Neuroscience 162 (2009) 14–22

REGION SPECIFIC GENE EXPRESSION PROFILE IN MOUSE BRAIN AFTER CHRONIC CORTICOTROPIN RELEASING FACTOR RECEPTOR 1 ACTIVATION: THE NOVEL ROLE FOR DIAZEPAM BINDING INHIBITOR IN CONTEXTUAL FEAR CONDITIONING

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Abstract—We have previously reported that repeated central administration of sub-anxiogenic doses of the corticotropin releasing factor 1 (CRF_1) agonist Cortagine, termed "priming," elicits a phenotype of increased anxiety-like behaviors in the elevated plus maze (EPM) and open-field test, and enhanced retention of contextual conditioned fear in C57BL/6J mice. Observed behavioral changes were functionally coupled to CRF_1-mediated elevated central cholecystokinin (CCK) tone in discrete brain regions. However, the changes in gene expression that mediated "priming"-induced behavioral and concurrent molecular changes in specific brain regions remained unknown. In the present study, a complementary DNA microarray analysis was used to investigate gene expression profiles in the hippocampus and prefrontal cortex (PFC) of C57BL/6J mice following the "priming" procedure. Here, we report that chronic stimulation of CRF_1, by i.c.v. administration of 10 ng Cortagine for five days, brought about alterations in the expression of a wide range of hippocampal (31 genes) and PFC (18 genes) genes, implicated in anxiety and aversive memory formation. These expression changes involved genes associated with signal transduction, neurotransmitter secretion, synaptic transmission, myelination, and others involved in the transport, biosynthesis, and binding of proteins. In particular, several genes of the protein kinase A (PKA) and protein kinase C (PKC) signaling cascades, known to be involved in synaptic plasticity, such as neurogranin, calmodulin 3, and the PKA regulatory subunit 1 b were found to be upregulated in the PFC and hippocampus of CRF_1 agonist "primed" mice. Moreover, we show pharmacologically that one of the newly implicated memory regulatory elements, diazepam-binding inhibitor (DBI) is functionally involved in hippocampus-dependent enhancement of contextual fear, a cardinal phenotypic feature of the "primed" mice. Finally, an interaction network mapping of the altered genes and their known interacting partners identified additional molecular candidates responsible for CRF_1-mediated hypersensitive fear circuitry.

Key words: CRF, priming, prefrontal cortex, hippocampus, anxiety, fear.

Numerous clinical observations suggest that prior exposure to stressful stimuli and a state of preexisting anxiety lowers the threshold for the elicitation of an anxiety response in the event that danger is detected (Hovatta and Barlow, 2008). In support of this notion, recent preclinical studies have demonstrated that chronic administration of sub-anxiogenic doses of a corticotropin releasing factor (CRF), and its related peptides, termed "priming," induces a state of increased sensitivity of an animal to subsequent stressful stimuli (Sajdyk and Gehlert, 2000; Rainnie et al., 2004; Sherrin et al., 2008). For example, repeated activation of the CRF receptors in the basolateral amygdala (BLA) with sub-threshold dose of CRF non-selective agonist urocortin1 (Ucn1) elicits high sensitivity to panicogenic sodium lactate and *N*-methyl-D-aspartate (NMDA) dependent anxiogenic responses in the elevated plus maze (EPM) and social interaction tests (Rainnie et al., 2004). Furthermore, in the absence of any additional Ucn1 treatment, these behavioral responses persist for more than 30 days (Rainnie et al., 2004). In a related study, we recently demonstrated that five days' administration of a sub-anxiogenic dose of CRF_1-selective agonist Cortagine (Tezval et al., 2004) results in an increase in cholecystokinin (CCK) mRNA and CCK_2 immunoreactivity in the basolateral amygdala and dentate gyrus of the hippocampus, with an associated increase in anxiety-related behaviors and enhanced ability to retain aversive memories (Sherrin et al., 2008). We suggested that our data provides a model for chronic, but mild stimulation of the CRF system, as may occur under stress conditions that elevate the central CCK tone and generate the phenotype of heightened anxiety.

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Abbreviations: aCSF, artificial cerebrospinal fluid; Aplp1, amyloid beta (A4) precursor-like protein 1; CCK, cholecystokinin; cDNA, complementary DNA; CRF, corticotropin releasing factor; cRNA, complementary RNA; Ct, threshold cycle; Ctnnb1, catenin beta; Dbi, diazepam binding inhibitor; EPM, elevated plus maze; FP, forward primer; Nrgn, neurogranin; PFC, prefrontal cortex; Prkar1b, protein kinase cAMP dependent regulatory type I beta; RP, reverse primer; Rps, ribosomal protein S; RT-PCR, real time polymerase chain reaction; Snca, synuclein A; TNFSF, tumor necrosis factor superfamily; Ttr, transthyretin; Ucn1, urocortin1; Vim, vimentin.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.04.012

However, the underlying molecular mechanisms involved in the crosstalk between central CRF-CCK neuropeptide systems, and the resulting behavioral phenotype, has remained unknown. The aim of the present study was to identify and characterize the gene expression profiles in the hippocampus and prefrontal cortex (PFC) of the C57BL/6J mice, by utilizing our "priming" paradigm. We report that i.c.v. administration of sub-anxiogenic dose of Cortagine for five days resulted in altered expression of 31 hippocampal and 18 PFC genes belonging to diverse functional groups. The altered genes and their interacting partners were then integrated into a gene interactome to get a wider perspective of CRF_1-induced behavioral and molecular sensitization of central fear circuitry. Finally, we identify down-regulation of diazepam binding inhibitor (Dbi) gene in the hippocampus as responsible for enhanced ability to retain aversive memories after chronic stimulation of CRF_1.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on 9 week old male C57BL/6J mice (Centre D'Elevage, Le Genest St Isle, France). The mice were individually housed and maintained on a 12-h light/dark cycle (lights on at 7 am) with access to food and water *ad libitum*. Animals were treated in accordance with National Institutes of Health guidelines, and procedures were approved by the University of Hawaii Animal Care. All efforts were made to minimize animal suffering. Additionally all experiments were carefully designed to reduce the number of mice used.

Cannulation and administration of drugs

Double guide cannulae were implanted with the help of a stereotactic holder following administration of 1.2% avertin anesthesia (0.02 ml/g i.p.) under aseptic conditions as previously described (Sherrin et al., 2008). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull by dental cement. The cannulae (C235; Plastics One, Roanoke, VA, USA) were placed into both lateral brain ventricles (anteroposterior −0.5 mm, lateral 1 mm, depth 2 mm) (Franklin and Paxinos, 2001). The animals were allowed to recover for 10 days before the experiments started. On the day of the experiment, bilateral injections were performed using an infusion pump (CMA/100, CMA/Microdialysis, Solna, Sweden) at a constant rate of 0.33 μl/min (final volume: 0.25 μl/side). Cortagine was synthesized as described previously (Tezval et al., 2004). It was initially dissolved in 10 mM acetic acid and diluted 1:2 with sterile saline (0.9% NaCl). The vehicle was made as above with the omission of Cortagine. For treatment with Cortagine, the C57BL/6J mice were divided into two groups. One group received a sub-effective dose of 10 ng (2.3 pmol) Cortagine i.c.v., whereas another group was administered vehicle. For behavior experiments we also used a commercially available DBI peptide fragment (Bachem, Torrance, CA, USA). This fragment, which is composed of 20 amino acids from glutamine (Gln) 51 to lysine (Lys) 70 of the human DBI sequence, was shown to be biologically active in mice (Dong et al., 1999). The DBI peptide fragment was dissolved in artificial cerebrospinal fluid (aCSF). The DBI dose used was 2.5 μg/0.5 μl (1.17 nmol), and was selected on the basis of dose response studies in mice (Dong et al., 1999; Manabe et al., 2001).

For microarray study on experimental days 1–4 both groups were injected with Cortagine or saline, respectively, and returned to their cages after each injection. On day 5 both groups were sacrificed 1 h after receiving a 5th injection of either saline or Cortagine. The number of mice per group was 6. For behavior experiments the same treatment procedure was employed except that both groups either received an i.c.v. injection of aCSF or DBI 15 min after the last injection of Cortagine or saline, and the EPM or contextual fear conditioning was performed 30 min afterward (Fig. 3). The number of mice per group was eight to 10 per treatment.

Brain tissue collection

Mice were killed by cervical dislocation 1 h after the 5th injection of either saline or Cortagine. The brains were immediately frozen in liquid nitrogen. Thick sections (500–600 mm) were sliced with a vibratome, and the PFC and hippocampus were dissected from these sections under a dissecting microscope, following delineations from the mouse brain atlas (Franklin and Paxinos, 2001). Tissues were stored at −80 °C until further used.

RNA isolation, reverse transcription and microarray analysis

Hippocampal and PFC total RNA was extracted using the SV total RNA isolation kit (Promega, Madison, WI, USA). RNA integrity was verified by measuring the A260/280 ratio on denaturing formaldehyde gels. Samples were pooled (n=2 mice per pool; n=3 pooled samples) for each treatment group and region and replicated on three chips. Target preparation and hybridization were according to the manufacturer's technical manual (Affymetrix, Santa Clara, CA, USA). Fifteen micrograms of total RNA was converted to complementary DNA (cDNA) using Superscript III for cDNA synthesis (Invitrogen, Karlsruhe, Germany). This was then converted to biotinylated complementary RNA (cRNA) (IVT labeling kit, Affymetrix) with Enzo High Yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). The synthesized cRNA was purified on an RNeasy column (Qiagen, Hilden, Germany). Purified cRNA was fragmented into segments of 40–200 nucleotide length by incubation in a fragmentation buffer (100 mM potassium acetate, 30 mM magnesium acetate, 40 mM Tris–acetate pH 8.1) for 20 min at 94 °C. The quality of cRNA amplification and cRNA fragmentation was monitored by micro-capillary electrophoresis (Bioanalyser, 2100, Agilent Technologies, Santa Clara, CA, USA). Targets were hybridized for 16 h at 45 °C to the mouse genome (MOE430A) Genechip (Affymetrix). The gene chips were washed and stained with streptavidin–phycoerythrin by using a fluidics system according to the procedure 2 protocols from Affymetrix. The chips were scanned with an Agilent GeneArray Scanner (Agilent Technologies, Palo Alto, CA, USA). From data image files, gene transcript levels (probe signal levels) were determined with the use of algorithms in the microarray suite 5.0 (Affymetrix).

Data normalization and statistical analysis

The average intensity of all probe sets was used for normalization and scaled to 100 in the absolute analysis for each probe array. Scaling procedure did not affect the global similarity between the samples. The Affymetrix MAS 5.0 scanner software provided individual probe readings. These were analyzed with dChip 3.1 (www.dchip.org) software (Li and Wong, 2001). This software fits a linear model across all chips. This allows the signal variation in each probe set to be partitioned into a specific hybridization-related signal vs. noise, and does so with increasing power relative to the number of chips. The approach also enables outliers to be flagged, providing a toll for quality control. The model-based method (Li and Wong, 2001) was used for probe selection and computing expression values. These expression levels were attached with standard errors, which were subsequently used to compute 90% confidence intervals of fold changes in two-sample or two-group comparisons. For each treatment group (naive, sa-