidance
Memory
Subtilisin
Proteases
Hippocampus

ABSTRACT

Since the serine protease subtilisin has been reported to generate a novel form of long-term depression (LTD) in rat hippocampal slices, the present work was designed to determine whether it has any effect on learning and memory processes. Rats were used to examine the effects of subtilisin, injected directly into the dorsal hippocampus, on task performance in a step-through inhibitory avoidance of a mild footshock. The administration of 100 ng of subtilisin into each hippocampus, immediately after training, was sufficient to induce a detectable learning deficit with a footshock stimulus of 0.5 mA. Higher doses produced dose-related impairments in memory consolidation. These effects were not the result of irreversible toxicity, since rats trained with a higher amplitude footshock (0.75 mA) were able to perform as control animals; therefore, the amnesic effect was not further evident. Furthermore, the administration of subtilisin before avoidance training did not produce any detectable effect on performance during the training or test sessions, indicating that neither acquisition nor consolidation was affected. It is concluded that the post-training administration of a serine protease inhibitor is able to produce robust deficits of memory consolidation consistent with its ability to generate LTD, raising the possibility that related molecules could play physiological or pathological roles in the modulation of learning and memory.

1. Introduction

In addition to the wide range of small molecules which affect neuronal plasticity in the central nervous system (CNS) by acting on amino acid or amine receptors, several large molecules, usually protein, have also been shown to induce or modulate the processes of long-term potentiation (LTP) or long-term depression (LTD). These macromolecules include serine proteases such as tissue plasminogen activator (tPA) [1], neuropsin [2–5] and neurotrypsin [6], all of which are known to be present in brain.

Both LTP and LTD have been linked with specific aspects of learning and memory processing, LTD being associated particularly with the exploration and familiarisation of novel environments [7]. It is therefore of interest to an understanding of these plastic phenomena that we have shown a form of LTD that can be produced by a novel serine protease, cadeprin, and by the closely related bacterial protease, subtilisin [8]. Since it is well established that CNS proteases can alter neuronal excitability, cell viability, and cellular morphology [9,10] and that altered expression or mutations in some of these enzymes may be associated with Alzheimer's disease [11] or schizophrenia [12], we have now sought to determine whether subtilisin has any modulatory influence on a learning paradigm in rodents.

2. Materials and methods

2.1. Animals and surgical procedures

Experiments with rats were performed in strict accordance with the Review Committee of the Veterinary School (CICUAL), University of Buenos Aires and the International Brain Research Organization (IBRO), and are in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Male Wistar rats weighing 180–250 g from our own breeding colony were housed six in a cage, under a 12-h light/dark cycle at 25 °C, with water and food available *ad libitum*. The animals were anesthetized by a ketamine–xylazine mixture (75 mg/kg and 10 mg/kg ip, respectively), and were bilaterally implanted with 27-gauge guide cannulae, positioned 2.00 mm above the CA1 region of the dorsal hippocampus at coordinates: AP: −4.3 mm; LL: ±4.0 mm; DV: −1.2 mm, from bregma [13]. The number of animals used is indicated in the legends to the figures of results.

* Corresponding author.
E-mail address: t.w.stone@bio.gla.ac.uk (T.W. Stone).
[1] Equal contribution to this paper.

0166-4328/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2010.12.027

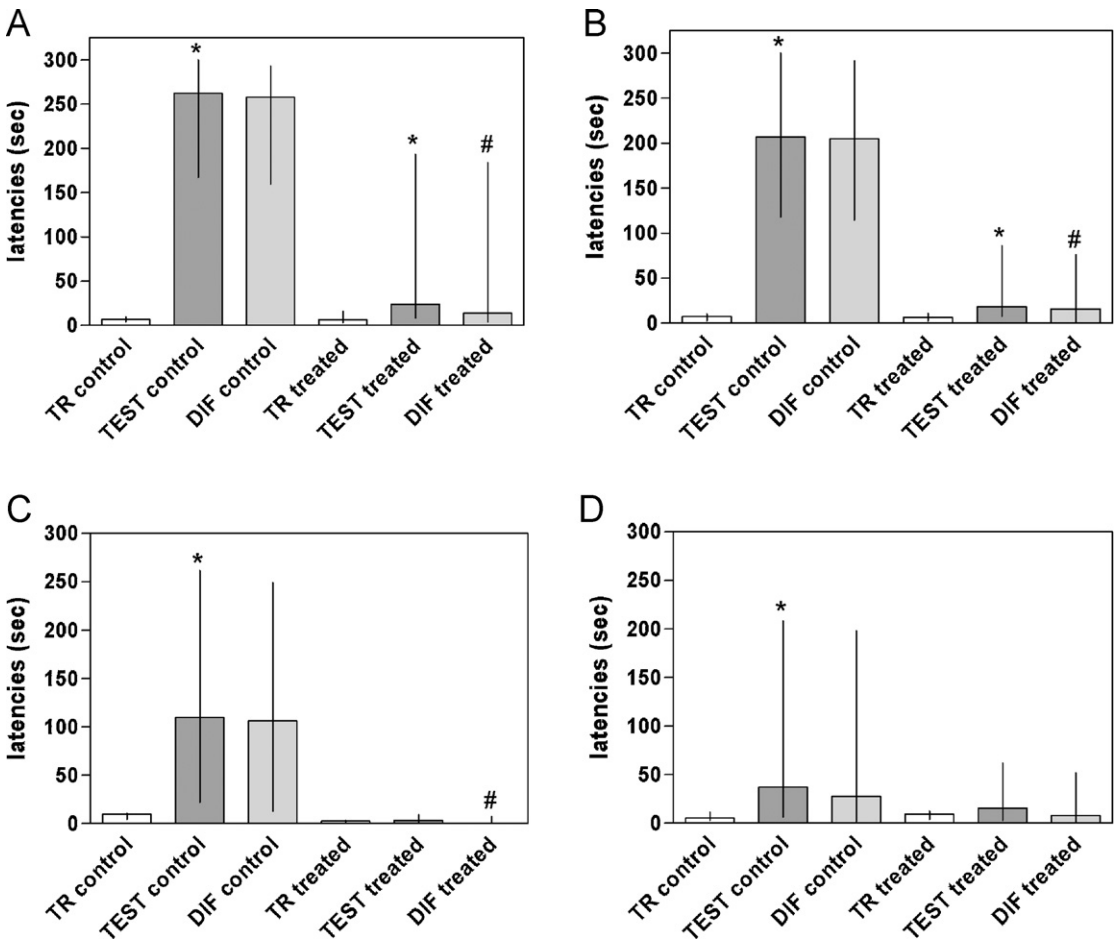

Fig. 1. Bar diagrams representing the latencies of entry to the dark compartment of a light–dark box shock-conditioned avoidance paradigm. Latencies are shown for control and treated rats. (median with interquartile ranges; *TR*: training session; *TEST*: test session; *DIF*: differences between test and training sessions). Panels are shown for treatment with subtilisin at a dose of (A) 100 ng, (B) 300 ng, (C) 1.5 μg, (D) 3 μg injected into each hippocampus. * significant difference relative to the training latencies ($p < 0.05$, Wilcoxon signed rank test after Kruskal–Wallis ANOVA for non-parametric samples). # significant difference relative to controls ($p < 0.05$, Mann–Whitney test after Kruskal–Wallis ANOVA for non-parametric samples). In (A) controls: $n = 11$, treated: $n = 13$; in (B) controls: $n = 12$, treated = 11; in (C) controls: $n = 12$, treated $n = 13$; in (D) controls: $n = 12$, treated: $n = 12$.

2.2. Behavioural tasks

2.2.1. Step-through inhibitory avoidance task

Since rats are intrinsically nocturnal animals preferring darkness, they will normally step-through from a lighted compartment to a dark one when an intervening door is opened. The administration of a mild foot-shock once the animal gets into the dark area was used to train rats to remain for longer periods in the light compartment by inhibiting a spontaneous preference.

2.2.1.1. Apparatus. The apparatus consists of a two-compartment box, one section with light and the other without. The dark compartment (30 cm wide × 30 cm long × 40 cm high) had black acrylic walls. The lighted compartment (15 cm wide × 20 cm long × 40 cm high) had white acrylic walls except for one which was transparent. A lamp of white light (40 W) was centrally positioned 30 cm above the floor of the lighted compartment. Between the two compartments there was a rectangular opening that could be closed by an opaque, vertically sliding door. The floors of both chambers were made of bronze rods of 3.00 mm in diameter, set 0.5 cm apart. The grid floor could be electrified.

2.2.1.2. Acquisition trial (training). The animal was placed in the lighted compartment facing the wall opposite to the door and 10 s later the vertical door was opened. Entrance latency to the dark compartment was recorded when all four paws had been placed inside the dark compartment. As the animal spontaneously moved into the dark compartment, the door was closed, and a mild electrical foot-shock (0.5 or 0.75 mA, as indicated in Section 3) was applied for 5 s. Training session was then terminated, the rat was removed from the dark compartment, bilaterally injected through the cannulae and returned to its home cage.

2.2.1.3. Retention trial (test). Retention was tested 24 h after the acquisition trial. In the test session, each rat was placed in the lighted compartment, as for the initial acquisition training, and 10 s later the vertical door was raised. The test latency was then recorded until the animal got into the dark compartment (up to a maximum of 300 s). If the rat did not go into the dark compartment within a 300 s period, the retention test was terminated and a ceiling score of 300 s was assigned. The difference between retention (test) and acquisition (training) latencies is considered as a measure of retention performance.

The results are expressed as the median of latencies measured before an animal enters the darkened box, with values shown for control animals (injected with vehicle) in their training session (TR control) and in the test session (TEST control), and the difference between these latencies (DIF control). Similarly, values are shown for animals used for subtilisin treatment in their training session (TR treated) and the test session (TEST treated), and the difference between these (DIF treated) (Figs. 1 and 2).

2.2.2. Open field test of locomotor and exploratory activities

To evaluate the possible effect of subtilisin upon locomotor activity and/or exploratory behaviour, the compound was administered into the dorsal hippocampus as described above, and 5 min later the rats were allowed to freely explore an open square field (measuring 60.0 cm long × 40.0 cm wide × 50.0 cm high) for 2 min (training). The floor of this arena was divided into 12 sectors of 15.0 cm × 13.3 cm each, and the number of rearings, groomings and crossings from one sector to another were counted in the initial training session and in the test session, performed 24 h later.

The results are expressed as the medians of the number of crossings or rearings by animals previously injected with vehicle (TR control and TEST control) or with subtilisin (TR treated and TEST treated) (Fig. 3).

2.3. Pharmacological treatment

Animals were injected bilaterally through the dorsal hippocampal cannulae with subtilisin A using the doses indicated in Section 3, administered in a volume of

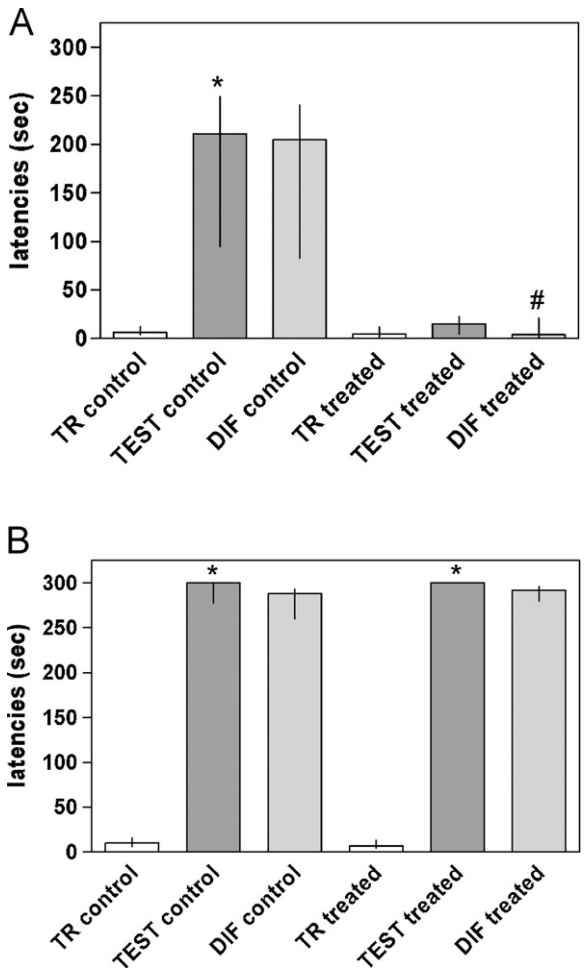

Fig. 2. Bar diagrams representing the latencies of entry to the dark compartment of a light–dark box shock-conditioned avoidance paradigm. Latencies are shown for control and treated rats. (median with interquartile ranges; *TR*: training session; *TEST*: test session; *DIF*: differences between test and training sessions). Panels are shown for treatment with subtilisin at a dose of 6 μg injected into each hippocampus with a footshock stimulus of (A) 0.5 mA and (B) 0.75 mA. * significant difference relative to the training latencies ($p < 0.05$, Wilcoxon signed rank test after Kruskal–Wallis ANOVA for non-parametric samples). # significant difference relative to controls ($p < 0.05$, Mann–Whitney test after Kruskal–Wallis ANOVA for non-parametric samples). In (A) controls: $n = 12$, treated: $n = 13$; in (B) controls: $n = 13$, treated = 15.

1 μl per side for 2 min. Injections were made 10 min before (open field task) or immediately after (step-through inhibitory avoidance task) the acquisition trial, as indicated in Section 3. Control animals were injected with the same volume of vehicle (0.9% saline). In one experiment of inhibitory avoidance task, subtilisin was injected 15 min before the acquisition trial.

2.4. Statistics

Test latencies were compared with training latencies using the Wilcoxon signed rank test, after a Kruskal–Wallis ANOVA for non-parametric samples, using $p < 0.05$ as the criterion for significance. Test-training difference in latencies were analysed with respect to saline control differences using the Mann–Whitney test, after a Kruskal–Wallis ANOVA for non-parametric samples, also using $p < 0.05$ as the criterion for significance. In the open field exploration task groups were compared by using Student's *t* test.

3. Results

3.1. Step-through inhibitory avoidance task

At the lowest dose of subtilisin used, 30 ng per hippocampus, both control and treated groups showed significant differences between test and training latencies, indicating that the animals learned and remembered the avoidance task ($p < 0.05$; controls $n = 11$; treated $n = 13$), and that subtilisin at this dose would not interfere with the learning or retrieval processes (median with [25,75] interquartile ranges of test-training latencies subtraction: for control animals, 288.2 s [132,296.4]; for treated rats, 91.65 s [9.89, 281.2]).

At the higher doses of 100 ng (Fig. 1A) or 300 ng (Fig. 1B) subtilisin per hippocampus, both treated animals ($n = 13$ and 11, respectively) and control animals ($n = 11$ and 12, respectively) showed statistically significant differences between the test and training latencies. This implies that both groups learned the task. However, there were also significant differences between control and treated groups when comparing the result of subtraction of test-training latencies, indicating that memory was significantly impaired in subtilisin-treated animals.

Higher doses of 1.5 μg (Fig. 1C) or 3 μg (Fig. 1D) subtilisin per hippocampus fully blocked the expression of inhibitory avoidance memory, leading to an apparent complete amnesia for the trained task. Control animals ($n = 12$ and 12, respectively) showed significant differences between test and training latencies, consistent with their learning and remembering of the task. In contrast, there were no significant differences between test and training latencies for the treated animals ($n = 13$ and 12, respectively) suggesting that they did not remember the avoidance task.

In order to examine the effects of increasing the strength of the avoidance foot-shock, an assay was also performed using 6 μg of subtilisin per hippocampus. As for the previous experiments, the median of test and training latencies at a 0.5 mA foot-shock (Fig. 2A) were significantly different for control animals ($n = 12$) confirming that they learned and remembered the task. On the other hand, medians of test and training latencies in the treated animals ($n = 13$) were not significantly different, showing that they appeared to be amnesic and would not remember the task. However, when the foot-shock strength was increased to 0.75 mA, a different pattern of responses emerged in which the increased stimulus overcame any deleterious or blocking effect of the protease. Both control ($n = 13$) and treated animals ($n = 15$) showed that the medians of their test and training latencies were significantly different (Fig. 2B), implying that animals in both groups were now able to remember the avoidance training.

In order to examine possible effects on acquisition session, rats were injected with 10 μg of subtilisin, 15 min before the training session, using a 0.5 mA foot-shock. Both control and treated groups learned the task, without significant differences between them, indicating that at this dose subtilisin did not seem to interfere with the learning or retrieval processes (test-training latencies for control rats:45.1 s [9.8, 282.9]; for treated rats:23.65 [8.44–267.8]; median [interquartile ranges]). The same dose of subtilisin (10 μg), when injected post-training, resulted in apparent amnesia (test-training latencies for control rats:275.1 [185.1–291.6]; for treated animals:12.2 [7.0–27.45]; median [interquartile ranges], $p < 0.05$; *n* controls = 14; *n* treated = 12).

3.2. Locomotor and exploratory activity

In the open field exploration test, the number of crossings was significantly lower in the test session compared to the training session for the control group, indicating habituation of the animals to the open field arena. In those animals injected with subtilisin (300 ng per hippocampus) before the first exposure to the open field (training session), the number of crossings in the second session (test session 24 h later) was not significantly different from that of the first session; therefore, there was no evidence of habituation in this group (Fig. 3A). Although the number of crossings in the training session appeared to be lower for treated ($n = 13$) com-

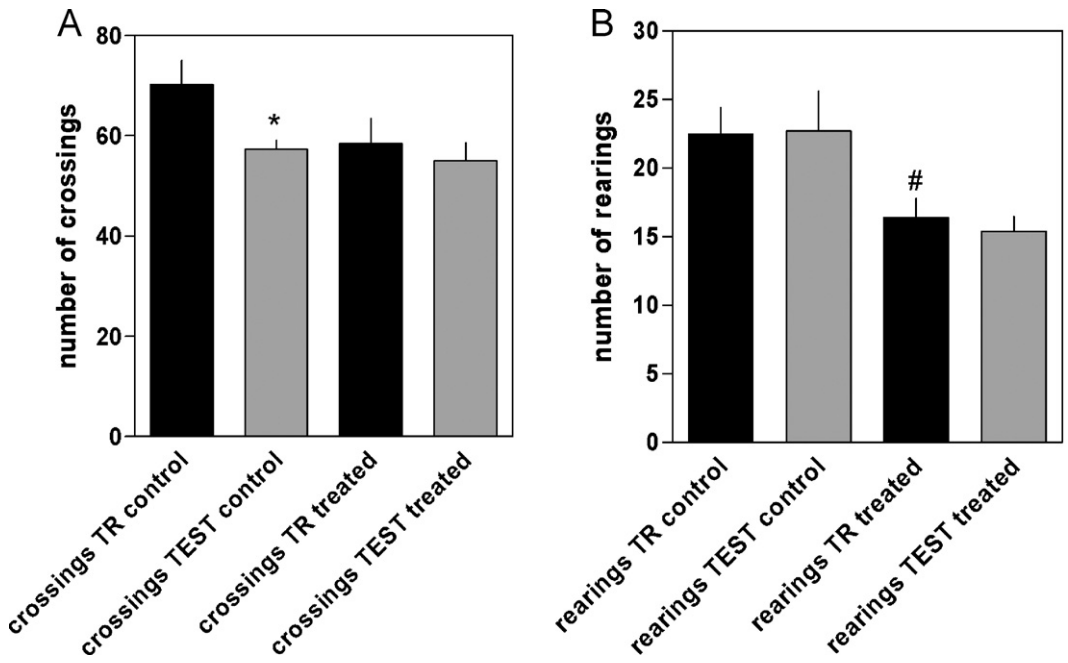

Fig. 3. Bar diagrams summarizing the number of times in a 2 min period, that animals cross into new sectors of an open-field arena (A) and the number of rearings (B) for control and treated rats. Subtilisin was injected at a dose of 300 ng into each hippocampus 5 min before training session. (mean ± SEM; *TR*: training session; *TEST*: test session). * significant differences relative to training session ($p < 0.05$, Student's paired t test). # significant differences relative to control group ($p < 0.05$, Student's unpaired t test). Controls: $n = 13$; treated: $n = 13$.

pared to control rats ($n = 13$), there were no significant differences between them.

There were significant differences between control and treated groups in the number of rearings in the training session (Fig. 3B), indicating less exploratory activity in the subtilisin-treated animals. However, there was no change in the number of grooming episodes (not shown).

3.3. Side effects

Some miscellaneous effects were noted in some of the rats after administration of subtilisin, including intense head shaking immediately after injection, lasting for a few seconds, immobility and hyporeflexia starting about 30 s after injection and lasting for 1–2 min, and signs of bleeding from the injection cannula (Table 1). Post mortem examination of the animals showed that some of them had experienced a subdural hemorrhage and hematoma around the infusion site, at doses of 1.5, 6 and 10 μg. However, there was no clear relationship between the occurrence of these side effects and the animals' behaviour or responses to subtilisin. It is most probable, therefore, that they reflect non-specific effects of a protease administration but are not effects that are likely to have interfered with the memory paradigms being examined.

Table 1
Side effects observed after injection of several doses of subtilisin into dorsal hippocampus of rats.

Dose (μg/side)	n[a]	Shake[b]	Immobility[c]	Bleeding[d]	Hemorrhage[e]
0.03	13	2	6	2	0
0.10	13	0	0	0	0
0.30	13	3	7	3	0
1.50	13	3	10	0	2
3.00	12	5	6	4	0
6.00	28	8	7	5	2
10.00	26	7	9	12	3

[a] n: number of animals treated with each dose.
[b] Shake: intense head shaking immediately after injection, lasting a few seconds.
[c] Immobility: mild lethargy and hyporeflexia (slower or absent accommodation reflex in an horizontal surface) starting about 30 seconds after injection and lasting for less than two minutes.
[d] Bleeding: signs of bleeding from the injection cannula, immediately after injection.
[e] Hemorrhage: subdural hemorrhage and hematoma around infusion site in post-mortem examination.

4. Discussion

The step-through avoidance task represents one of the simpler experimental paradigms in the study of learning processes. Its value has been well-established in a wide range of experimental conditions, although recent evidence suggests that some pharmacological differences are demonstrable using relatively minor variations in the experimental equipment or design [14]. Because of this, it is vital to consider which control groups would be appropriate to test pharmacological effects in this and similar one-step tasks which are mediated by the hippocampus [15]. Since the absolute values are modified by minor changes in equipment, experimental design, individual variability in animal behaviour, seasonal variations, etc., it is obligatory to use reference groups without drug administration to compare these control groups between them, to be considered as the experimental population. Besides the necessary dose–response assays, it is useful and also necessary to compare different shock intensities to investigate the activity of the compound, as well as choosing the adequate moment/period for drug administration, to discriminate the affected process.

There were no significant differences between training latencies for all the groups of animals used in this study. Hence, all the rats behaved in a rather comparable way. Similarly, although pre-training injected rats seemed to have a low performance in the avoidance task, there were not significant differences in test latencies for control groups.

The bilateral, intrahippocampal, administration of subtilisin at doses of 100 ng or greater, injected immediately post-training, had clear effects on memory parameters when a footshock of 0.5 mA amplitude was used. The effects produced with this post-training paradigm of subtilisin administration would be consistent with

impairments in memory consolidation, as it begins immediately after or during acquisition and lasts for several hours. Since subtilisin has already been found by us to produce LTD in hippocampal slices [8], the results suggest that LTD, rather than LTP, may bear a strong functional relevance to consolidation in this experimental situation of step-through avoidance.

When the strength of the deterrent footshock was increased by 50%, the training schedule was strong enough to overcome the amnesic effect and to prevent any significant change in memory consolidation by subtilisin. This result is crucially important as it shows that subtilisin has not generated a non-specific or irreversible toxic effect in the hippocampus, but has merely expressed its activity in the learning protocol to a degree which can be overcome by a greater sensory motivation. It is feasible that this happened in much the same way that a competitive antagonist can be displaced from a receptor site by increased concentrations of a competing agonist and/or by recruiting more synapses/circuits.

When a pre-training intra-hippocampal administration of subtilisin was examined for comparison, there was no evidence of an impairment in learning behaviour during the training session, or in memory performance in the test session. This is also an important control experiment indicating that the effect of subtilisin is exerted on the consolidation of memory rather than on the acquisition of the task. This result also suggests that the subtilisin effect did not last until the consolidation was taking place, as performance of the rats was not different from saline-injected animals.

The effect of subtilisin on performance in the exploration of the open field arena, suggests that the depression of hippocampal function was also able to give rise to a minor exploratory activity and loss of habituation. This would be entirely consistent with the idea that hippocampal learning is a major contributor to the spatial components of this phenomenon, and would therefore be expected to mirror the results obtained in the avoidance paradigm.

As reviewed in Section 1, there is ample evidence for responses to a number of serine proteases on brain function, including hippocampal plasticity and learning behaviour. There are also reports indicating potential pathological roles for these enzymes in disorders of brain function. The over- or under-expression of serine proteases or their inhibitors in brain can affect neuronal excitability or viability [10]. Some studies suggest that altered expression or mutations of endogenous proteases may contribute to Alzheimer's disease [11], schizophrenia [12] or some forms of mental retardation [16]. The present results strengthen the view that physiological situations, or pathological conditions, which increase the production of serine proteases, may have deleterious effects on cognitive processes. Since subtilisin is a major secretory product of many environmental and commensal bacteria, it may contribute to abnormal neural function during and following infections with these organisms.

References

[1] Qian Z, Gilbert ME, Colico MA, Kandel ER, Kuhl D. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature 1993;361:453–7.
[2] Chen Z-L, Yoshida S, Kato K, Momata Y, Suzuki J, Tanaka T, et al. Expression and activity-dependent changes of a novel limbic serine protease gene in the hippocampus. J Neurosci 1995;15:5088–97.
[3] Shimizu C, Yoshida S, Shibata M, Kato K, Momota Y, Matsumoto K, et al. Characterization of recombinant and brain neuropsin, a plasticity-related serine protease. J Biol Chem 1998;273:11189–96.
[4] Komai S, Matsuyama T, Matsumoto K, Kato K, Kobayashi M, Imamura K, et al. Neuropsin regulates an early phase of Schaffer-collateral long-term potentiation in the murine hippocampus. Eur J Neurosci 2000;12:1479–86.
[5] Tamura H, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S, et al. Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. J Physiol (Lond) 2006;570:541–51.
[6] Frischnecht R, Fejtova A, Viesti M, Stephan A, Sonderegger P. Activity-induced synaptic capture and exocytosis of the neuronal serine protease neurotrypsin. J Neurosci 2008;28:1568–79.
[7] Kemp A, Manahan-Vaughan D. Hippocampal long-term depression and long-term potentiation encode different aspects of novelty acquisition. Proc Nat Acad Sci USA 2004;101:8192–7.
[8] MacGregor DG, Mallon AP, Harvey AL, Young L, Nimmo HG, Stone TW. Group S8A serine proteases, including a novel enzyme cadeprin, induce long-lasting, metabotropic glutamate receptor-dependent synaptic depression in rat hippocampal slices. Eur J Neurosci 2007;26:1870–80.
[9] Monard D. Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth. Trends Neurosci 1988;11:541–4.
[10] Hirata A, Yoshida S, Inoue N, Matsumoto-Miyai K, Ninomiya A, Taniguchi M, et al. Abnormalities of synapses and neurons in the hippocampus of neuropsin-deficient mice. Mol Cell Neurosci 2001;17:600–10.
[11] Diamandis EP, Scorilas A, Kishi T, Blennow K, Luo LY, Soosaipillai A, et al. Altered kallikrein 7 and 10 concentrations in cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia. Clin Biochem 2004;37:230–7.
[12] Vawter MP, Weickert CS, Ferran E, Matsumoto M, Overman K, Hyde TM, et al. Gene expression of metabolic enzymes and a protease inhibitor in the prefrontal cortex are decreased in schizophrenia. Neurochem Res 2004;29:1245–55.
[13] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press; 1998.
[14] Blake MG, Boccia MM, Baratti CM. Behavioral differences on memory retrieval between two variants of step-through inhibitory avoidance task in mice. Neurosci Lett 2008;444:102–5.
[15] Martel G, Jaffard R, Guillou J-L. Identification of hippocampus-dependent and hippocampus-independent memory components in step-down inhibitory avoidance tasks. Behav Brain Res 2010;207:138–43.
[16] Molinari F, Rio M, Meskenaite V, Encha-Razavi F, Auge J, Bacq D, et al. Truncating neurotrypsin mutation in autosomal recessive non-syndromic mental retardation. Science 2002;298:1779–81.