 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

The entorhinal cortex regulates blood glucose level in response to microinjection of neostigmine into the hippocampus

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Submitted: November 19, 2004 *Accepted: February 18, 2005*

Key words: **entorhinal cortex; ibotenic acid; neostigmine; hippocampus; glucose**

Neuroendocrinol Lett 2005; **26**(3):225–230 **PMID:** 15990726 NEL260305A06 ©Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJECTIVE: Microinjectin of neostigmine, an inhibitor of acetylcholine esterase, into the rat hippocampus elicited stress-like responses reflected by the release of adrenocorticotropic hormone (ACTH) and blood glucose elevations. The entorhinal cortex is regarded as an interface between the hippocampus and neocortex. The current study was designed to examine the role of the entorhinal cortex in regulation of blood glucose elevation induced by hippocampal neostigmine injection.

MARERIAL AND METHODS: We produced the entorhinal cortex lesions in 9 week-old male Wistar rats by the bilateral injections of the cell-selective neurotoxin, ibotenic acid (15 μg / μl). Two weeks after the injections, neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected into the rat hippocampus in a volume of 1 μl for 1 min using a CMA/100 microinjection pump.Plasma ACTH levels were measured by radioimmunoassay. Plasma glucose concentrations were determined by the immobilized enzyme membrane/H_2O_2 method with a compact glucose analyzer Antsense II (Bayer Medical Co.Ltd,Tokyo, Japan).

RESULTS: Compared with sham-operated control rats, the entorhinal lesions produced by ibotenic acid significantly attenuated the elevations of blood glucose evoked by the microinjection of neostigmine into the hippocampus. However, no significant difference of plasma ACTH in response to the injection was observed between the entorhinal-lesioned rats and controls.

CONCLUSION: The results of the present study indicate that the entorhinal cortex plays a role in the central nervous systems regulation of blood glucose and may be involved in a stress response presumably via an alternative pathway.

Abbreviations:

ACTH	adrenocorticotropic hormone
EC	entorhinal cortex
CNS	central nervous systems
CRH	corticotropin releasing hormon
PVN	paraventricular nucleus
HPA	hypothalamic-pituitary-adrenal
BNST	bed nucleus of the stria terminalis

Introduction

Stress is common to all living creatures regardless of differences in its quality or intensity. The imposition or perception of environmental or physical change, negative or positive, elicits a spectrum of physiologic changes that can be construed as adaptive to the organism. Prominent among these is the release of glucocorticoids by the adrenal glands, which serves to alert the organism to environmental or physiologic changes and to preserve homeostasis. Levine and Ursin [10] provided a definition of stress that consists of three elements: stimulus input, central processing system, and response output; with biological and psychological processes viewed as integral parts of the general homeostatic principle. The brain perceives inputs of various stressors and responds via the nervous, endocrine and immune systems, which are called stress responses [17]. In this sense, the brain plays a role in governing the stress responses. Elevations of corticotropin-releasing factor, ACTH and glucocorticoids are the main features of reactions to diverse and acute stressful stimuli [2, 24]. During stress, neurons of the hypothalamic paraventricular nucleus (PVN) release corticotropin-releasing hormone into the pituitary portal circulation, and ACTH secreted from the anterior pituitary gland in response to corticotropin-releasing hormone, stimulates the secretion of glucocorticoids from the adrenal gland. This constitutes the hypothalamic-pituitary-adrenal (HPA) axis, which is the major regulator of neuroendocrine stress responses [1,5,7,11,13,15]. Involvement of the limbic system in neuro-endocrine responses to some stressors has been documented. A wealth of evidence suggests that the hippocampal cholinergic system is involved in some stress responses [9,12,21]. In particular, the cholinergic system in the hippocampus plays a role in regulating the peripheral metabolism of glucose and catecholamines [7,21]. Under stress, the release of acetylcholine in the hippocampus increases, which coincides with the elevation of plasma glucose and catecholamines [19]. In our previous experiments, we observed that the administration of neostigmine, an acetylcholine esterase inhibitor, into the hippocampus elevates the levels of blood glucose and ACTH. Thus, we concluded that the microinjection of neostigmine into the hippocampus is a potential experimental model for acute stress responses [7,8]. The entorhinal cortex is a gateway to the hippocampus. Many sensory inputs and other information reach the hippocampus via the entorhinal cortex. It receives inputs from the neocortex, including the temporal and frontal lobes, amygdala and olfactory bulbs [3]. Information enters the hippocampal formation via the entorhinal cortex and exits via the fornix. Also, the entorhinal cortex is the primary supplier of converging neocortical sensory input to the ipsilateral dentate gyrus of the hippocampal formation [20]. We previously reported on the involvement of the entorhinal cortex in the stress response to immobilization [22]. Lesions in this area produced by ibotenic acid attenuate ACTH elevation during immobilization stress but not during insulin-induced hypoglycemia. The aim of this study was to investigate the role of the entorhinal cortex in stress responses. We produced bilateral entorhinal lesions using ibotenic acid in rats, and observed the peripheral responses of stress markers induced by microinjections of neostigmne into the hippocampus.

Material and Methods

Subjects: We used 9 week-old male Wistar rats (200–300 g) for the experiment. The animals were individually housed under standard laboratory conditions in temperature-controlled rooms (25 °C), and were maintained under a 12 h light/dark cycle (light on at 06.00) with food pellets and water available *ad libitum*. The rats were cared for in accordance with the ethical guidelines approved by the Animal care and Use Committee of Nagoya University.

Experimental protocol: Rats were randomly assigned to one of two major groups: unlesioned or lesioned.

The rats in each group were then divided into two subgroups: Group 1: unlesioned neostigmine-injected, Group 2: unlesioned saline-injected, Group 3: lesioned neostigmine-injected, and Group 4: sham-operated rats, neostigmine-injected.

Surgery: The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and mounted in a stereotaxic frame (Narishige Scientific Instruments Laboratory, Tokyo, Japan). For insertion of the stainless steel needle, the skull was exposed and a burr hole was drilled overlying the injection coordinates. Ibotenic acid was injected through a stainless steel needle (outside tip diameter of 28 μm), which was connected to a 1.0 μl syringe via a 30 cm tube filled with the injection solution. Coordinates for the entorhinal cortex were calculated relative to Bregma with the incisor bar set at –3.30 mm. The coordinates used were anterior-posterior –6.04 mm, medial-lateral ±6.50 mm and dorsal-ventral 7.00 mm from the skull surface in accordance with the atlas of Paxinos and Watson [14]. Entorhinal cortex lesions were produced by pressure-injection of 0.1 μl of ibotenic acid (15 μg / μl in 0.9% NaCl, Sigma Chemical Co., St Louis, MO, USA) bilaterally over 5 min. The tip was allowed to remain in the brain for 5 min after injection to minimize dorsal diffusion of the drug along the needle tract. Sham-operated rats were treated in an identical manner to the ibotenic acid-lesioned rats but were injected with the same volume of saline without ibotenic acid.

A recovery period of 7 days was given to the above operated rats (Group 3, 4), otherwise all rats were anesthetized one week before the experiment to stereotaxically implant a guide cannula (Bas, Tokyo, Japan) into the left dorsal hippocampus at the following coordinates: anterior-posterior –2.0 mm, medial-lateral 1.5 mm, dorsal-ventral 3.5 mm in accordance with the Paxinos and Watson atlas [14] one week before the experiments.

The day before the experiments, the rats were anesthetized with diethyl ether (Kanto Chemical Co. Inc, Tokyo, Japan), and a catheter was inserted into the jugular vein for repeated blood sampling. A 2 cm longitudinal incision was made in the neck directly over the trachea. The underlying muscles were separated using blunt dissection and the right jugular vein was catheterized with Silastic tubing (Shiniest Polymer, Nagoya, Japan) filled with heparinized saline. The catheter was threaded through the vein over a distance of 2.5 cm, which allowed the tip of the cannula to rest in or near the atrium. The free end of the catheter was plugged with a knot and the catheter exteriorized and secured at the back of the neck with a special cap.The rats were kept in individual cages with free access to water and food.

Procedures: Two weeks after developing entorhinal cortex lesions, saline containing neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected in a volume of 1µl for 1 min using a CMA /100 microinjection pump (BSA, Tokyo, Japan) through the guide cannula into the left dorsal hippocampus of free moving rats. To determine the plasma concentration of ACTH and glucose, blood was intermittently sampled (0.8 ml), starting at time 0, just before injection, and at 10, 30, 60 and 120 min after. To minimize the effect of volume loss, an equal volume of heparinized saline was retuned to the general circulation at each sampling. The blood samples were kept on ice, centrifuged, and the plasma was removed and stored at –20 °C in 400 µl aliquots for subsequent determination of ACTH by radioimmunoassay [16]. Plasma glucose concentrations were determined by the immobilized enzyme membraine/H_2O_2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) (21). All experiments were completed between 10.00 h and 13.00 h to minimize variability resulting from circadian rhythm. Two hours after neostigmine injection, the rats were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). They were postfixed over night and cryoprotected in phosphate-buffered saline containing 30% sucrose for 2 days.

Histological verification: For the verification of lesions and the effect of the vehicle, the postfixed brains were frozen with powdered dry ice and serial sections 20 µm in thickness were processed from the region of the entorhinal cortex and mounted on glass slides. Selected regions were stained with Cresyl Violet to assess the extent of the lesions in the entorhinal cortex.

Data and statistical analysis: All sections were assessed by means of microscopic examination using an Olympus BX50 microscope (Tokyo, Japan). Photographs were made of representative lesions and vehicle injection sites in the entorhinal cortex. Blood glucose concentrations were expressed as means ±S.E.M, and differences between the four experimental groups were assessed using repeated measures of one factor ANOVA. Plasma ACTH levels were measured by radioimmunoassay [16], and plasma glucose concentrations were determined by the immobilized enzyme membrane/H2O2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) [18].

Results

Figure 1 shows Nissle staining of representative sections including the entorhinal cortex. The significant loss of neurons accompanied by extensive glial proliferation was observed in the entorhinal cortex sections of animals that received ibotenic acid injections (Group 3) (Fig. 1D,E). Animals that received injections of vehicle in the entorhinal cortex did not show any histological signs of neuronal damage (Fig. 1B,C). Figure 2 shows the ACTH and blood glucose concentrations for the lesoined and unlesioned rats following microinjectin of neostigmine into the hippocampus. Figure 2A shows the blood glucose concentration after microinjection of neostigmine into the hippocampus. For Group 1, the plasma concentration of glucose increased after 10 min and reached a peak after 60 min. The saline injected group (Group 2) showed no effect. For the lesioned groups, blood glucose levels for Group 3 were significantly lower than those of Group 4 (Fig. 2A). ANOVA showed that there was a statistically significant difference among the groups ($p<0.0001$), and Scheffe's post–hoc analysis indicated that ibotenic acid lesions significantly attenuated blood glucose release evoked by the microinjection of neostigmine into the hippocampus (Fig. 2A). No significant difference was observed in the plasma ACTH concentration between Group 1, 3 and 4 after the microinjection of neostigmine into the hippocampus (Fig. 2B).

Discussion

In the current study, we have discovered that the entorhinal cortex is involved in the regulation of stress-like responses induced by hippocampal neostigmine injection. The lesions in this area significantly attenuated the blood glucose elevation but did not affect ACTH secretion. No significant difference of weight was observed before and after lesion.

During stress, an adaptive or compensatory response by the organism is activated to sustain homeostasis. Stress induces adaptation through the produc-

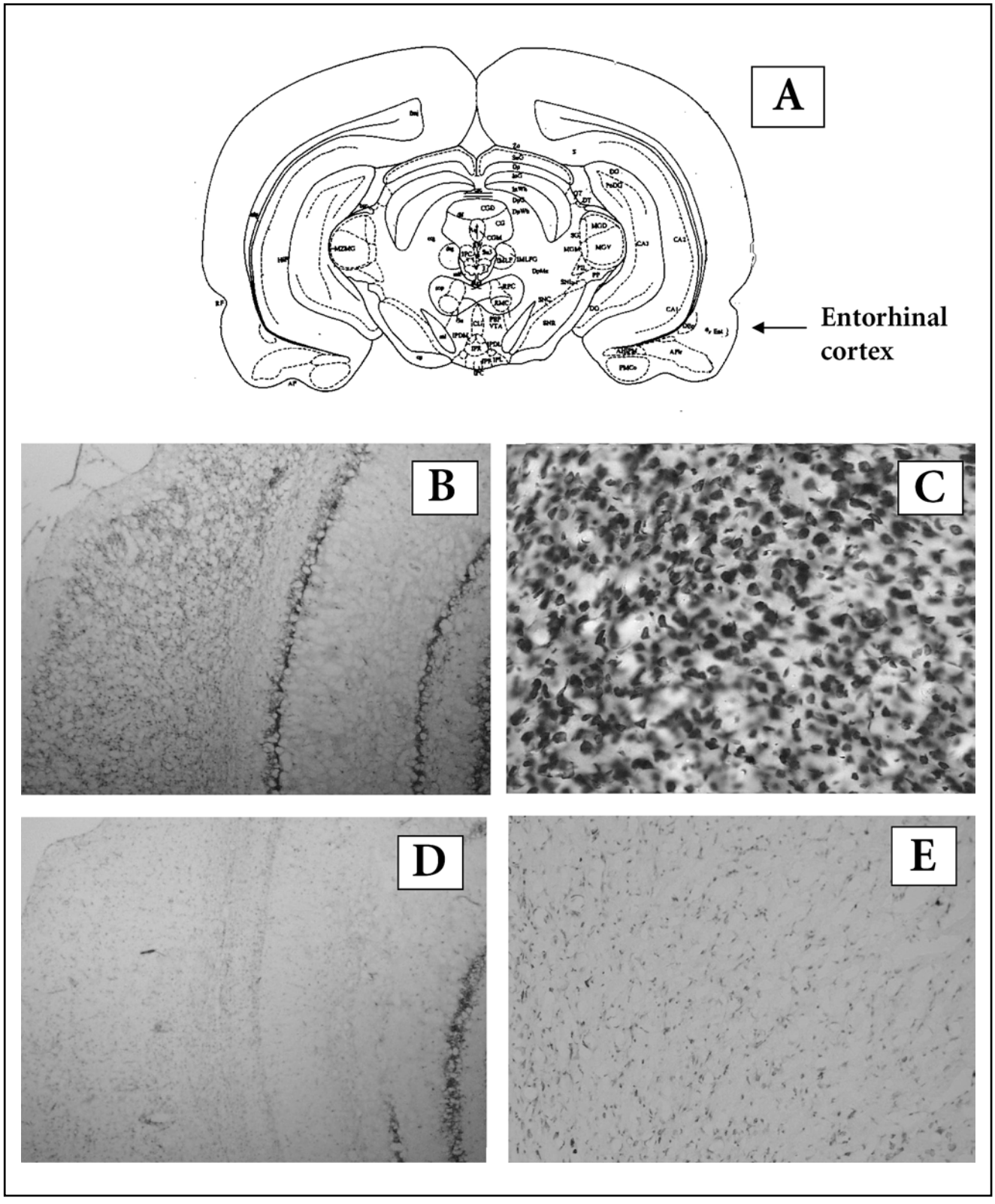

Figure 1.
(A): Schematic representation of a bilateral entorhinal cortex lesion.
Photomicrographs of coronal sections through the entorhinal cortex stained with Cresyl violet illustrating the extent of damage:
(B) Vehicle injection into the entorhinal cortex. (C) Enlarged view of that shown in (B).
(D) Ibotenic acid injection into the entorhinal cortex. (E) Enlarged view of that shown in (D).
The section was sampled from the injection sites where the maximal amount of cell damage was observed.

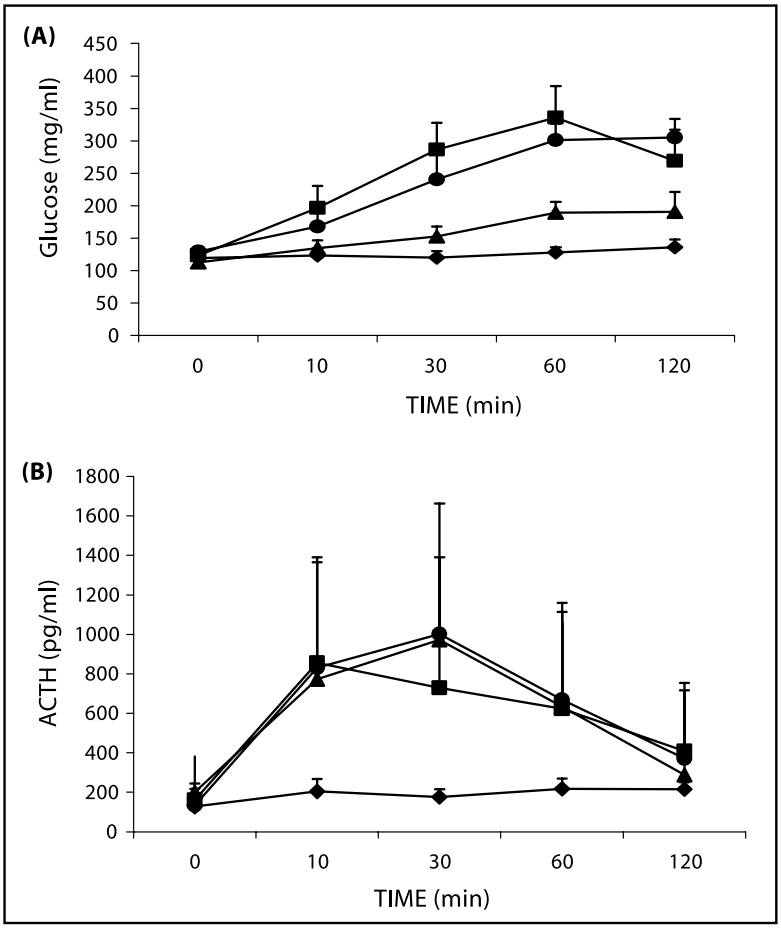

Figure 2.
Plasma ACTH and blood glucose concentrations in rats microinjected by neostigmine into the hippocampus.
(A) Blood glucose concentrations. Repeated ANOVA showed that entorhinal-lesioned rats had significantly lower concentrations than unlesioned rats ($p<0.0001$)
■, Group 1: unlesioned neostigmine-injected (n=6);
◆, Group 2: unlesioned saline-injected (n=5);
▲, Group 3: lesioned neostigmine-injected (n=11);
●, Group 4: sham-operated, neostigmine-injected (n=6).
(B) Plasma ACTH concentrations. Repeated ANOVA showed no significant difference between entorhinal lesioned rats and rats who had saline injected into the entorhinal cortex.
■, Group 1 unlesioned neostigmine-injected (n=6);
◆, Group 2: unlesioned saline-injected (n=5);
▲, Group 3: lesioned neostigmine-injected (n=6);
●, Group 4: sham-operated, neostigmine-injected (n=5).

tion of various mediators such as adrenal steroids, catecholamines, cytokines, and tissue mediators [13]. Stress-related signals in the central nervous system initially act upon the hypothalamus. From there, signals, which respond to stress stimuli in the central nervous system, reach the peripheral nervous system through several pathways. Two main ones are; (1) autonomic neurons and the adrenal medulla system, which release catecholamines; and (2) the hypothalamic-pitutitary-adrenal system, known as the HPA axis, which releases glucocorticoids. The secretion of adrenocortical glucocorticoids is driven by the release of ACTH from corticotropes in the anterior pituitary gland. Neurons in the PVN are the most potent structures capable of inducing ACTH release in response to stress through the release of corticotropin-releasing factor [24].

Microinjections of neostigmine into the hippocampus produce hyperglycemia associated with the secretion of plasma cathecholamines, which showed similarities to stress responses. Regarding the mechanism responsible for the neostigmine-induced elevation of plasma glucose, at least four pathways had been hypothesized; (1) secreted epinephrine may directly act on the hepatic release of glucose, (2) epinephrine may induce the release of glucagon, (3) direct neuronal control in the pancreas causes glucagon secretion, (4) direct innervation in the liver induces glucose release [6]. Our previous studies showed that ACTH release is accompanied by c-fos expression, a universal marker of neuronal activation, in the PVN of the hypothalamus [24]. We also found that the bed nucleus of the stria terminalis (BNST) is involved in the regulation of ACTH release in response to hippocampal neostigmine injection [4,23]. This structure receives inputs directly from the ventral hippocampus area and sends a heavy axonal projection into neuroendocrine cell regions of the PVN [5,23]. Although lesions of the BNST attenuated the elevation in ACTH, and c-fos expression in the PVN induced by hippocampal neostigmine injection, blood glucose elevation was not altered by the BNST lesions [23,24]. This suggests that glucose and ACTH are regulated differently within the brain, and that BNST is not involved in the glucose regulation pathways.

The entorhinal cortex occupies a key position in the limbic system, functioning as a relay station between the hippocampus and neocortex. The entorhinal cortex is a major gateway for sensory information into the hippocampal formation. In the current study, the entorhinal cortex lesion attenuated blood glucose elevation but did not affect the ACTH response. These results suggest that the entorhinal cortex plays role in blood glucose regulation and not in ACTH regulation when the hippocampal cholinergic system is activated.

We previously found that the entorhinal cortex is involved in the stress response to immobilization but

not to insulin-induced hypoglycaemia [22]. Lesions in this area attenuate ACTH release induced by immobilization but have no effect on the blood glucose response. These findings are not compatible with what we observed in this study using hippocampal neostigmine injections. Although the hippocampal neostigmine–injection model shows similarities to the stress responses in terms of ACTH and glucose profiles in the plasma, the model may activate a pathway in the brain different from what is activated in response to immobilization.

Recent evidence suggests that various stressors activate different regulatory pathways. According to Herman and Cullinan [5], stressors can be divided into two categories. One category is processive (emotional/ psychological) stressors, which activate cortical and limbic areas before the PVN is activated. Signals from multiple sensory modalities are processed in these structures prior to final elaboration of the stress response. The other group is systemic (physical) stressors, which directly threaten the survival and activate the PVN through the ascending catecholaminergic pathway from the brainstem. According to Herman and Cullinan's definition immobilization stress is processive stress. Since the brain pathways activated in the hippocampal neostigmine-injection model were different from the responses to immobilization, this model may show similarities to other types of stress responses. Further investigation should be performed to elucidate the mechanism responsible for this. In conclusion, the present study showed the role of entorhinal cortex in regulating stress response induced by microinjection of neostigmine and the relationship between the entorhinal cortex and hippocampus in stress responses.

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