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Corticotropin-releasing hormone regulates common target genes with divergent functions in corticotrope and neuronal cells

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ABSTRACT

As a key regulator of the neuroendocrine stress axis and as a neuromodulator in the brain, the neuropeptide corticotropin-releasing hormone (CRH) plays an important role in various diseases of the central nervous system. Its cognate receptor CRH receptor type 1 (CRHR1) is a potential novel target for the therapeutic intervention in major depressive disorder. Therefore, a more precise understanding of involved intracellular signaling mechanisms is essential. The objective of this project was to identify specific target genes of CRHR1-mediated signaling pathways in the corticotrope cell line AtT-20 and in the neuronal cell line HN9 using microarray technology and qRT-PCR, respectively. In addition, we assessed the capacity of validated target genes to directly impact on CRHR1-dependent signaling using reporter assays. Thereby, we identified a set of CRHR1 downstream targets with diverging and cell type-specific roles which strengthen the role of CRH and CRHR1 as dynamic modulators of a variety of signal transduction mechanisms and cellular processes.

1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the stress response of the organism at multiple levels. A key component of the HPA system is the 41-amino acid neuropeptide corticotropin-releasing hormone (CRH). CRH plays a central role in coordinating the neuroendocrine, autonomic, and behavioral responses to stress (Vale et al., 1981). Increased and prolonged activation of the HPA axis has been associated with various psychiatric disorders (Tsigos and Chrousos, 2002). In addition, CRH is released at extrahypothalamic sites where it acts as a neuromodulator regulating neuronal activity. The hypothalamic CRH system modulates endocrine and metabolic responses to stress, whereas extrahypothalamic regions mediate the behavioral adaptation to stress (DeSouza, 1985; Koob, 1999). To understand the neuroendocrine and neuromodulator function of CRH in mood disorders, the CRH system has been extensively studied applying genetically engineered gain- and loss-of-function mouse models which underscore its importance for the development of stress-related disorders (Deussing and Wurst, 2005; Müller and Holsboer, 2006; Lu et al., 2008; Refojo et al., 2011; Dedic et al., 2011).

The complex mechanisms leading to changes in behavior initiated by CRH might be based on different signaling mechanisms. The functions of CRH are mainly mediated by the CRH receptor type 1 (CRHR1), which is widely expressed in the mammalian brain and the pituitary, with high levels found in the cerebral cortex, cerebellum, amygdala, hippocampus, olfactory bulb and anterior pituitary (Van Pett et al., 2000). CRH receptors belong to the secretin family of G protein-coupled receptors and are capable of activating different G proteins and signaling cascades upon ligand-binding. The dominant CRHR signaling pathway in endogenous cells and recombinant cell lines is the activation of the adenylyl cyclase (AC)–protein kinase A (PKA) pathway via $G_{\alpha s}$ (Aguilera et al., 1983; Olianas et al., 1995). But dependent on species, tissue and cell type, CRH receptors are capable to activate multiple signaling pathways including $G_{\alpha q}$/PLC, AKT/PI3-kinase, NOS/guanylyl cyclase, NFκB or NURR1/NUR77 (Hauger et al., 2006; Kovalovsky et al., 2002).

Comparable to the *in vivo* situation within the pituitary gland, CRH has major effects on the AtT-20 cell line via the CRHR1 (Rosendale et al., 1987; Iredale and Duman, 1997). Therefore, mouse pituitary corticotrope AtT-20 cells are an established cell culture model

Abbreviations: AC, adenyly cyclase; aRNA, amplified RNA; CRE, cAMP responsive element; CRH, corticotropin-releasing hormone; CRHR, corticotropin-releasing hormone receptor; FCS, fetal calf serum; GO, gene ontology; HPA, hypthalamic-pituitary-adrenal; MAPK, mitogen activated protein kinase.

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for CRHR1 signaling research. Peeters et al. (2004) as well as Trümbach et al. (2010) identified new target genes of CRHR1 signaling in AtT-20 cells by using microarray technology, but never investigated the functional role of these target genes in CRHR1-controled signal transduction itself (Peeters et al., 2004; Trümbach et al., 2010). Furthermore, the precise signaling mechanisms of CRHR1 in neuronal cell types are not fully understood yet.

Therefore, this study intended to identify target genes of CRH and CRHR1 in corticotrope cells and to investigate their function in the regulation of CRHR1-dependent pathways in corticotrope as well as neuronal cells. To this end, we investigated the transcriptional responses after CRHR1 stimulation at various time points in corticotrope cells. In the first instance, we applied microarray technology to compare the time-dependent gene expression profiles of CRH-treated and control AtT-20 cells. A subset of candidate genes, which are regulated in a CRH-dependent manner and are related to cAMP, mitogen activated protein kinase (MAPK) or epidermal growth factor receptor (EGFR) signaling, was chosen to address, in a second step, the direct impact of these candidate genes on CRHR1 signaling itself using reporter assays. Furthermore, some of the regulated genes we found in AtT-20 cells were also regulated by CRH in neuronal HN9 cells. We observed that the activation of CRHR1 leads to expression changes of common genes independent of cellular context in corticotrope and neuronal cells. However, differences in the direction of regulation and in the function of the candidate genes indicate distinct CRHR1-dependent signaling mechanisms determined by the cellular identity.

2. Materials and methods

2.1. Cell cultures and treatments

AtT-20 cells and HN9 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured under standard culture conditions in Dulbecco's modified eagle medium (DMEM; Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Invitrogen) and antibiotics (Invitrogen). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. After FCS deprivation for 18 h cells were treated in each experiment with human/rat CRH (100 nM in 0.01 M acetic acid) (Bachem, Heidelberg, Germany) for 1, 3, 6, 12 and 24 h, respectively (Trümbach et al., 2010). The efficacy of the CRH treatment in AtT-20 cells was approved by increased levels of ACTH in the cell culture medium and by increased Pomc mRNA expression as determined by qRT-PCR (Supplementary Fig. S1A and B). For both cell types untreated controls were harvested at according time points.

2.2. RNA isolation and microarray hybridization

AtT-20 cells were harvested and total RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's protocol. Amplified RNA (aRNA) synthesis and labeling were performed with the Amino Allyl MessageAmp™ aRNA Kit (Ambion, Austin, Texas) following the manufacturer's protocol. RNA and aRNA quality was controlled by gel electrophoresis. To exclude dye bias, a dye-swap approach was chosen, i.e. one half of aRNA was coupled to mono-reactive Cy3 and the other half to Cy5 N-hydroxysuccinimid (NHS) esters (Amersham). The labeled aRNA samples were hybridized on Max Planck Institute of Psychiatry (MPIP) 24 k mouse cDNA arrays (Max Planck Institute of Psychiatry, Munich, Germany) (Deussing et al., 2007). Six technical replicates were performed meaning three for each control/CRH-treated dye-coupling combination, and scanned on a PerkinElmer Life Sciences ScanArray 4000 laser scanner (Rodgau–Jügesheim, Germany).

2.3. Data normalization and selection of candidate genes

Microarray data were quantified by the fixed circle method using QuantArray (PerkinElmer Life Sci-ences). The bottom 10% of the scan intensity values were defined as background and erased. Raw data were then normalized using a nonlinear regression method according to Yang et al. (2002) and subjected to a t-test for significantly differential expression (Yang et al., 2002). The obtained p-values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure (Benjamini and Hochberg, 1995).

To identify the strongest regulated genes, simple cut-off criteria with p-value < 0.05, |Z-score| $\geqslant$ 1.837 and |fold regulation| $\geqslant$ 1.5 were applied. The Z-score is an additional statistical criteria and is calculated with the $\log_2$[ratio] of the mean expression values divided by the standard deviation. With a |Z-score| $\geqslant$ 1.837 accept2 = true and fulfills the statistical cut-off. Additionally, raw signal intensity > 950 was set as threshold ensuring the practicability of independent confirmation by qRT-PCR.

2.4. Quantitative real-time PCR

To validate the differential expression of selected candidate genes the annotation of the genes was verified by sequencing of the corresponding array clones (Sequiserve, Vaterstetten, Germany). cDNA of independently untreated and 100 nM CRH-treated AtT-20 and HN9 cells was analyzed by qRT-PCR using the LightCycler® FastStart DNA MasterPLUS SYBR Green I reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol and using different oligonucleotide primers (Errfi_fwd 5′-CAA-TCT-GAA-CTC-CCC-TGC-TC-3′, Errfi1_rev 5′-CTT-GAT-CCT-CTT-CAC-GCT-GTC-3′, Gapdh_fwd 5′-CCA-TCA-CCA-TCT-TCC-AGG-AGC-GAG-3′, Gapdh_rev 5′-GAT-GGC-ATG-GAC-TGT-GGT-CAT-GAG-3′, Pak3_fwd 5′-GAA-CAG-AAG-AAG-AAC-CCA-CAA-G-3′, Pak3_rev 5′-TAC-AGG-AGG-AGC-CAA-AGG-AG-3′, Rgs4_fwd 5′-TGC-AAG-CAA-CAA-AAG-AGG-TG-3′, Rgs4_rev 5′-CTG-GGC-TTC-ATC-AAA-ACA-GG-3′). The experiments were performed in duplicates in a LightCycler®2.0 (Roche Diagnostics, Mannheim, Germany) with the following PCR settings: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation (95 °C for t_D = 10 sec), annealing (T_A = 56–65 °C for t_A = 4–5 s) and elongation (72 °C, t_E = 7–13 sec). At the end of every run a melting curve (50–95 °C with 0.1 °C/sec) was measured to ensure the quality of PCR products. Crossing points (Cp) were calculated by the LightCycler®Software 4.0 (Roche Diagnostics, Mannheim, Germany) using the absolute quantification fit points method. Threshold and noise band were set in all compared runs at the same level. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Therefore, the real PCR efficiency was calculated from an external standard curve. The data was normalized to the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and Hypoxanthine-guanine phosphoribosyltransferase (Hprt, data not shown), and related to the data of untreated AtT-20 and HN9 cells, respectively.

RT-PCR for detection of CRHR1 and CRHR2 in AtT-20 and HN9 cells was performed using primers CRHR1_fw 5′-GCC-GCC-TAC-AAC-TAC-TTC-CA-3′; CRHR1_rev 5′-CAG-AAA-ACA-ATA-GAA-CAC-AGA-CAC-3′ and CRHR2_fw 5′-GGC-AAG-GAA-GCT-GGT-GAT-TTG-3′; CRHR1_rev 5′-GGC-GTG-GTC-CTG-CCA-GCG-3′.

2.5. Plasmids

pSG5-IcerIIγ was a kind gift of Paolo Sassone–Corsi (Liu et al., 2006) and was subcloned into pcDNA3.1 (Invitrogen) by restriction digest. pcDNA3-Nf2_16 and pcDNA3-Nf2_17 were kindly provided by David H. Gutmann (Sherman et al., 1997) and pcDNA3.1-HA-Pak3a and pcDNA3.1-HA-Pak3b by Jean–Vianney Barnier (Rousseau et al., 2003). For pcDNA3.1-Errfi1 and pcDNA3.1-Rgs4 the coding se-