Neuroscience 218 (2012) 185–195

CHOLECYSTOKININ KNOCK-DOWN IN THE BASOLATERAL AMYGDALA HAS ANXIOLYTIC AND ANTIDEPRESSANT-LIKE EFFECTS IN MICE

C. DEL BOCA, P. E. LUTZ, J. LE MERRER, P. KOEBEL AND B. L. KIEFFER *

Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Centre National de Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale/Université de Strasbourg, Illkirch-Graffenstaden, France

Abstract—Cholecystokinin (CCK) is a neuropeptide widely distributed in the mammalian brain. This peptide regulates many physiological functions and behaviors, such as cardio-respiratory control, thermoregulation, nociception, feeding, memory processes and motivational responses, and plays a prominent role in emotional responses including anxiety and depression. CCK-expressing brain regions involved in these functions remain unclear and their identification represents an important step towards understanding CCK function in the brain. The basolateral amygdala (BLA) is strongly involved in emotional processing and expresses high levels of CCK. In this study we examined the contribution of CCK expressed in this brain region to emotional responses in mice. To knockdown CCK specifically in the BLA, we used stereotaxic delivery of recombinant adeno-associated viral vectors expressing a CCK-targeted shRNA. This procedure efficiently reduced CCK levels locally. shCCK-treated animals showed reduced levels of anxiety in the elevated plus-maze, and lower despair-like behavior in the forced swim test. Our data demonstrate that CCK expressed in the BLA represents a key brain substrate for anxiogenic and depressant effects of the peptide. The study also suggests that elevated amygdalar CCK could contribute to panic and major depressive disorders that have been associated with CCK dysfunction in humans.

Key words: cholecystokinin, baso-lateral amygdala, depression, anxiety, adeno-associated viral vector, short-hairpin RNA.

INTRODUCTION

Cholecystokinin (CCK), first identified as a gastrointestinal hormone (Mutt and Jorpes, 1968), is also highly expressed within the brain (Meziane et al., 1997). Neural CCK has been implicated in a wide range of physiological processes such as emotional and motivational states, thermoregulation, nociception and cognition (Beinfeld, 2001). Pre-pro-CCK is cleaved in smaller biologically active fragments acting as neuromodulatory peptides (Rotzinger et al., 2010). CCK peptides activate two closely related G-protein coupled receptors, CCK_1 (CCK_1-R) and CCK_2 (CCK_2-R), the latter being predominant in the brain (Noble et al., 1999).

In human, high levels of CCK have been associated with motivational loss and panic disorders (Hebb et al., 2005), and a role for CCK has been proposed in the induction of anxiety and depression (Shindo and Yoshioka, 2005; Berna et al., 2007), with CCK_2-R being the main effector of CCK anxiogenic properties (Bradwejn and Koszycki, 2001; Eser et al., 2011). In rodents, CCK_2-R agonists increase levels of anxiety-like responses in several behavioral paradigms (Rotzinger et al., 2010), while conversely systemic CCK_2-R antagonists attenuate stress-induced anxiety-like behavior (Wang et al., 2011). In addition, CCK_2-R antagonists show antidepressant-like properties: systemic injection of a CCK_2-R antagonist decreased behavioral despair in the forced swim test, and this effect was synergistic with the activity of the enkephalinase inhibitor RB101 (Hernando et al., 1996). Furthermore, chronic blockade of CCK_2-R normalized hypothalamic–pituitary–adrenal axis hyperactivity and normalized the increase in despair-like behavior elicited by repeated social defeat (Becker et al., 2008). Concordant with pharmacological studies, genetic approaches (Noble and Roques, 2002) reveal that CCK_2-R knock-out (KO) mice display decreased levels of anxiety (Raud et al., 2005). Moreover, transgenic mice over-expressing CCK_2-R showed increased anxiety, as well as prolonged HPA axis activity following acute stress (Chen et al., 2010). Finally, antagonistic interactions between CCK and opioid systems have been established in the modulation and expression of stress-related behaviors, opposing memory-enhancing and anxiogenic effects of CCK to amnesic and anxiolytic effects of opioid peptides (Hebb et al., 2005). These data also implicate CCK in reward, motivation and addictive behaviors (Lu et al., 2002; Pommier et al., 2002; Mitchell et al., 2006).

CCK and CCK-Rs are widely distributed throughout the rodent brain and may modulate emotional behaviors at

*Corresponding author. Address: IGBMC, Parc d'innovation 1 rue Laurent Fries, BP 10142, 67404 Illkirch Cedex, France. Tel: +33-(0)8-65-56-93; fax: +33-(0)8-65-56-04.
E-mail address: briki@igbmc.fr (B. L. Kieffer).

Abbreviations: ANOVA, analysis of variance; BCIP, bromo-chloro-indolyphosphate; BLA, basolateral amygdalaCCK, Cholecystokini; CPA, conditioned place aversion; EPM, elevated plus-maze; FST, forced swim test; ISH, *in situ* hybridization; KO, knock-out; LD, light/dark box; NBT, Nitroblue tetrazolium; OF, open field; PBS, phosphate buffered saline; RSA, retrosplenial agranular cortex; RT, room temperature; shRNA, short-hairpin RNA; TS, tail suspension test.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.05.022

several brain sites, notably in olfactory bulb, cerebral cortex, hippocampus, amygdala, thalamus and ventral tegmental area (Meziane et al., 1997; Cain et al., 2003). The amygdalar complex is implicated in the generation of emotional states, stress coding and associative learning (LeDoux, 2000), as well as retrieval of aversive memories (Frenois et al., 2005). In rat, local pharmacological studies have suggested a role for CCK_2-R in the basolateral amygdala (BLA) in anxiogenic effects of CCK (Belcheva et al., 1994; Frankland et al., 1997). To our knowledge however, the role of CCK expressed in the BLA has not been investigated by genetic approaches. In the present study, we used a siRNA-based strategy to locally knock-down CCK. We injected in the BLA a recombinant viral vector encoding a short hairpin RNA (shRNA) targeting CCK and then examined behavioral consequences on emotional responses.

EXPERIMENTAL PROCEDURES

AAV_2 viral vectors construction

A shRNA was designed to target the m*cck* gene (5′-TC AGTGACTCCCAGACCTAATGTT-3′). Recombinant adeno-associated virus serotype 2 (AAV_2)-shCCK viral vectors were generated expressing enhanced green flourescent protein (eGFP) and shCCK (AAV_2-shCCK) under the control of CMV and mU6 promoters respectively. Control vectors encode either for eGFP alone (AAV_2-eGFP) or for eGFP and a scramble shRNA (AAV_2-shScramble). The shScramble sequence selected (5′-GTTGGCTCCTAGCAGATCCTA-3′) has no match *in silico* in the mouse genome. AAV_2 vectors were generated by triple transfection of AAV-293 cell line (Stratagene) using (i) either pAAV-eGFP, pAAV-eGFP-shScramble or pAAV-eGFP-shCCK, (ii) pAAV-RC (Stratagene) containing *rep* and *cap* genes of the AAV_2 and (iii) pHelper (Stratagene) encoding for the adenoviral helping functions. Following 2 days cells were collected, lysed and treated with Benzonase (50 U/ml, sigma, 30 nm, 37 °C). Viral vectors were purified by iodixanol gradient ultracentrifugation (Zolotukhin et al., 2002) followed by dialysis and concentration against Dulbecco phosphate buffered saline (PBS) using centrifugal filters (Amicon Ultra-15 Centrifugal Filter Devices 50 K). Viral particles were quantified by real time PCR using a plasmid standard pAAV-eGFP. To achieve comparable working concentrations, viruses were diluted in Dulbecco-PBS buffer to a final concentration of 3×10^{11} viral genomes *per* ml (vg/ml) and finally stored at −80 °C until use.

Behavioral experiments

Animals. Male C57BL/6J mice provided by Charles River (Lyon, France) were used for all the experiments. Mice were aged 8 weeks at the beginning of the experiments and housed 3–4 *per* cage in a 12 h dark/light cycle (light from 7 am to 7 pm), under controlled conditions of temperature and humidity. Food and water were available *ad libitum*. Experimental procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the local ethical committee (Comité Régional d'éthique en matière d'expérimentation animale de Strasbourg, CREMEAS, 2003-10-08-[1]-58).

Drugs. For surgery mice were anaesthetized using ketamine/xylazine (Virbac/Bayer, 100/10 mg/kg). Morphine hydrochloride (Francopia), naloxone hydrochloride (a non-specific opioid antagonist, Sigma) and anesthetics were dissolved in sterile isotonic saline (NaCl 0.9%). All the doses refer to salt weight and were administered in a volume of 10 ml/kg.

Experimental procedure. Experiment 1. 12 naïve mice were unilaterally injected into the BLA with either AAV_2-shCCK ($n = 6$) or AAV_2-eGFP ($n = 6$) viral vectors. To qualitatively assess their silencing activity, we performed *in situ* hybridization (ISH) using Dig-labeled CCK-cRNA probes. To confirm the stability of CCK knock-down over time, Dig-ISH was performed either 2 or 6 weeks following surgery ($n = 3$ mice/time point/viral vector).

Experiment 2. 56 mice (2 independent cohorts) were bilaterally injected into the BLA with either AAV_2-shCCK or AAV_2-shScramble vectors (Cohort 1, $n = 16$ mice/group; Cohort 2, $n = 12$ mice/group) and 5 weeks later analyzed for emotional responses. Animals from the second cohort were processed only throughout tests for which an effect was observed in the first cohort. Twenty-four hours after the last behavioral experiment, all mice were sacrificed and brains analyzed for injection accuracy and viral spread. Finally, we used Dig-ISH to qualitatively confirm the pattern of down-regulation ($n = 3$ mice/condition) and [^{35}S]-ISH to quantify the intensity of CCK knock-down in both shCCK- and shScramble-injected mice ($n = 5$ mice/condition).

Surgery and virus delivery. Anesthetized mice were placed in a stereotaxic frame (Unimécanique, France). The skull was exposed and incisor bar adjusted such that bregma and lambda were at the same height. Stereotaxic injections were performed according to the Mouse Brain Atlas (Paxinos and Franklin, 2001). To this aim, a 5 µl microsyringe (SGE Analytical Science, Australia) was mounted to a micro-drive pump (Harvard apparatus, France) and connected by a PE-10 polyethylene tubing (Harvard apparatus, France) to a stainless-steel injector needle (0.47 mm external diameter). 1.5 µl of purified AAV_2 viral vectors were delivered into the BLA uni- or bilaterally in experiment 1 and 2, respectively. Injection speed was 0.1 µl/min, and the needle was slowly withdrawn 10 min after delivery. Following surgery, mice were single housed for 48 h to recover and then placed back in their original home cages.

Behavioral testing. We evaluated the effects of local CCK knock-down in four categories of behaviors: anxiety-like behavior, despair-like behavior, aversive place conditioning, and withdrawal syndrome, starting 5 weeks after surgery. The design of the behavioral test battery (see Fig. 1) was adapted from previous reports (McIlwain et al., 2001; Duangdao et al., 2009) and tests were ordered from less to most stressful as follows: elevated plus-maze (EPM), open field (OF), light/dark box (LD), forced swim (FST), tail suspension tests (TS), naloxone-induced conditioned place aversion (CPA), and naloxone-precipitated withdrawal. Elevated plus-maze was placed at the beginning of the battery as recommended by Voikar et al. (2004). The inter-test intervals were selected to allow the mice to fully recover between tests. A 3-days interval was chosen between tests of the same category, a 5-days interval separate anxiety from despair behaviors, and 7-days intervals were placed before and after the naloxone-induced CPA. All behavioral tests were performed between 8am and 1 pm.

Elevated plus-maze. The EPM was a plus-shaped maze elevated 52 cm from base, with black Plexiglas floor, consisting of two open and two closed arms (37 × 6 cm each) connected by a central platform (6 × 6 cm). The experiments were conducted under low-intensity light (10 lux). Movement and location of the mice were analyzed by an automated tracking system (Videotrack; View Point, Lyon, France). Each mouse was placed on the central platform facing a closed arm and observed for 5 min. Anxiety-like behavior was assessed by measures of the time spent and number of entries in closed and open arms of the maze, and related time and activity ratios (time spent or number of entries in open arms/total time spent or number of entries in arms). Risk-taking behavior was evaluated by the percentage of time spent in the distal part of the open arms (time spent in the last 1/3 of the open arm/total time in arms) and the number