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Research report

Electrophysiological evidence against a neurotransmitter role of corticotropin-releasing hormone (CRH) in primary somatosensory cortex

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Abstract

The possible neurotransmitter role of corticotropin-releasing hormone (CRH) was studied in the primary somatosensory cortex of the rat. Electrical activity of single neurones was recorded in layers II–VI of cortex, and in the region of the locus coeruleus. Iontophoresis and pressure ejection were employed to locally apply CRH, and changes in spontaneous, synaptically driven and iontophoretically driven firing were examined. In the cortex, of 62 neurones recorded most (51) were completely unaffected by high and prolonged current/pressure ejections of CRH. Depression of firing was occasionally seen (8 of 62), while a very few (3) were weakly excited. Of 25 cells studied with vibrissal stimulation to evoke excitatory synaptic responses, responses in two cells were depressed and in two they were enhanced. Activity that was evoked by iontophoretic ejection of excitatory amino acids, such as glutamate, was depressed in 6 of 40 cells (none were enhanced). Such effects as were seen were weak and often difficult to reproduce. The effect of CRH on depressions produced by GABA was also tested in four experiments. No effects on the amplitude or duration of the depressions were observed. In contrast recordings made in the midbrain, in the region of the locus coeruleus, resulted in over half the neurones (11 of 20) showing clear reproducible excitatory responses to CRH applications. Solutions used in the experiments were analysed using chromatography, radioimmunoassay and bioassay, and no significant degradation of the peptide was found compared with the synthetic standard (CRH (1–41)). The data provide evidence against CRH acting as a neurotransmitter or modulator in primary cortex, suggesting that the CRH which is localized in certain types of cortical cells is involved in other processes. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Barrel cortex; Peptide; Neuromodulator; Microiontophoresis; Single cell recording; Rat

1. Introduction

Hypothalamic corticotropin-releasing hormone (CRH) is known to regulate the release of corticotropin from the anterior pituitary [14]. However, CRH immunoreactivity has also been observed more widely outside the hypothalamus [11], and more recently has been localized to bipolar neurons of layers II–III in the neocortex of the rat [8]. More detailed studies revealed that CRH-immunoreactive neurons made asymmetric contacts, largely on dendritic spines [9]. Thus, a neurotransmitter or neuroregulator role is suggested at these excitatory synapses. Such a role was supported by earlier findings. For example intracerebroventricular injection of CRH evokes behavioural [13] and autonomic [7] effects. Electrophysiological studies (reviewed by Siggins [12]) indicate that CRH has predominantly excitatory actions in the locus coeruleus, the solitary tract complex, the hippocampus and some regions of the hypothalamus. One study applied CRH iontophoretically to single neurons recorded extracellularly in various forebrain (including cortical) areas [6]. In the forebrain cortex almost half the neurons showed increases in spontaneous firing during iontophoresis of CRH. To our knowledge no studies have been carried out in primary sensory cortex.

The purpose of the present study was to examine what effects CRH might have on the spontaneous and evoked firing of primary somatosensory (SI) barrel cortical cells. This could provide information about the type of transmitter role CRH might play in its location at specific synapses in neocortex. The rat barrel cortex provides a suitable model in the study of primary cortical function. Given the recent anatomical data of Lowenstein et al. [8,9] it might

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be expected that excitatory amino acid-mediated neurotransmission would be modulated by CRH, so experiments included iontophoretic application of excitatory amino acids in the testing protocol. The results of such a study could indicate the normal functional role of CRH in primary sensory cortex. Some of these data have been communicated in abstract form [3].

2. Methods

Thirteen male Wistar rats weighing between 390–700 g were used in the experiments. Recordings were made under urethane anaesthesia using seven-barrelled micropipettes in which the centre barrel was used to record extracellular action potentials. Side barrels contained Na L-glutamate (0.5 M, pH 8.5), CRH (0.5 mM, pH 6.5, obtained from Bachem California), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (0.01 M in 0.075 M NaCl, pH 8), (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) (0.1 M, pH 8), γ-amino-*N*-butyric acid (GABA) (0.5 M, pH 3.5), 1 M NaCl for current balancing and for current controls, and Pontamine Sky Blue (2% in 0.5 M Na acetate) for histological localization of recording sites. The micropipette measured approximately 15 μm across, and drug barrels typically had resistances measuring 10–100 MΩ. Samples taken from CRH barrels of used micropipettes were tested for levels of CRH. Negative currents (range 30–200 nA) or pressure (range 15–33 psi) by a Neuro Phore, Medical Systems, (IP-2 and PPM-2 modules respectively) were used to apply CRH to single recorded neurones. A retaining current of 5 nA was used to retain CRH, while 12 nA was used to retain the dicarboxylic amino acids and -12 nA for GABA. Stock solution of CRH was made up with approximately 2.4 mg ml^{-1}, and kept frozen until the day of use.

An effect of CRH that produced a deviation of more than 30% from the baseline response was considered to be a significant effect. ANOVA and selected *t*-tests were also used. A minimum current of 60 nA, and pressure of 15 pounds per square inch (psi), applied for at least 2 min were used to determine that CRH had no effect.

Synaptic activation of recorded cells was achieved using an air-jet played over the receptive field of the cell, or by applying a voltage across a piezoelectric bimorph attached to a glass capillary tube (in the end of which a vibrissa was inserted) (see Cahusac [2] for further details).

Immunoreactive CRH (1–41) was measured using a specific radioimmunoassay (RIA), as described in detail previously [5]. In order to test if CRH samples used in the electrophysiological experiments were biologically active, we used AtT20 D16-16 mouse corticotropic tumour cells and measured immunoreactive adrenocorticotropic hormone (IR-ACTH) released by these cells. The AtT20 D16-16 cell culture conditions and treatment with secretagogue was performed as described previously [4]. ACTH released into the culture medium was measured using a specific radioimmunoassay provided by The National Hormone and Pituitary Program (NHPP, Baltimore, USA) [10]. The basal release was 4.5 ± 0.8 ng ml^{-1} well^{-1} ($\pm$standard error of the mean) of IR-ACTH, and the plateau response was reached at 10^{-7} M to 10^{-6} M with synthetic CRH (1–41), with a release of 9.2 ± 0.6 ng ml^{-1} well^{-1} of IR-ACTH. The integrity of CRH (1–41) used in the electrophysiological experiments was also assessed using gel filtration chromatography. A Sephadex G50 column (0.9×1 m^2) was used and eluted in 0.05 M phosphate buffer containing 1% Polypep (Sigma). Fraction volume was 1 ml. The fractions were assayed using the CRH (1–41) RIA.

3. Results

A total of 62 neurones were studied throughout layers II–VI of SI cortex. The firing of most neurones studied (51 of 62) was completely unaffected by high and prolonged current/pressure (median 100 nA/30 psi) applications of CRH. In Fig. 1 is shown an example of a neurone that was activated by a piezoelectric stimulus to the vibrissa in the centre of the neurone's receptive field. No statistically significant effect was observed despite the high (200 nA) iontophoretic application current applied for 2 min. When

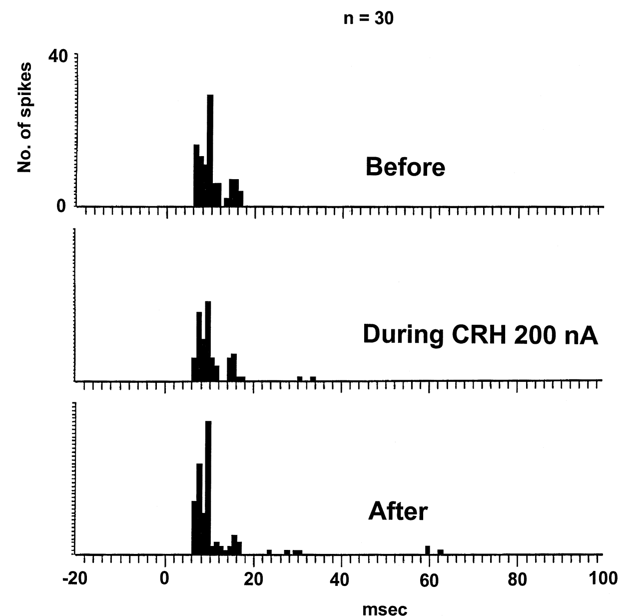

Fig. 1. Responses of a SI neurone to stimulation of the vibrissa in the centre of the receptive field were relatively unaffected (small reduction of 21%) by a high current (200 nA) of CRH applied for 2 min. Stimulation of the vibrissa was provided by a piezoelectric stimulator device whose onset time is shown at time 0. Each peristimulus histogram consisted of 30 trials. The reduction did not reach the criterion of 30%, and the statistical analysis (one-way ANOVA on Before, During and After CRH responses) did not obtain a statistically significant effect (i.e., $p > 0.05$).

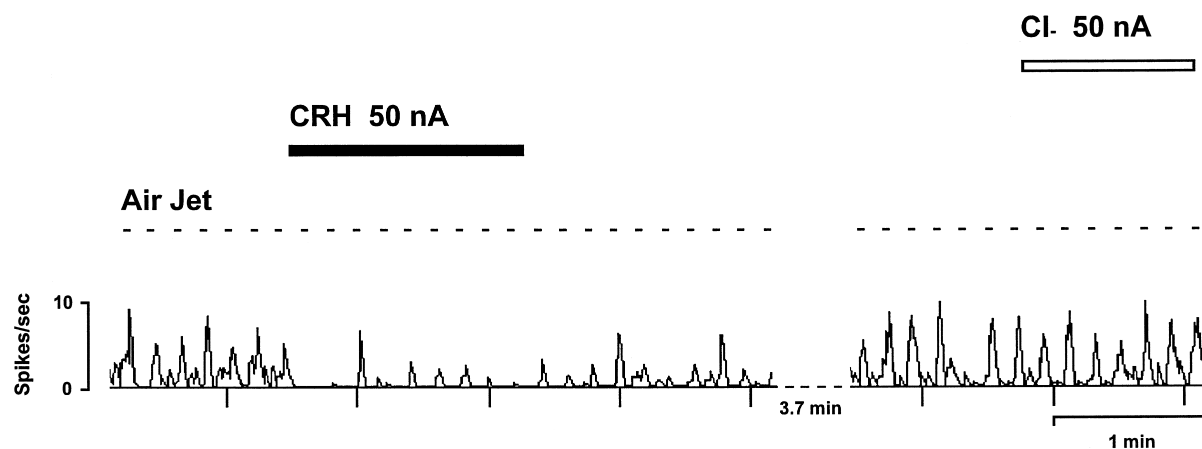

Fig. 2. An example of the depressant effect of CRH on the responses of a cell to air jet applied to the vibrissa receptive field. During a repeated cycle of air jet CRH was applied iontophoretically at 50 nA, as shown by the black bar above the trace. The depressant effect lasted some 5 min. A current control at the end of the record, indicated by the open bar, shows that a negative iontophoretic current (ejection of Cl^- ions from NaCl solution) did not reproduce the same effect. The reduction of response was 64% of control. The spontaneous firing was also reduced. This example illustrates the largest depressant effect on vibrissal responses that was seen in this study.

an effect was seen during the application of CRH this was more likely to consist of depression of firing (8 of 62 cells). Only three neurones displayed significant excitation during CRH application.

Of the 25 cells (of a total of 62) activated by vibrissal stimulation, responses were depressed in two cells (see Fig. 2) and enhanced in two cells. Early (5–8 ms) and late epochs (after 10 ms) were examined as it was possible that CRH might differentially affect direct thalamocortical synaptic and intracortical synaptic responses [1]. No such differential effects were observed. In 13 cells that exhibited spontaneous firing, this firing was increased in two cells and decreased in one cell during CRH applications. In some experiments continuous excitatory amino acid iontophoretic ejection (0–50 nA) was used to drive the firing rate of the cell during the study of the effects of CRH (e.g., see Fig. 3). In other experiments short pulse ejections (10–20 s, 30–60 nA) of amino acids were used (for example AMPA followed by glutamate in repeated cycle). In these experiments using glutamate and/or AMPA to excite the cell, such activity was depressed in 6 of 40 cells tested; and in none was this activity enhanced (see Fig. 3 for an example of glutamate driven activity that was unaffected by CRH). However, all these effects on excitatory transmission, whether on excitatory amino acid driven, spontaneous or stimulus-evoked, were very weak and were often difficult to reproduce. Similar results were obtained for 12 cells using pressure ejection to apply CRH (e.g., see Fig. 3). (For 3 of the 12 cells both iontophoresis and pressure ejection were tested).

One possibility was that CRH might influence inhibitory transmission. In order to test this CRH was applied during depressions produced by repeated cycle iontophoretic applications of GABA. In four such experiments CRH had no effect on the amplitude or duration of depressions induced by iontophoretic GABA (see Fig. 3), includ-

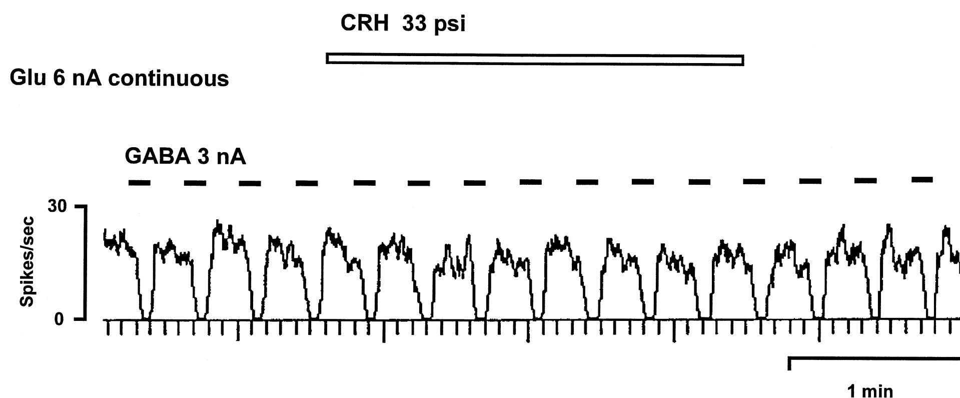

Fig. 3. Lack of effect of CRH on GABA evoked depressant responses. The SI cortical cell was driven by a continuous current of glutamate (6 nA), and GABA was applied at regular intervals (3 nA, black bars above trace). The maximal pressure used to apply CRH (33 psi, open bar) consistently failed to evoke any change in the amount or duration of depression induced by GABA.

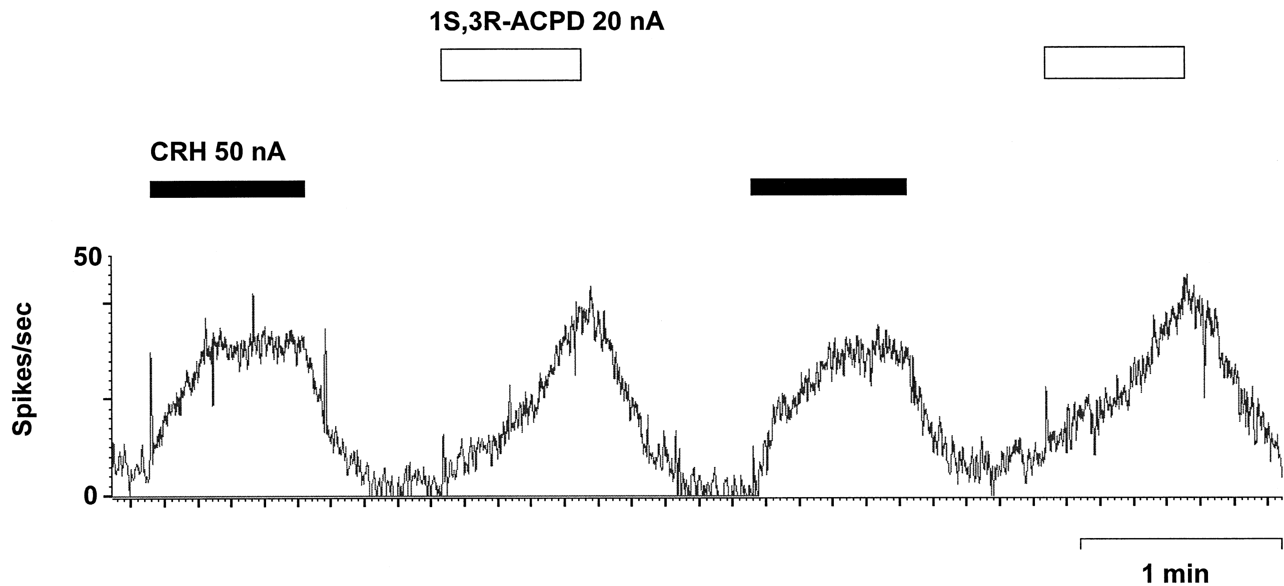

Fig. 4. Excitatory effect of iontophoretically applied CRH (filled bars) to a cell recorded in the region of the locus coeruleus. Note the characteristically slow onset and offset of the effect. The excitatory effect of the metabotropic glutamate receptor agonist 1*S*,3*R*-ACPD (open bars) is shown for comparison, and had a longer onset and offset time course.

ing two experiments where sub-maximal depressions were produced by GABA.

In four animals recordings were made in the region of the locus coeruleus (rLC) (as indicated by dye marks retrieved in the histology). In three of these animals recordings were first made in the cortex prior to the rLC, using the same pipette for both penetrations. Of 20 neurones recorded in rLC, 11 displayed clear excitations to iontophoretically applied CRH (median 50 nA). Fig. 4 shows a typical result of excitations produced by CRH in the rLC. By contrast none of the 10 cortical cells studied prior to the rLC were affected by CRH applications (median 200 nA).

The chromatography profile and elution position of the CRH-IR peak of samples of CRH-containing solutions used in the experiments was identical to that for synthetic CRH (1–41) used as standard. The specific RIA indicated that 1.8 mg ml^{-1} of CRH was present in the stock solution, whereas in samples taken from used electrodes following an experiment (more than 2 h duration) there was 1.9 mg ml^{-1} of CRH (not statistically different from each other). This indicates that the levels of CRH were similar in the frozen stock solution as in the solutions used for iontophoresis and pressure ejection, and that no, or insignificant, degradation occurred. The biological activity of the CRH was also confirmed using a bioassay. The results of this assay are given in Table 1. The assay results indicate that the CRH in the solutions used for electrophysiology was intact, and therefore that the lack of effect seen on the firing activity of most neurones studied was not due to any degradation in the biological efficacy of the peptide.

Table 1
Analysis of CRH specimens used in the experiments

Secretagogue used	Secretagogue concentration			
	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
CRH (1–41)	4.8 ± 0.3	6.2 ± 0.3	8.1 ± 1.0	9.2 ± 0.6
Stock	4.6 ± 0.4	5.9 ± 0.3	7.9 ± 0.8	8.1 ± 0.5
From pipette	4.4 ± 0.6	6.0 ± 0.9	8.5 ± 0.7	8.2 ± 1.0

Amounts of IR-ACTH released by synthetic CRH (1–41) (used as a standard control), a sample of the stock solution (kept frozen), and a sample extracted from a pipette used for an experiment that lasted more than 2 h. The biological activity of the samples used for the experiments was not different to that produced by the synthetic standard, and there was no difference between the stock and pipette samples. The values of IR-ACTH are given as ng ml^{-1} $well^{-1}$ $\pm$standard error of the mean. Apart from one difference at 10^{-6} M (synthetic CRH (1–41) and Stock), all comparisons lie within one standard error of the respective means.

4. Discussion and conclusions

This study has shown that locally applied CRH has little or no effect on the firing of most neurones in SI cortex. There were few and inconsistent effects on synaptically evoked responses. There was also little or no affect on the excitatory responses evoked by iontophoretically applied excitatory amino acids, nor any effect on the depressions produced by iontophoretic GABA. The weak effects of CRH were not related to the cortical layer in which the recordings were made. This contrasts with the striking layer-dependent effects of metabotropic glutamate agonist actions in this part of cortex [2]. There were also no differential effects on short vs. long latency response components, indicating that receptors for CRH were not selectively located at direct thalamocortical synapses vs. intracortical synapses.

Recordings made from the region of the locus coeruleus produced clear excitatory effects of CRH in more than half of the cells tested. This was consistent with other electrophysiological studies carried out in this region [15]. It indicates that the micropipettes were releasing the peptide and that the peptide could have electrophysiologically observable effects. As such these positive findings in rLC validate the negative findings in SI cortex, and confirm that CRH was ineffective when applied to cells of primary cortex.

Many peptides are known to be unstable when used in experimental procedures at room temperature over many hours. The analyses of the samples, both of stock solution and of solution removed from a pipette following an experiment, clearly demonstrated that CRH was not only present in a similar concentration to that initially prepared but also that the CRH was biologically still efficacious (see Table 1). These data show that the CRH, used under these experimental conditions, was not significantly degraded.

Recent work has identified other peptides related to CRH, for example urocortin [16] which has 45% sequence identity with CRH. It is possible that there was cross-reactivity with antibodies for one of these peptides in the previous studies aimed at localizing CRH in the cortex [8,9]. Urocortin is known to act at type-2 CRH receptors, at which CRH has less affinity. Further studies will need to determine the exact distribution of these other peptides in the cortex, and if necessary, determine their actions on the firing of cortical neurones.

Although in a minority of SI cells (13%) studied there appeared to be depression of firing and in even fewer cells (5%) some excitation occurred, these effects were difficult to reproduce and were all very weak (see Fig. 2 for the strongest effect observed). The present data indicate that CRH does not act as a neurotransmitter in primary somatosensory cortex. It is also unlikely to act as a neuromodulator since no reproducible effects were seen on synaptically evoked firing, nor on firing evoked by iontophoretically applied excitatory amino acids, nor on depressions produced by iontophoretically applied GABA. This suggests that CRH does not modulate excitatory or inhibitory cortical circuits in SI cortex. No differential effect of CRH was seen on presumed thalamocortical and intracortical excitatory transmission, as determined by analysis of short latency and longer latency synaptic responses. These data contrast with that previously obtained using iontophoretically applied CRH in rat forebrain cortex [6], although they did not examine possible effects of CRH on synaptic responses. In that study, 31 cortical neurones were recorded, of which almost half were excited (using a 30% change criterion). In addition they found that 13% of neurones were depressed and the remaining 39% were unaffected. This suggests that the role of CRH differs in different parts of cortex, in particular between primary and association cortices. In the present study the most common effect seen was depression (in 13% of neurones), and it is therefore conceivable that CRH might act as some kind of inhibitory neurotransmitter or modulator in SI cortex. However, it must be emphasized that the depressant effects observed here were extremely weak. It seems more likely that CRH is involved in the longer term regulation of signalling processes, or in trophic, tropic or other mechanisms underlying synaptogenesis and synapse retraction.

Acknowledgements

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