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Citation
Koutmani, Y, P K Politis, M Elkouris, G Agrogiannis, M Kemerli, E Patsouris, E Remboutsika, and K P Karalis. 2013. Corticotropin-releasing hormone exerts direct effects on neuronal progenitor cells: implications for neuroprotection. Molecular Psychiatry 18(3): 300-307.

Published Version
doi:10.1038/mp.2012.198

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IMMEDIATE COMMUNICATION

Corticotropin-releasing hormone exerts direct effects on neuronal progenitor cells: implications for neuroprotection

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Neurogenesis during embryonic and adult life is tightly regulated by a network of transcriptional, growth and hormonal factors. Emerging evidence indicates that activation of the stress response, via the associated glucocorticoid increase, reduces neurogenesis and contributes to the development of adult diseases. As corticotropin-releasing hormone (CRH) or factor is the major mediator of adaptive response to stressors, we sought to investigate its involvement in this process. Accordingly, we found that CRH could reverse the damaging effects of glucocorticoid on neural stem/progenitor cells (NS/PCs), while its genetic deficiency results in compromised proliferation and enhanced apoptosis during neurogenesis. Analyses in fetal and adult mouse brain revealed significant expression of CRH receptors in proliferating neuronal progenitors. Furthermore, by using primary cultures of NS/PCs, we characterized the molecular mechanisms and identified CRH receptor-1 as the receptor mediating the neuroprotective effects of CRH. Finally, we demonstrate the expression of CRH receptors in human fetal brain from early gestational age, in areas of active neuronal proliferation. These observations raise the intriguing possibility for CRH-mediated pharmacological applications in diseases characterized by altered neuronal homeostasis, including depression, dementia, neurodegenerative diseases, brain traumas and obesity.

Molecular Psychiatry (2013) 18, 300–307; doi:10.1038/mp.2012.198; published online 5 February 2013

Keywords: adult; CRH/CRF; neurogenesis; neuronal progenitors; stem cells; stress

INTRODUCTION

Living organisms maintain their physiological homeostasis during development as well as in adult life against constant challenges by internal and environmental stimuli. Although differentiated cells account for the majority of homeostatic functions, stem cells contribute critically to the whole process either by generation of differentiated cells or by changing their own function to adapt to the altered tissue/organ demands. Thus, in a number of tissues, including the nervous system, tissue-specific stem cells persist throughout life and give rise to new cells in order to meet the demands of turnover and injury-induced cell loss. Along these lines, emerging evidence has linked changes in adult neurogenesis to the pathogenesis, and often to the success of therapeutic regimens, of major diseases such as depression.2–6 Neurogenesis occurs constitutively in the embryonic brain and, as has been confirmed lately, to a lesser extent in specific niches of the adult brain.7,8 In the adult human and rodent brains, proliferation of neural stem/progenitor cells (NS/PCs) persists throughout life in areas such as the subventricular zones (SVZs) and the subgranular hippocampal zones.9 Neurogenesis involves a tightly controlled process of spatiotemporal neuronal proliferation and programmed cell death9 achieved by the orchestrated action of a network of transcription and growth factors. Additional intrinsic factors, such as secreted molecules, and environmental stimuli impact significantly on the potential of NS/PCs for proliferation, differentiation and survival, with mechanisms we start to understand better.10–14 For example, excess levels of circulating glucocorticoid, such as during prolonged, unopposed stress, are associated with suppressed proliferation12 and decreased survival of NS/PCs in the hippocampus.15,16

The adaptive response to challenges, otherwise stress or ‘fight-or-flight’ response,17 is a well-preserved process intimately associated with survival and development. In mammals, it is driven by the activation of the hypothalamic–pituitary–adrenal axis and the catecholaminergic system.18 The critical step in the development of the stress response is the activation of the neuropeptide corticotropin-releasing hormone (CRH) or factor.19 This response is self-limited as return back to homeostasis is linked to decrease in CRH neurons activation back to their basal state.20 CRH is expressed early in the developing mouse brain, such as on E13.5 in forebrain and on E10 in the cerebellum21,22 correlating temporally with the birth of the first neurons from the neural progenitor cells of the ventricular layer.22 In addition, in both the developing and the adult brain, CRH is expressed in the neurogenic niche of the hippocampal granular zone, a well-characterized neurogenic area.23 In the central nervous system, CRH has been shown to function as a neurotransmitter/neuromodulator.24 The first indication that CRH may be implicated in processes related to neuronal development and/or differentiation derived from the altered expression of genes involved in myelination and cell proliferation in transgenic mice overexpressing CRH.25 CRH has been also implicated in the differentiation of noradrenergic neurons in the locus coeruleus during brain development.26 CRH acts via binding to CRH receptor-1 (CRH-R1) and -2 (CRH-R2), members of the G-protein-coupled receptors (GPCRs) family.27 Several GPCRs have been

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Received 4 October 2012; accepted 9 October 2012; published online 5 February 2013
implicated in neurogenesis and apoptosis in site- and/or developmental stage-restricted manners. In line, neuroprotective effects of CRH have been described during development, in primary neuronal cultures exposed to toxic factors, or in vivo following oxidative stress. Here, we present evidence that CRH regulates neurogenesis, an effect that could be considered as part of the adaptive response of the nervous system to various challenges. This regulatory role of CRH is in line with late reports on the contribution of CRH in the maintenance of peripheral tissue homeostasis following metabolic or inflammatory stimuli. In particular, we show that CRH through its receptor-1 (CRH-R1) is implicated in the control of proliferation and apoptosis of NS/PCs both in vitro and in vivo. Most importantly, CRH seems sufficient to reverse the glucocorticoid-mediated suppression of proliferation and the associated induction of apoptosis in NS/PCs. Finally, we demonstrate the expression of CRH receptor(s) in the neurogenic areas of mouse and human brain. These data indicate a novel role of CRH and renders CRH as a potential therapeutic target for central nervous system-associated neurological disorders and diseases.

MATERIALS AND METHODS

Human tissues

Human embryos from 13 weeks old (n = 4) were obtained from Alexandra Hospital and from Medical School, University of Athens, Greece. The ethics committees of the participating university and hospital approved tissue collection. Handling of tissue was carried out in accordance with all regulations of the institutional ethics committees.

Animals

Animals housing and care were according to NIH and EU guidelines. The Ccrh null mouse line was generated as described previously. Ccrh+/– mice were raised in C57/Bl6 background and were obtained by crossing of Crh+/– mice and C57/Bl6 mice. In the adult mouse, we found Crh receptor expression in the lineage of neuronal progenitors of adult SVZ, a very active neurogenic area, including quiescent radial glia-like cells (nestin+/GFAP +) (Figures 2b–b′ and c–c′), transient amplifying progenitor cells (Mash1 +) (Figures 2d–d′) and migrating neuroblasts (DCX +) (Figure 2e–e′).

The above findings suggested the possibility that the CRH/CRHRs system may be involved in neurogenesis. We tested this hypothesis in primary cultures of NS/PCs isolated from the cortex of embryonic day 13.5 mouse brain. We first identified expression of CRH receptors in NS/PCs, by reverse transcriptase PCR (Figure 2f) and by immunocytochemistry (Figure 2g–g′). As shown by double staining for nestin and CRHRs, the great majority of nestin-positive NS/PCs (80%) co-express CRHRs (Figure 2g–g′).

Statistical analysis

Results are expressed in mean ± s.e.m. Data were analyzed by two-tailed, unequal, equal variance Student t-test and defined as p < 0.05.

RESULTS

Evidence for altered neurogenesis in the CRH-deficient mice

To assess the impact of CRH in mouse neurogenesis, we used the Ccrh null mouse with global CRH deficiency, and the corresponding glucocorticoid insufficiency. We used heterozygote pregnancies taking advantage of the fact that in that case all embryos, independent of their genotype, are exposed to similar glucocorticoid levels of maternal origin. Proliferation was assessed by immunohistochemistry of multiple tissue sections following BrdU administration on E14.5 for 2 h. As shown, significantly fewer BrdU-positive NS/PCs were identified in the proliferating layers of the cerebral cortex of Ccrh–/– mice compared with their littermate wild-type mice (Figures 1a and c). Furthermore, TUNEL analysis revealed significant increase in TUNEL + cells in the ventricular zones (VZ) and SVZ of the Ccrh–/– mice (Figures 1b and d). As glucocorticoid insufficiency of the Ccrh–/– mice was ‘corrected’, these findings reflect the direct effects of Ccrh+/– deficiency on neurogenesis in the developing mouse brain.

Expression of CRH receptors in neural progenitor cells of developing and adult mouse brain

Next, we assessed the expression of CRHRs in the developing mouse brain. Immunohistochemical analysis revealed a broad and strong reactivity for CRH receptors in mouse neuroepithelium on E14.5 (Figures 2a–a′). As shown (Figure 2a), CRH receptors were specifically expressed on NS/PCs, as indicated by the colocalization with the neuronal stem cell marker nestin. In the adult mouse, we found CRH receptors in the lineage of neuronal progenitors of adult SVZ, a very active neurogenic area, including quiescent radial glia-like cells (nestin+/GFAP +) (Figures 2b–b′ and c–c′), transient amplifying progenitor cells (Mash1 +) (Figures 2d–d′) and migrating neuroblasts (DCX +) (Figure 2e–e′).

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Distinct signaling pathways mediate the effects of CRH/CRH-R1 on NS/PCs

Binding of CRH to CRH-R1 induces the activation of cAMP and, in several cells and tissues, it is shown to engage additional signaling pathways, including mitogen-activated protein kinase (MAPK) and PI3K/Akt.20,28 To elucidate the contribution of these pathways in the neuroprotective effects of CRH, we applied specific inhibitors. As shown, PD98059, a MAPK inhibitor, blocked the CRH-induced increase of BrdU-positive NS/PCs, while co-treatment with wortmamzin, that blocks activation of the PI3 kinase, had no effect (Figures 4a and c). In contrast, wortmanin blocked the anti-apoptotic effect of CRH, whereas no effect of PD98059 was detected (Figures 4b and d). These findings demonstrate that two distinct intracellular signaling pathways, MAPK and PI3K/Akt, are specifically involved in the proliferative and anti-apoptotic effects of CRH/CRH-R1 on NS/PCs.

Expression of CRH receptors in proliferating zones of the developing human brain

To assess the potential implications of our findings in humans, we assessed the expression of CRH receptors in the developing human brain. We performed immunostainings with specific antibody that recognizes both CRH receptors (CRHRs). As shown, we detected wide expression of CRHRs in the fetal human brain from 13 weeks embryo (Figure 5a). Most importantly, there were high levels of expression staining in the ventricular and SVZ (Figures 5b–c), both rich in proliferating cells as revealed by staining for Ki67 (Figure 5c). Our findings demonstrate for the first time CRH receptors in the human brain in the proliferating neuronal cells from early fetal age and provide evidence for the possible relevance of our findings in human neurogenesis.

**DISCUSSION**

In this study, we demonstrate that CRH, identified as the major mediator of the stress response and glucocorticoid release in mammals, exerts protective effects on mouse neural progenitors/stem cells. We show that CRH receptors are expressed in neurogenic areas of fetal and adult mouse brain and that CRH-R1 mediates the above effects of CRH, via activation of distinct signaling pathways, MAPK and PI3K. We also provide evidence that CRH can oppose the neurotoxic effects of excess glucocorticoids on neuronal progenitors. Finally, we demonstrate that CRHRs are expressed in the dividing human fetal brain cells.

We found compromised neuronal proliferation and increased rates of apoptosis in the Crh–null (Crh–/–) fetal mouse brain as compared with wild-type tissue (Figure 1). CRH receptors are broadly expressed in the developing mouse neuroepithelium, particularly in actively proliferating, nestin-positive NS/PCs (Figure 2), around the time window that neurogenesis occurs. Significant expression of CRH receptors in neurogenic niches persisted in adult brain, with the strongest staining in the SVZ/rostal migratory stream. This area is considered critical for recovery from ischemia.
generation of GABAergic neurons and olfaction. As shown (Figure 2), a good proportion of all distinct cell types in the lineage of adult neurogenesis, including GFAP+ and nestin/GFAP (white arrowheads), Mash1 (red, d-d') and neuralblastic marker DCX (red, e-e’) in several areas is shown. Co-expression of CRHRs (green) and the neural progenitor markers Mash1 (red, d-d') and neuroblast marker DCX (red, e-e’) in several areas is shown. Co-expression of CRHRs and the neural progenitor marker nestin. While the majority of neural progenitor cells express both CRHRs and nestin (white arrows), some cells are selectively positive only to nestin (yellow arrows). 4’6-diamidino-2-phenylindole (DAPI) was used as counterstain. Scale bar = 100 μm (a), 50 μm (b-e), 200 μm (g). Figures in the right panel (a’, g’) or lower panels (b’-e’) are magnifications of the figures shown in the left or upper panel, respectively. aSVZ, anterior SVZ; CP, cortical plate; LV, lateral ventricle; pia, pial surface; RMS, rostral migratory stream.
as tissue-specific factors, co-expression of CRH-R2 and/or additional ligands, the cell type, that is, primary versus stable cell line. Neural mitogenic signaling has been primarily associated with the activation of tyrosine kinase receptors, whereas emerging evidence points to similar effects following activation of several GPCRs. CRH receptors belong to the class B subfamily of GPCRs that in addition to cAMP activation, in several tissues or cells, act through induction of NFκB, MAPK and PI3K. Modulation of neuronal survival analogous to that we found for CRH has been described for PACAP, VIP and MIP-2 ligands of other GPCRs. Our present data suggest that the dual effects, mitogenic and anti-apoptotic, of CRH on neural progenitor populations are specific and achieved via distinct signal transduction pathways, MAPK and PI3K, respectively (Figure 4). These findings were replicated in the SH-SY5Y cells, that provided a tool to show specific effects of CRH in cell cycle events and induction of cyclin D1 (Supplementary Figure S1). A similar mechanism has been postulated for the neuroprotective effects of VEGF. Using the same system, we found inhibition of the activation of caspase-3 by CRH (Supplementary Figure S1). On the other side, there is a body of reports on the hazardous effects of CRH/CRHR1 in the brain in association with chronic stress and the corresponding increase in glucocorticoid. Glucocorticoid receptors are expressed in the developing neuroepithelium, and the proliferation of NS/PCs has been decreased dramatically following dexamethasone or corticosterone treatment. Furthermore, high levels of glucocorticoid has been considered as the major cause of the stress-induced neuronal death, evidenced, for example, by reduction of the volume of the dentate gyrus during chronic stress. Here, we demonstrate blockade of these effects of glucocorticoid following co-treatment with CRH (Figure 3). Based on the above, it is possible that CRH and glucocorticoid have exerted opposing effects on the proliferation of NS/PCs. Along these lines, it was recently shown glucocorticoid inhibit cyclin D1, an effect opposite to our current findings with CRH. To our knowledge, this is the first study that demonstrates specific effects of CRH on physiological neurogenesis and the mechanisms involved. In agreement with our results, it has been shown that connexin 43, a factor expressed in fetal brain and involved in neurogenesis, mediates the neuroprotective effects of CRH. It is in support of the intriguing possibility of the applicability of our findings in human neurogenesis, the identification of CRHR in human fetal dividing neurons.

**Figure 3.** Effect of CRH in dexamethasone-treated NS/PCs proliferation and apoptosis. (a) Representative figures of 5-bromo-2-deoxyuridine (BrdU)-labeled cells (red) counted 24 h after treatment with or without CRH after pretreatment with dexamethasone. Dexamethasone was added in cell culture 1 h before CRH. 4′-6-diamidino-2-phenylindole (DAPI) staining was applied for visualization of total cell abundance. Scale bar = 200 μm. (b) Graph that depicts total number of the BrdU-positive cells count in 180 × 180 μm² areas. Data are shown as mean ± s.e.m. (n = 4). *P < 0.01, **P < 0.01 versus non-CRH-treated cells. (c) Effect of CRH on dexamethasone-treated NS/PCs apoptosis induced by serum deprivation for 24 h. Representative images of terminal transferase dUTP nick-end labeling (TUNEL)-stained NS/PCs (red) combined with DAPI nuclear staining (blue) after treatment with or without CRH and/or dexamethasone. Scale bar = 200 μm. (d) Graph depicts quantification of the TUNEL-positive cells count in 180 × 180 μm² areas. Data represent the mean ± s.e.m. (n = 4). *P < 0.05, **P < 0.01.
Furthermore, detailed analysis of the expression of the CRHR1 and CRHR2 genes in human tissues showed expression for both in the hippocampus to levels similar or even higher than in the amygdala, one of the main areas for CRH action. In a recent study, looking at the effect of CRH specifically in hippocampal pyramidal cells, the authors suggested that physiological release of low levels of CRH seem to be required for normal function of differentiated neurons.

In summary, our findings demonstrate stimulatory effects of CRH on mouse neurogenesis and indicate a direct homeostatic role for CRH in antagonizing the negative effects of glucocorticoid in neuronal survival. Our working hypothesis is that CRH exerts direct, beneficial effects on neuronal progenitors, via its specific receptor CRH-R1. These effects are unmasked in states of severe stress owing to the prolonged and significant rise in glucocorticoid and the associated inhibition of CRH expression. More studies are needed to provide further insights on the role of this peptide in human neuronal stem and progenitor cells. Our study raises the possibility for potential therapeutic application of CRH/CRHR1 in the treatment of brain and neurodegenerative disorders by support of specific neuronal actions.

CONFLICT OF INTEREST
The authors declare no conflict of interest

ACKNOWLEDGEMENTS
This work was supported by intramural funding of BRFAA to KPK and PKP and a Regpot Grant (TransMed) from the EU (KPK).
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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)