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Normal Anxiety, Fear and Depression-related Behaviors in Mice Lacking α-Calcitonin Gene-Related Peptide

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Calcitonin gene-related peptide (CGRP) expressing neurons are distributed widely throughout the central and peripheral nervous systems. Due to its distribution and pharmacological studies, CGRP has been implicated to be involved in anxiety, fear and depression. In this study, αCGRP-knockout mice were used to assess the consequences of removing this neuropeptide to the mice behaviors. αCGRP-knockout mice performed equally as well as wild type mice in the light-dark transition test and in the elevated plus maze test of anxiety. αCGRP-null mice behaved similarly as wild-type mice in the Porsolt swim test of depression. They also exhibited normal learning and memory in the fear conditioning tasks. It is concluded that αCGRP is not essential for mice to be able to perform these tests, despite the presence of αCGRP in the relevant regions of the brain.

Key Words: CGRP, Mouse, Behavior, Anxiety, Fear, Depression

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide that are widely expressed in the central and peripheral nervous systems (Amara et al, 1985; Skofitsch & Jacobowitz, 1985c; Kruger et al, 1988). Although CGRP has been implicated in numerous physiological processes including peripheral vasodilation (Brain et al, 1985), cardiac acceleration (Sigrist et al, 1986), a reduction of gastric acid secretion (Lenz et al, 1985) and nicotinic receptor activity at the neuromuscular junction (Takami et al, 1986), the precise physiological roles of CGRP remain to be elucidated.

Because CGRP-positive neurons are prevalent throughout the brain regions known to be associated with anxiety including the nucleus accumbens, central amygdaloid nucleus (Skofitsch et al, 1985; Saria et al, 1992) and fewer CGRP binding sites are identified in the central amygdaloid nucleus of the alcohol-preferring rats than non-alcohol preferring rats (Hwang et al, 1995), CGRP has been considered to be involved in the anxiolytic effects of ethanol. Likewise, the presence of CGRP in amygdala and ventral tegmental area (Skofitsch & Jacobowitz, 1985; Deutch & Roth, 1987; Marcos et al, 1999) suggests that CGRP are involved in fear and depression related behaviors. Consistent with this possibility, intracerebroventricular administration of CGRP potentiated fear-related behaviors (Poore, 1996) and administration of human $CGRP_{8-37}$ attenuated freezing to an auditory conditioned stimulus (Kocorowski & Helmstetter, 2001), suggesting that CGRP may play an important role in fear-related behaviors. CGRP concentrations in the cerebrospinal fluids were found to be significantly increased in the patients with depressive disorders compared to control groups (Mathe, 1994), suggesting CGRP's role in depression.

The overlapping expression pattern for the α- and β-CGRP (Amara et al, 1985), the multiple CGRP receptor subtypes (Kapas & Clark, 1995; Aiyar et al, 1996), and the lack of specific CGRP antagonists (Dumont et al, 1997) have complicated studies on the biological relevance of this neuropeptide. To assess the role of endogenous αCGRP in the brain, anxiety, depression and fear-related behaviors were examined in mice with targeted deletion of the expression of αCGRP.

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METHODS

Animals

The generation of αCGRP mutant mice have been described previously and the successful generation of αCGRP-knockout mice was confirmed by RNase protection assay and immunohistochemistry (Lu et al, 1999); αCGRP-null heterozygotes were backcrossed with wild-type C57BL/6 mice for 10 generations to generate a congenic strain for phenotypic comparisons to wild-type C57BL/6 control mice. Animals were housed in a temperature and humidity-controlled environment with a 12-hour light/dark cycle and all mice had *ad libitum* access to food and water. Mice of both genders (8~13 weeks of age) were used for the described studies as indicated. All behavioral experiments were performed between 9 : 00 a.m. and 3 : 00 p.m. Experimental protocols were performed in accordance with the regulations of the Vanderbilt Institutional Animal Care and Use Committee.

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; CS, conditioned stimulus; US, unconditioned stimulus.

Light-dark transition test

The light/dark transition test consisted of a cage (27.4×27.4×20 cm) equally divided into two by a black partition containing a small opening (MED-associates). One chamber was open and brightly illuminated, whereas the small chamber was closed and dark. Mice were placed into the illuminated side of the apparatus and allowed to move freely between the two chambers for 10 min. The total number of transitions, time in the dark side and distance traveled were recorded.

Elevated plus maze

The plus-maze consisted of two open arms (30×5 cm) and two enclosed arms of the same size, with 15 cm high, transparent walls. The arms and central square were made of black plastic plate and elevated to a height of 50 cm from the floor. In order to minimize the likelihood of animals falling from the apparatus, 3-mm high plexiglass edges were provided for the open arms. Arms of the same type were arranged at opposite sides to each other. Each mouse was placed in the central square of the maze, facing one of the open arms (5×5 cm). During a 10 min test period, the behavior of the mouse was recorded by Image EP software. The number of entries onto, and the time spent on, (a) open and (b) enclosed arms were recorded. The maze was cleaned with alcohol after each trial. For data analysis we employed the following three measures: the percentage of open arm entries, the percentage of time spent on the open arms, and the total number of arm entries. Data acquisition and analysis were performed automatically, using Image EP software.

Porsolt forced swim test

Each mouse was placed in a Plexiglas cylinder (diameter, 15 cm), which was filled with water (22°C, 7.5 cm height), for a trial period of 10 min. Data acquisition and analysis were performed automatically, using Image FZ software. Immobility was determined by the software in a similar way described in the methods for contextual and cued fear conditioning. Images were captured at 1 frame per second. When the amount of area changed between two successive frames was below certain threshold (i.e. 10 pixels), it was judged as 'immobile'. 'Immobility' lasting less than defined time threshold (cf. 2 sec) was discarded. Percentage time spent in immobile posture was calculated for each 30 seconds bin.

Context- and auditory-cued fear conditioning

Each mouse was placed in a test chamber (20×16×36 cm) inside a sound-attenuated chamber (MED associates) and allowed to explore freely for 2 min. A 4.5 kHz pure tone (conditioned-stimulus; CS), which served as the conditioned stimulus, was presented for 30 sec followed by a mild (2 sec, 0.5 mA) foot shock (unconditioned stimulus; US), which served as the conditioned stimulus. Two more CS-US pairings were presented with 2 min inter-stimulus interval. Context testing was conducted 24 hours after conditioning in the same chamber. Auditory-cued testing with altered context was conducted 48 hours after conditioning using triangular box (25×25×30 cm) made of white opaque plexiglass, which was located in different room. Orange extract odor was added to change context. Data acquisition, control of stimulus (i.e. tones and shocks) and analysis were performed automatically, using Image FZ software. Images were captured at 1 frame per second. For each pair of successive frames, exclusive OR was carried out in order to measure amount of area (pixels) by which subject moved in context- and auditory-cued fear conditioning test. When the amount of area was below certain threshold (i.e. 10 pixels), it is judged as 'freezing'. When the amount of area equals or exceeds the threshold, it was judged as 'non-freezing'. Optimal threshold (amount of pixels) to judge freezing was determined by adjusting it to the amount of freezing measured by human observation. One frame/sec of frame capturing rate yields good agreement with results obtained by human. 'Freezing' lasting less than defined time threshold (i.e. 2 sec) was discarded. Percentage freezing was calculated for each 30 seconds bin. Images were captured at 2.5 frames/second for 6 seconds from 2 seconds before shock presentations to 2 seconds after termination of the shocks to assess the reactivity to shocks by measuring distance traveled during shock presentation.

Image analysis

Mouse behavioral responses in the elevated plus maze test, contextual and cued fear conditioning, social interaction test, and Porsolt forced swim test were recorded and analyzed by Image EP, Image FZ, Image SI and Image FZ, respectively. The applications were based on the public domain NIH Image program (developed by Wayne Rasband at the U.S. National Institute of Mental Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and were modified for each test by Tsuyoshi Miyakawa (available through O'Hara & Co., Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted, using StatView (SAS institute). Data were analyzed by two-tailed t test, two-way ANOVA, or two-way repeated measures ANOVA. Values in tables and graphs were expressed as mean ∀standard error of mean (S.E.M.).

RESULTS

Light-dark transition test

To test anxiety-related behavior, the light-dark transition test was performed (Fig. 1). Light-dark transition test is to titrate the tendency of mice to explore a novel environment versus the aversive properties of a brightly lit open field (Crawley & Goodwin, 1980; Blumstein & Crawley, 1983). Two measures commonly used to examine anxiety-related responses in the light-dark box are the latency to enter the dark and the total number of transitions between the light and dark chambers. We found no significant differences between wild-type and αCGRP-null mice in either the latency to enter the dark or the total number of transitions between the dark and light chambers of the testing apparatus.

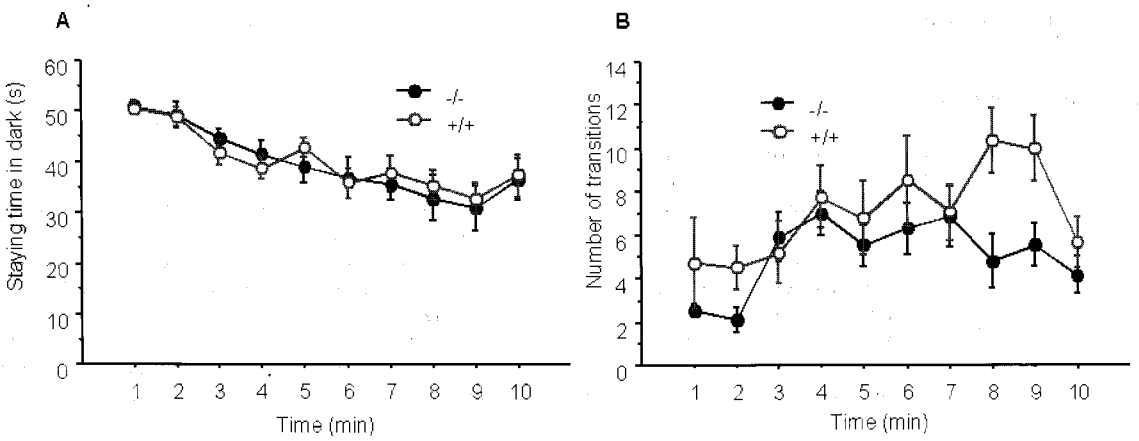

Fig. 1. Analysis of anxiety-related behaviors in the light-dark transition test. Data are mean values±S.E.M. for time to enter the dark side (A) and total number of transitions (B) for wild-type (+/+; n=20) and αCGRP-null mice (−/−; n=20) during the 10-min test. Data are presented as ten 1-min intervals.

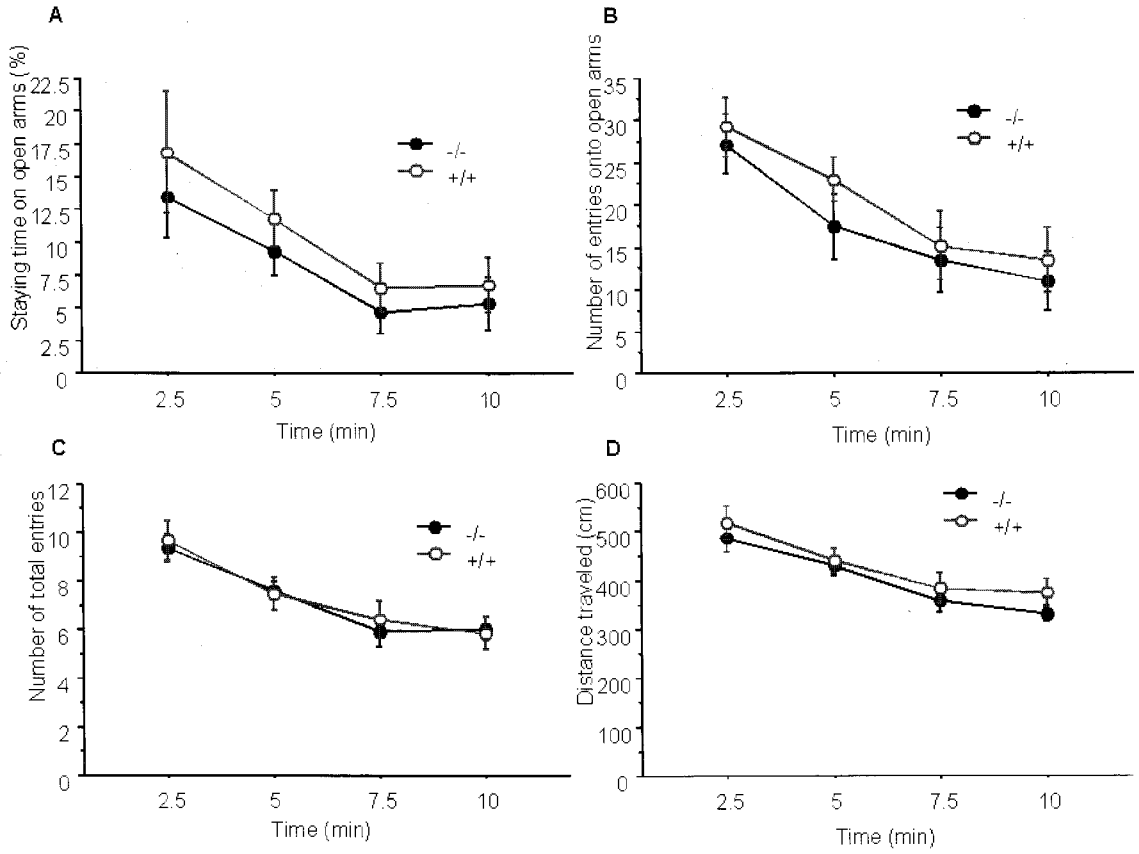

Fig. 2. Analysis of anxiety-related behaviors using the elevated plus maze. Data are mean values±S.E.M. for the staying time on open arms (A), the number of entries to open arms (B), the number of total entries (C) and total distance traveled (D) for wild-type (+/+; n=20) and αCGRP-null mice (−/−; n=20) during the 10-min test. Data are presented as four 2.5-min intervals.

Elevated plus maze

As another measure of anxiety-related behavior, we used the elevated plus maze test (Fig. 2). In this behavioral paradigm, the mice rests on the same naturalistic conflict between the tendency to explore a novel environment and the aversive properties of a brightly lit, open area (Handley & Mithani, 1984; Pellow et al, 1985). The mutant mice demonstrated no abnormal behaviors compared to wild-type littermates in the staying time on open arms, the

number of entries onto open arms, the number of total entries and the total distance traveled.

Porsolt forced swim test

To examine the defect of depression-related behavior in the mutant mice, we used the Porsolt forced swim test (Fig. 3). In this behavioral test, initially the mice swim in the water, apparently seeking an escape route. After some time, the mouse stops swimming and floats on the surface of the water, appearing to have given up the search for an escape route that has been suggested to represent an index of depression and this immobility was reduced by tricyclic antidepressants and atypical antidepressants validating that this assay is effective for analyzing depression-related behavior (Porsolt et al, 1977; Plaznik & Kostowski, 1987). We found no significant difference in the immobility time between wild-type and knockout mice, indicating endogenous αCGRP is not essential for generating depression-related behaviors.

Context- and auditory-cued fear conditioning

To assess fear-related behavior using a Pavlovian model of learning and memory, context- and auditory-cued conditioning experiments were performed. When confronted suddenly with a bright light, a painful stimulus, or a predator, the animal may stop and stand perfectly still. "Freezing", measured as total immobility, can therefore be used as an indicator of learning and memory (Blanchard & Blanchard, 1969; LeDoux, 1996). As shown in Fig. 4, the knockout mice displayed similar levels of fear-related

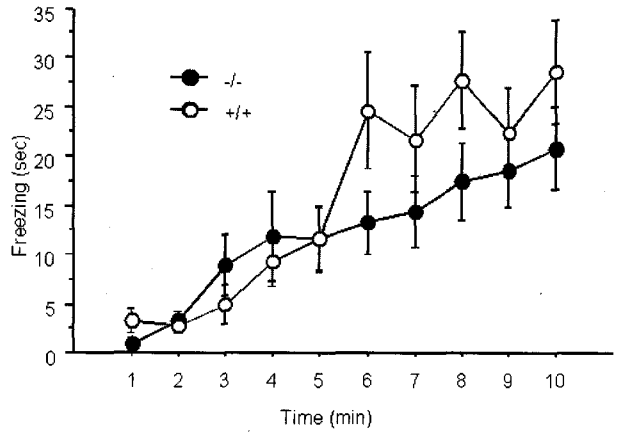

Fig. 3. Analysis of depression-related behaviors in the Porsolt forced swim test. Data are mean values±S.E.M. for immobilized time for wild-type (+/+; n=20) and αCGRP-null mice (−/−; n=20) across the 10-min test. Data are presented as ten 1-min intervals.

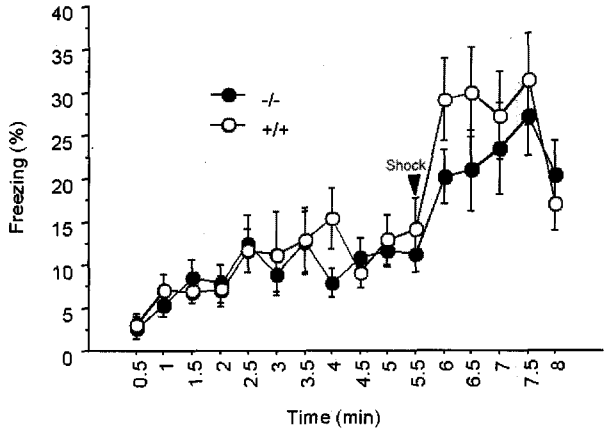

Fig. 4. Analysis of fear-related behaviors in conditioning training. The percent intervals freezing during the context- and cued-conditioning training data are shown. Data are expressed as mean values±S.E.M. during the 8-min training period and data are presented as 0.5-min intervals for wild-type (+/+; n=20) and α CGRP-null mice (−/−; n=20).

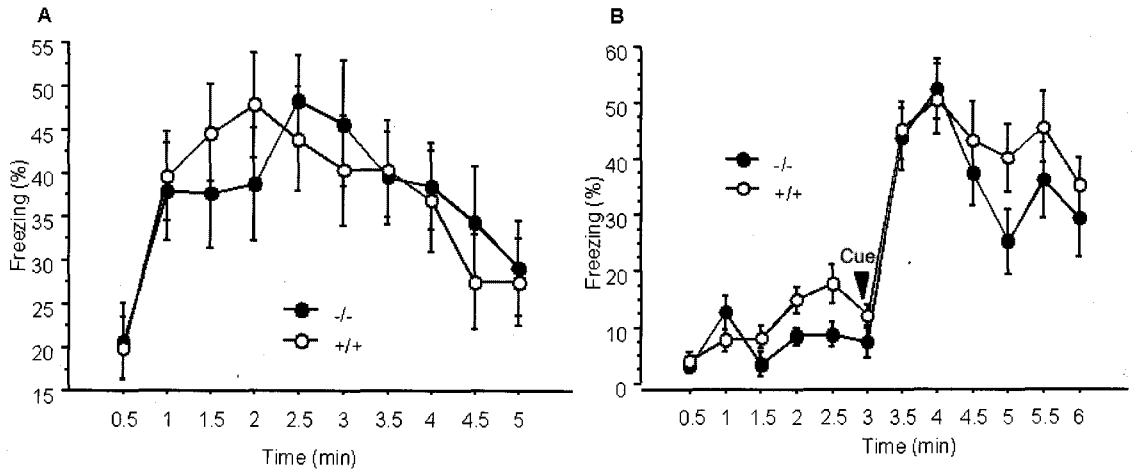

Fig. 5. Analysis of fear-related behaviors in context- and auditory-cued fear conditioning test. The percent intervals freezing during the contextually conditioned fear test (A), and cued conditioning test (B) are shown. Data are expressed as mean values±S.E.M. during the 5-min (A) and 6-min test (B) and data are presented as 0.5-min intervals for wild-type (+/+; n=20) and αCGRP-null mice (−/−; n=20).

behavior (freezing) after electric shock, when compared to wild-type mice. The mutant mice also demonstrated no difference in freezing time from wild-type littermates in the context- and the auditory cued conditioning test (Fig. 5).

DISCUSSION

Despite the data concerning the roles of αCGRP in many behavioral responses, mice homozygous for the αCGRP-null mutation demonstrated no obvious behavioral phenotypic differences from wild type animals. The present studies specifically demonstrated that αCGRP-null mice exhibited normal responses in the tasks for anxiety, fear, learning, memory and depression-related behaviors, suggesting that αCGRP is not essential for such functions or that compensatory mechanisms may exist to overcome the absence of this peptide.

If the absence of behavioral deficits in αCGRP-null mice results from developmental or compensatory alterations, one of the compensatory molecules may be a closely related CGRP isoform such as β-CGRP, which differs from the αCGRP by only three amino acids in the mouse (Lee & Emeson, unpublished data). Previous studies have demonstrated that α- and β-CGRP have similar biological properties in many physiological systems (Firth & Pipkin, 1989; Beglinger et al, 1991). However, immunohistochemical analysis of αCGRP null animals have shown an ablation of αCGRP-ir from regions where αCGRP expression is known to predominate and no detectable compensatory increase in β-CGRP expression was observed (Lu et al, 1999).

The confounding effects of environmental influences on alterations in behavioral phenotypes have recently been recognized. An example of environmental influences is provided by a study by Crabbe et al. in which different laboratories reported different behavioral phenotypes for the same inbred and mutant mice, despite strictly controlling both environmental variables and experimental procedures (Crabbe et al, 1999). One way to reduce environmental influences is to perform behavioral experiments on 2 or 3 litters, each containing all genotypes to be examined, over a period of several months and to use a standard battery of tests. In our experiment, we used twenty wild type and twenty homozygous mutant mice. All animals were male littermates in an attempt to minimize the environmental influences in behavioral testing.

Another factor that limits behavioral studies is the potential for the genetic background to modify behavioral phenotypes. In the mouse, it may take a year or more to transfer a mutation to a different genetic background by successive back-crossing to obtain a congenic strain, and these genetic backgrounds can modify the initially observed phenotype with the original genetic background (Threadgill et al, 1995; Crabbe et al, 1996; Kelly et al, 1998; Phillips et al, 1999) and therefore complicate the interpretation of behavioral phenotypes. It has been suggested that the behavioral assessment of mouse mutants should include a comparison with one or two inbred mouse strains (such as C57BL/6J and DBA/2J). This will provide a 'running baseline' with which results at any given time are compared (Tarantino et al, 2000; Veasey et al, 2000). Therefore, before making any conclusion about the role of αCGRP on behaviors, it would be better to do the behavioral tests on a different genetic background.

In conclusion, despite the wealth of data concerning the roles of αCGRP in multiple behaviors, αCGRP null mice showed no obvious behavioral phenotypic differences from wild-type animals. The present studies specifically demonstrated that αCGRP null mice exhibited normal behavior regarding anxiety, fear, learning, memory and depression-related behaviors suggesting that αCGRP is not required for these behavioral responses or that compensatory mechanisms may exist to overcome the absence of this peptide.

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