Increased levels of cocaine and amphetamine regulated transcript in two animal models of depression and anxiety

Sara Wiehager a, Daniela I. Beiderbeck b, Susanne H.M. Gruber c, Aram El-Khoury c, Jackie Wamsteeker a, Inga D. Neumann b, Åsa Petersén a,⁎,1, Aleksander A. Mathé c,1

a Translational Neuroendocrine Research Unit, BMC D11, Department of Experimental Medical Science, Lund University, Lund, Sweden
b University of Regensburg, Behavioural and Molecular Neuroendocrinology, Regensburg, Germany
c Karolinska Institutet, Clinical Neuroscience, Psychiatry M56, Karolinska University Hospital Huddinge, Stockholm, Sweden

A R T I C L E   I N F O

Article history:
Received 27 July 2008
Revised 12 February 2009
Accepted 16 February 2009
Available online 27 February 2009

Keywords:
Flinders Sensitive Line
High anxiety behavior
PAG
Hypothalamus
Rat

A B S T R A C T

The neurobiological bases of mood disorders remain elusive but both monoamines and neuropeptides may play important roles. The neuropeptide cocaine and amphetamine regulated transcript (CART) was shown to induce anxiety-like behavior in rodents, and mutations in the human CART gene are associated with depression and anxiety. We measured CART-like immunoreactivity (-LI) in genetic rat models of depression and anxiety, i.e., the Flinders Sensitive Line (FSL) and rats selected for High Anxiety-related Behavior (HAB) using a radioimmunoassay. CART-LI was significantly increased in the periaqueductal grey in FSL rats, whereas in the HAB strain it was increased in the hypothalamus, both compared with their respective controls. No line-dependent changes were found in the hippocampus, striatum or frontal cortex. Our results confirm human genetic studies indicating CART as a neurobiological correlate of depression and anxiety, and suggest that its differential regulation in specific brain regions may play a role for the behavioral phenotypes.

© 2009 Elsevier Inc. All rights reserved.

Introduction

The heterogeneity of mood disorders suggests that multiple brain circuits and neurotransmitters are implicated in their pathogenesis. Although knowledge of the neurobiological basis for emotion regulation is limited, brain regions such as prefrontal cortex, hippocampus, striatum, amygdala and hypothalamus have been shown to be affected in depression and anxiety (Berton and Nestler, 2006; Krishnan and Nestler, 2008). These brain regions are interconnected via complex circuits where dopaminergic input from the ventral tegmental area, serotonergic input from the dorsal raphe and noradrenergic input from locus coeruleus mediate important functions. Furthermore, neuropeptides, e.g., neuropeptide Y (NPY), arginine vasopressin (AVP) and corticotropin releasing hormone (CRH) are likely to be important players together with interactions with cytokines in the pathogenesis of depression and anxiety (Landgraf, 2001; Mathé et al., 2007; Anisman et al., 2008; Krishnan and Nestler, 2008). Recent studies indicate that hypothalamic peptides that regulate energy metabolism and feeding may also be involved in depression and anxiety (Berton and Nestler, 2006; Krishnan and Nestler, 2008). Cocaine and amphetamine regulated transcript (CART), a recently described neuropeptide, is expressed in neurons in the hypothalamus that project to regions thought to be affected in these disorders such as the frontal cortex, the hippocampus, the striatum, and the periaqueductal grey (PAG) (Kuhar et al., 2002; Rogge et al., 2008). In the human hypothalamus, CART immunoreactive neurons are present in the paraventricular and supraoptic nuclei of the hypothalamus, the dorsomedial hypothalamus, the lateral part of the arcuate nucleus, the periventricular nucleus, and the lateral hypothalamic area (Elias et al., 2001). The distribution is similar in rodents where the CART immunoreactive neurons co-express neuropeptides involved in energy homeostasis such as tyrotophin regulating hormone, melanin concentrating hormone, dynorphin and neuropeptide (Murphy et al., 2000; Elias et al., 2001). Central injections of CART into rodents increase anxiety via activation of the hypothalamic–pituitary–adrenal (HPA) axis (Koylu et al., 2006; Stanek, 2006). Mutations in the CART gene in humans are associated with depression and anxiety (Miraglia del Giudice et al., 2006). CART has recently been suggested as a novel therapeutic target for treating depression (Pae et al., 2007).

Several animal models of depression and anxiety have emerged that mimic important features of these human conditions. Flinders Sensitive Line (FSL) is a genetic animal model of depression originally established in the process of selective breeding of Sprague–Dawley rats for their hypersensitivity to the anticholinesterase compound diisopropylfluorophosphate (DFP) (Overstreet et al., 2005). It was then realized that the hypersensitive FSL rats display a number of characteristics similar to those of depressed patients, e.g., stress vulnerability, cognitive deficits, lower body weight, impaired sexual

⁎ Corresponding author. Fax: +46 46 2223436.
E-mail address: Asa.Petersen@med.lu.se (A. Petersén).
1 Shared last authors.
Available online on ScienceDirect (www.sciencedirect.com).
0969-9961/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2009.02.010
behavior, elevated REM sleep and increased immobility in the Porsolt forced swim test compared to their control line, the Flinders Resistant Line (FRL) (Overstreet et al., 2005). As demonstrated in several laboratories, the FSL model does not display elevated anxiety-like behavior (Schiller et al., 1991; Overstreet, 2002; Braw et al., 2006). In contrast, high anxiety-related behavior (HAB) rats and their low anxiety-related behavior (LAB) counterparts are selectively bred for differences in their behavior on the elevated plus maze (Landgraf and Wigger, 2002). The behavioral phenotype in HAB rats including anxiety- and depression-like behavior, increased stress susceptibility and impaired social behaviors (Veenema and Neumann, 2007) is correlated with increased expression of AVP in the hypothalamus, due to a single nucleotide polymorphism in the promoter of the AVP gene (Landgraf et al., 2007; Murgatroyd et al., 2004). Also, CRF expression is elevated within the hypothalamus of HAB rats (Bosch et al., 2006). Both, AVP and CRF are important regulators of the HPA axis which shows elevated responsiveness to acute stressors in HAB rats (Landgraf et al., 1999). Hence, whereas the HAB rat models a combined state of depression and anxiety, the FSL rat recapitulates a depressive phenotype without the comorbidity of anxiety. In this study, we therefore used these two genetic animal models to test the hypothesis that CART-like immunoreactivity (-LI) may be altered in specific brain regions in depression with or without the comorbidity of anxiety.

Materials and methods

Animals

Adult male FSL and FRL rats from the colonies maintained at the Karolinska Institute and male HAB and LAB rats from the colonies maintained at the University of Regensburg were used between the age of 12 and 14 weeks. All rats were housed 4/cage at a constant room temperature (22 ± 1 °C) in a 12 h light/dark cycle (light on at 6:00 am) with ad libitum access to pellets and water. The experimental procedures met the guidelines approved by the Ethical Committees for Animal Protection of Stockholm and of the government of Oberpfalz, respectively, and were conducted in accordance with the Karolinska Institute’s and NIH Guidelines for the Care and Use of Laboratory animals.

Behavioral analysis

All animals used for radioimmunoassay analysis were tested two weeks prior to sacrifice either in the Porsolt forced swim test (PST; 2 consecutive days, FSL/FRL rats, Petersén et al., 2008) or the forced swim test (1 day, HAB/LAB rats, Ebner et al. 2005). On the first of the two test days for PST, all animals were gently placed individually in a Plexiglas cylinder filled with 20–26 °C water at a depth that makes it impossible to reach the bottom with hind paws (28–30 cm). The animals were removed from the water after 15 min, and dried before they returned to their home cages. The water was changed after each session. On the next day, the same experimental procedure was repeated for 6 min. This session was video-recorded and an observer, blind to the rat strain being tested, subsequently scored the behavior of the animals. According to the criteria of Porsolt et al. (1977), the rat was judged to be immobile when it floated passively, making only small movements to keep its nose above the surface. Increased immobility in the PST is interpreted as representing signs of psychomotor retardation and/or despair in the depressive state.

The same HAB and LAB rats that were tested in the forced swim test were also assessed using the elevated plus maze (EPM) as described previously (Bosch et al., 2006). The EPM analysis was performed one week prior to the PST. FSL rats have repeatedly been shown not to exhibit increased anxiety and were therefore not tested on the EPM in this study (Schiller et al., 1991; Overstreet, 2002; Braw et al., 2006). The EPM consisted of two opposing open (50 × 10 cm; 100 lx) and two opposing closed (50 × 10 × 40 cm; 20 lx) arms, which are connected by a common central area (10 × 10 cm). The apparatus was made of dark grey plastics and was elevated to a height of 80 cm above the floor. Before each trial, the maze was cleaned with water containing low concentration of a detergent. Rats were placed individually in the center square facing a closed arm and were allowed to explore the maze for 5 min. Behavior was recorded with a video camera mounted above the platform and scored by a trained observer pressing pre-set keys on a PC (Plus-maze version 2.0; Ernest Fricke). An open/closed arm entry was defined as both fore-paws of the rat being on the respective arm of the EPM. The following parameters of anxiety-related behavior were measured: the percentage of time spent on the open arms [time on open arms / (time on open arms + time in closed arms)] × 100% and the percentage of open arm entries [open arm entries / (open + closed arm entries)] × 100%.

Tissue processing

Rats were sacrificed at the age of 14 weeks (n = 7–10/group). Rats from the different breeding lines were randomly taken out of their cage and transferred to another room where they were directly sacrificed with CO2 before brains were quickly removed. Brains were then immersed for 0.5–1 min into isopentane (−40 °C) and subsequently stored at −80 °C. The brains were later cut into 2 mm slices on ice using a brain blocker and the following brain regions were carefully dissected according to the brain atlas by Paxinos and Watson (1998): hypothalamus, frontal cortex, PAG, hippocampus and striatum by using neuro punches and scalpel. Tissues for peptide measurements were first weighed and then prepared as extracts by homogenization of the dissected brain tissue, boiling in acetic acid and then in water, and finally by lyophilization as described previously (Husum et al., 2008).

Radioimmunoassays

CART-LI was determined in the extracts prepared from different brain regions using a commercially available 125I radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals, Belmont, CA, USA). Duplicate samples were assayed and levels were determined against a known standard.

Immunohistochemistry

Another set of 14-week old FSL and FRL rats as well as HAB and LAB rats were perfusion fixed with 4% paraformaldehyde (n = 5 genotype). Eight series of coronal sections of the brains were cut on a freezing microtome at a thickness of 40 μm. Free floating sections were processed for immunohistochemistry for CART. Briefly, free floating brain sections from one series were first washed in 0.1 M KPBS, quenched in 3% H2O2 and 10% methanol for 15 min and washed in KPBS again. They were pre-incubated in 5% normal goat serum (NGS) with 0.3% Triton X in KPBS for 1 h and then the primary antibody against CART (anti-CART 1:4000, made in rabbit, generated by Prof M. Kuhar, Emory University, Atlanta, GA, USA) in 5% NGS/0.3% Triton X in KPBS was added. The sections were incubated overnight in room temperature. They were then washed twice in KPBS and once in 5% NGS/0.3% Triton X in KPBS and a biotinylated secondary antibody (goat anti-rabbit IgG 1:200, Vector Laboratories Inc. Burlingame, CA, USA) with diaminobenzidine (DAB) chromagen (Vector Laboratories Inc.). The sections were washed again before they were mounted on gelatin-coated slides. The dried slides were dehydrated in 70–99% ethanol before they were cleared with xylene and finally coverslipped with DPX.
Stereology

The total number of CART immunopositive neurons in the hypothalamus was estimated on blind-coded slides using stereology with a modified version of the fractionator method (West et al., 1991). The stereology program Visopharm integrator system was used together with a microscope (Nikon eclipse 80i). The hypothalamic area was masked with the guide of a rat brain atlas at 4× magnification (Paxinos and Watson, 1998). Approximately 250 cells were counted per brain using systematic random sampling at 60×. The total number of CART immunopositive cells in the hypothalamus of each animal was calculated using the following formula (based on the optical fractionator principle):

\[ N = \sum Q \times 1/f \times \text{number of series} \]

where \( N \) is equal to the total number of neurons per brain; \( \sum Q \) is the number of neurons counted per rat and \( f \) is the fraction of the hypothalamic area analyzed. In our study, 20% of the whole hypothalamic area was assessed \((f=20\%)\) and there were 8 series. Hence, as each section was cut at a thickness of 40 μm, the interval between the sections in one series was 320 μm.

Statistical analysis

Data were analyzed with unpaired Student’s t-tests or Pearson’s r for correlation analyses using the Statview 5.4 package (Abacus concepts, Berkeley, CA, USA) and JMP (Cary, NC, USA). Data are presented as mean ± SEM.

Results

Behavioral phenotypes of depressive and anxiety states in FSL and HAB rats

As indicative of depression-like behaviour in the PST, FSL rats displayed more immobility compared with FRL rats on day 2 of the PST (Fig. 1A; \( p<0.05 \)). HAB rats also displayed increased immobility in the during forced swimming (Fig. 1B; \( p=0.001 \) versus LAB). On the EPM, the high level of anxiety-related behavior could be confirmed in HAB rats as they spent less time on the open arms of the EPM (Fig. 1B; \( p<0.001 \)) and had less entries into the open arms of the EPM compared with LAB rats (Fig. 1B; \( p<0.05 \)).

Increased levels of CART-LI in the PAG of FSL rats

A significant increase in CART-LI was found in the PAG of FSL compared with FRL rats (Fig. 2A; \( p<0.05 \)) but no significant difference was found in the hypothalamus (Fig. 2A). A trend toward an increased level was found in the frontal cortex of FSL compared with FRL rats (Table 1) \((p=0.056)\). No significant differences in CART-LI were detected in the hypothalamus (Fig. 2A), the hippocampus or the striatum (Table 1) between FSL and FRL rats. CART levels in the PAG did not correlate with the level of immobility in the PST in FSL and FRL rats (Pearson’s \( r^2 0.00, \) n.s.).

Increased levels of CART-LI in the hypothalamus of HAB rats correlate with anxiety-like behavior

A significant increase in CART-LI was found in the hypothalamus of HAB compared with LAB rats (Fig. 2B; \( p<0.05 \)). No significant difference in CART-LI was found in the PAG (Fig. 2B), the frontal cortex, the hippocampus or the striatum between HAB and LAB rats (Table 1). CART levels in the hypothalamus correlated negatively with the percentage time spent on the open arms \((r^2 0.46, p<0.05)\) as well as with the percentages of entries into open arms \((r^2 0.40, p<0.05)\). In contrast, no correlation was found between CART-LI and the level of immobility in the PST in HAB or LAB rats.

No differences in the number of CART immunopositive neurons in the hypothalamus

Stereological analysis of the estimated total number of CART immunopositive neurons in the hypothalamus of FSL (11,200 ± 500 neurons) and FRL (12,400 ± 700 neurons) rats revealed no significant differences (Figs. 3A, B). There were also no significant differences in the estimated number of CART immunoreactive neurons between HAB (8900 ± 600 neurons) and LAB rats (8800 ± 500 neurons) (Figs. 3C, D). Neurons expressing CART in the hypothalamus project

Table 1

<table>
<thead>
<tr>
<th>Region</th>
<th>FRL</th>
<th>FSL</th>
<th>LAB</th>
<th>HAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>9 ± 1</td>
<td>13 ± 2</td>
<td>2 ± 0</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>43 ± 8</td>
<td>56 ± 15</td>
<td>3 ± 0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Striatum</td>
<td>4 ± 1</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

FSL: Flinders Sensitive Line; FRL: Flinders Resistant Line; LAB: low level of anxiety-related behavior; HAB: high level of anxiety-related behavior.
to the PAG (Sita et al., 2007) where fibers can be visualized using immunohistochemistry. No qualitative differences in the intensity of CART fibers could be detected between the different groups.

Discussion

CART has recently been implicated as a therapeutic target for depression due to its putative role in the regulation of signal transduction pathways of brain derived neurotrophic factor and neurotransmitters such as serotonin (Pae et al., 2007). The main findings in this report are that CART-LI was selectively increased in the PAG of the genetic rat depression model FSL and in the hypothalamus of the genetic HAB rat that models both elevated anxiety- and depression-related behaviors. Interestingly, CART-LI in the hypothalamus correlated with anxiety-like behavior in HAB and LAB rats. Our results confirm data on human subjects showing that individuals with a CART mutation exhibit increased anxiety and depression (Miraglia del Giudice et al., 2006) and further strengthen the notion that CART may be one of the neurobiological correlates of depression and anxiety. Our study also indicates that the differential regulation of CART in two models of these two disease categories may be important in causing the different behavioral phenotypes.

Published data indicate that PAG is one of the sites of neurobiological changes occurring in anxiety and depression (Graeff et al., 1993; Cannon et al., 2007; Wörtwein et al., 2006; Husum et al., 2008). Our results confirm data on human subjects showing that individuals with a CART mutation exhibit increased anxiety and depression. Our study also indicates that the differential regulation of CART in two models of these two disease categories may be important in causing the different behavioral phenotypes.

Published data indicate that PAG is one of the sites of neurobiological changes occurring in anxiety and depression (Graeff et al., 1993; Cannon et al., 2007; Wörtwein et al., 2006; Husum et al., 2008). Our results confirm data on human subjects showing that individuals with a CART mutation exhibit increased anxiety and depression. Our study also indicates that the differential regulation of CART in two models of these two disease categories may be important in causing the different behavioral phenotypes.

Increased activity in the hypothalamus has been found in patients with panic disorder as measured by increased cerebral blood flow (Boshuisen et al., 2002). HAB rats, characterized by increased AVP and CRF expression in the hypothalamus (Bosch et al., 2006; Murgatroyd et al., 2004) show increased hypothalamic c-fos induction following exposure to mild anxiety stimuli (Salchner et al., 2003). Moreover, CART injected icv in rodents induces anxiety-like responses in the EPM test (Chaki et al. 2003). Thus, we suggest that the increased level of hypothalamic CART contributes to the anxious phenotype of male HAB rats. The mechanisms by which CART exerts its effects are not fully understood. Interestingly, icv injections of CART activate the HPA axis and induce c-fos expression in CRH-positive, but not in the CRH-expressing neurons in the hypothalamus (Smith et al., 2004; Vrang et al., 2000). Thus, CART could contribute to the elevated CRH activity found in HAB rats (Bosch et al., 2006) and additive effects of increased

Fig. 3. No differences in the number of CART immunopositive neurons in the hypothalamus. Representative photomicrographs of CART immunopositive neurons in the hypothalamus of FSL (A, A’), FSL (B, B’), LAB (C, C’) and HAB (D, D’) rats. Scale bars 500 and 50 μm respectively.
levels of CART, CRF and AVP may cause the upregulation of the HPA axis in HAB rats (Landgraf et al., 1999). Supporting our findings of increased levels of CART in the hypothalamus of rats with high anxiety is a recent study that demonstrated transient upregulation of CART in hypothalamic nuclei following ethanol withdrawal, which leads to anxiety-like behavior (Dandekar et al., 2008a). Taken together, the present result further supports changes in the activity of hypothalamic neuropeptide signaling as being important in causing the specific phenotype in the HAB model.

Although CART-LI differed in the PAG of FSL/FRL rats and in the hypothalamus of HAB/LAB rats, no differences in the number of CART immunoreactive neurons were found in the hypothalamus in the two animal models. Also, there appeared to be no major change in the intensity in CART fibers in the PAG but the innervation in this region was not quantified. Increased levels of CART-LI using RIA in the hypothalamus and PAG were, therefore, not a consequence of an increased number of neurons in the HAB and FSL model, respectively, but may rather be due to increased gene expression or reduced breakdown of the peptide. Consistent with such reasoning are previous findings of increased expression of neuropeptide Y and somatostatin following antidepressant treatments (Jiménez-Vasquez et al., 2007; Zachrisson et al., 2005a,b). No differences in the levels of CART-LI using RIA were found in the striatum, the cerebral cortex or the hippocampus in this study. This does not rule out that changes may be present in subregions of these brain areas in the HAB and FSL models. CART mRNA has recently been shown to be downregulated in the frontal cortex of rats with anhedonia after being exposed to chronic mild stress (Orsetti et al., 2008). In another rat model of depression produced by social isolation, reduced CART immunoreactivity was found in the hypothalamus, whereas it was increased in the amygdala (Dandekar et al., 2008b). It is however possible that these latter models recapitulate signs of stress rather than clinical depression. Importantly, it has previously been shown that exposure of male rats to PST can induce CART expression in the hypothalamus, as assessed using western blots of hypothalami removed 10 min after the behavioral test (Balkan et al., 2006). This upregulation could not be found in brains processed for immunohistochemistry for CART, and was thought to be due to the upregulation of the HPA axis found acutely after the PST (Balkan et al., 2006; Gozen et al., 2007). The corticosterone response has been shown to peak 30 min after the PST and return to baseline after around 120 min (Connor et al., 1997), which renders it highly unlikely to affect CART levels assessed two weeks after the PST which was the design in our study. We therefore suggest that the changes in CART-LI found in the HAB and FSL models constitute their trait features. Further studies, including EPM and forced swim test on separate experimental subjects, to replicate current results and elucidate the underlying mechanisms are warranted.

In conclusion, we have found significantly increased levels of CART-LI in the PAG in the FSL model of depression and in the hypothalamus of the HAB model of both anxiety and depression. These results confirm the results from genetic studies on human subjects showing increased depression and anxiety in individuals with a CART mutation (Miraglia del Giudice et al., 2006) and suggest that the region-specific alteration in CART expression could be a potentially important factor in differentiating between various psychopathological phenotypes.

Acknowledgments

This work was supported by the Swedish Medical Research grants M2206–6238 (AP) and 10414 (AAM), the Karolinska Institutet (AAM), the Sjöbring foundation and the Royal Physiographic Society (AP) as well as the Deutsche Forschungsgemeinschaft (IDN) and BMBF (IDN). We are grateful to Prof. Kuhar for the donation of the antibody against CART.

References


