The main effect of cocaine- and amphetamine-regulated transcript (CART) peptide on hypothalamic neuronal activity depends on the nutritional state of rats

Helga Davidowa [a], Yuzhen Li [a,c] & Andreas Plagemann [b]

[a] Johannes-Mueller-Institute of Physiology, Charité – University Medicine Berlin, GERMANY
[b] Clinic of Obstetrics, Charité – University Medicine Berlin, GERMANY
[c] Research Laboratory of Pathophysiology, Institute of Basic Medical Sciences, PLA General Hospital, Beijing, CHINA

Correspondence to: Dr. Helga Davidowa
Johannes-Müller-Institut für Physiologie
Charité – Universitätsmedizin Berlin
Campus Charité Mitte
Tucholskystr. 2, D-10117 Berlin, GERMANY
PHONE: +49 30 450528111
FAX: +49 30 450528962
EMAIL: helga.davidowa@charite.de

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Abstract

OBJECTIVES: The anorectic and catabolic action of CART is primarily mediated by the hypothalamus. The study proved the hypothesis that neurons of the hypothalamic regulatory system of body weight differentially react to CART in dependence of the nutritional state of the animal: overweight, fed or short-term fasting.

DESIGN AND SETTING: Single unit activity was extracellularly recorded in brain slices. The action of CART was studied in brains of 1. overweight adult rats previously subjected to early postnatal overfeeding in small litters (SL), compared to control litters, 2. normal rats that were deprived of food for 24 h, compared to fed rats.

RESULTS: Hypothalamic dorsomedial neurons of controls, but not SL rats were significantly excited by CART, ventromedial neurons of SL rats were significantly inhibited. Also neurons of hungry rats were significantly inhibited.

MAIN FINDINGS: Controls and overweight SL as well as fed and hungry rats differed significantly in the neuronal effects of CART. The predominant effect of the peptide did neither depend on weight nor on age of animals, but on neonatal development or nutritional state.

CONCLUSION: The increase in inhibition by CART of ventromedial and dorsomedial neuronal activity could in vivo contribute to increased food intake and reduced energy expenditure of overweight SL as of hungry rats. Since leptin is able to change synaptic wiring and the expression of excitatory and inhibitory synapses already within short time, the increased expression of inhibitory responses to CART may reflect a general mechanism in adaptation of neuronal regulatory systems to the nutritional state, in fed, adult small-litter rats acquired during the postnatal critical differentiation period, thus leading to permanently altered function.

ORIGINAL ARTICLE

Abbreviations:

ACSF	–artificial cerebrospinal fluid
ANOVA	–analysis of variance
CART	–cocaine- and amphetamine-regulated transcript
CL	–control litter
CRH	–corticotropin-releasing hormone
DMD	–dorsomedial hypothalamic nucleus, dorsal part
DMH	–dorsomedial hypothalamic nucleus
DMV	–dorsomedial hypothalamic nucleus, ventral part
f	–fornix
GABA	–γ-aminobutyric acid
mRNA	–messenger ribonucleic acid
mt	–mammillothalamic tract
SD	–standard deviation
SL	–small litter
SNK	–Student-Newman-Keuls test
sp	–spikes
VMH	–ventromedial hypothalamic nucleus
VMHDM	–ventromedial hypothalamic nucleus, dorsomedial part
3V	–3rd ventricle

Introduction

Cocaine- and amphetamine-regulated transcript (CART) [1] peptide is highly expressed in brain and peripheral organs [2, 3]. It is involved in various physiological processes, the most investigated is its role in energy homeostasis [4]. Substantial amounts of CART mRNA and CART peptide are found in hypothalamic nuclei involved in body weight regulation [5, 6]. CART has been shown to have an anorectic and catabolic action [7–10], although direct injection into hypothalamic nuclei could induce an orexigenic effect [11]. The synthesis of CART peptide is influenced by leptin and by the metabolic state of animals [7, 12]. The action of CART on body weight regulation seems partly to be mediated by corticotropin-releasing hormone (CRH) and the hypothalamo-pituitary axis [13–16]. In overweight, hyperleptinemic rats, the action of leptin and CRH on neuronal activity is changed, seemingly contributing to the overweight disposition of the animals [17, 18]. Hypothalamic paraventricular neurons of the parvocellular part known to contain neurons producing CRH were activated by CART in normal rats, but mainly inhibited in overweight rats previously subjected to early postnatal overfeeding in small litters [19]. Such rats are hyperphagic [20, 21]. They appear to express an elevated set point of body weight and become hyperleptinemic, hyperinsulinemic and hyperglycemic [20, 22, 23]. We raised the hypothesis that in such neonatally overfed rats CART may have a changed effect also on neurons of other parts of the hypothalamic regulatory system expressing leptin receptors, [24] e.g., the ventromedial and dorsomedial nucleus that have influence on the vegetative nervous tone. Furthermore, as leptin has been shown to have not only trophic action during development [25], but to change synaptic wiring also within short periods [26], we raised the hypothesis that the effect of CART on neuronal firing may be changed also in food deprived animals compared to fed ones.

Materials and methods

The experiments were performed in the offspring of an outbred colony strain of Wistar rats (Charles River Laboratories, Sulzfeld, Germany). The animals were kept under standard conditions with normal 12-h light: 12-h dark cycle. To induce early postnatal overnutrition, the primary litter size was reduced on the third day of life to only three pups per litter (small litters, SL). In control litters (CL), the size was adjusted to 12 neonates per mother. The dams were singly housed during pregnancy and with the litter during lactation until weaning at day 21. They had free access to tap water and pellet diet (commercial control diet for rats; Altromin, Lage, Germany; Code 1314, energy content: 12.5 kJ/g, respectively Code 1326, 11.9 kJ/g, after the fourth week of age). Males of each litter size were randomly assigned to the electrophysiological studies presented here. In a second series, some of normal rats were deprived of food for 24 h (from 8 h a.m. until the next morning) and then neuronal activity was studied. All procedures were carried out in accordance with the guidelines for the care of animals [27] and approved by the local Animal Care and Use Committee (G 0028–96 and T 139–99). The rats were decapitated under ether anesthesia. Coronal slices (350 or 400 μm) were cut with a vibroslicer from the brains submersed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF, containing (in mM): NaCl 129, KCl 3, $NaHCO_3$ 21, $CaCl_2$ 1.6, $MgSO_4$ 1.8, NaH_2PO_4 1.25, glucose 10; pH 7.4). The slices that also contained surrounding nuclei were placed into an interface chamber for storage. They were continuously perfused with oxygenated ACSF (2 ml/min) at 35 ± 0.1°C. The electrophysiological studies were performed in a second chamber after equilibration of about 2 h. Action potentials of spontaneously firing neurons were extracellularly recorded with ACSF-filled glass microelectrodes (8–20 MOhm) from the dorsomedial part of the ventromedial hypothalamic nucleus (VMHDM), the dorsal (DMD) or seldom ventral part (DMV) of dorsomedial hypothalamic nucleus (DMH) [28]. The fornix and the 3rd ventricle served as landmarks for introduction of the electrode. They were used together with the mammillothalamic and the optic tract for determination of the coronal section (Bregma –2.3 mm until –3.6 mm [28]). The localization of neurons examined was marked in these sections. Only neurons with regular baseline activity for at least five minutes were studied, whereas units that ceased firing shortly after detection were excluded from further investigations. Stored as frozen aliquots of concentrated stock solutions, CART (55–102) fragment (rat) [7] (purchased from Bachem, Heidelberg, Germany) was freshly dissolved in warmed ACSF before administration in drops of 50 μl upstream to the slice directly into the experimental interface chamber. CART was applied in a concentration of 1, 10 and/or 100 nM. The drug was further diluted about 40 times by the perfusion medium (chamber volume about 250 μl) before reaching the examined neuron. Such doses were shown to have behavioural effects [8, 13]. Only one neuron per slice was studied. The data are

presented as means ± SD. We used the paired t-test for determination of a significant activity change within a neuronal population, and ANOVA with following Student-Newman-Keuls test (SNK) for determination of differences between groups. We evaluated spontaneous variation of firing and the neuronal responses by counting the spikes/s of representative periods 100 s or 200 s before (twice) and under the influence of each drug. The effects were determined by calculating the difference between the rate of baseline firing and the drug-induced firing. For individual neurons, a change in the discharge rate by at least 20% was considered to be a response. For neurons discharging in rates below 1.2 spikes/s, a difference of 0.4 spikes/s was considered a response. These values exceeded those of spontaneous variation in firing. The proportions of responsive neurons in both groups were compared using the Chi2-test. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

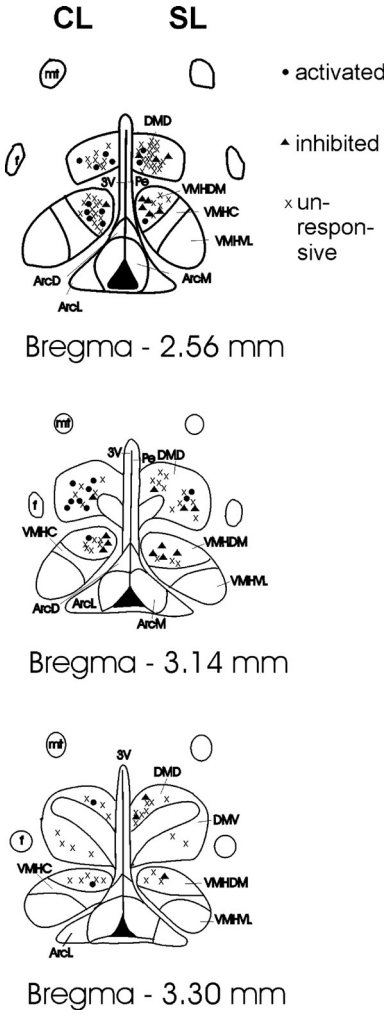

Fig. 1. Location of neurons studied in controls (CL) and small-litter (SL) rats and marked in three sections of the brain. The parts of the frontal sections correspond to the Stereotaxic atlas [28], the distance to Bregma is given. The neurons are marked as activated, inhibited and unresponsive to CART in the dorsomedial nucleus, its dorsal (DMD) or ventral part (DMV) and the dorsomedial part of the ventromedial hypothalamic nucleus (VMHDM). The slices contain also surrounding nuclei as the arcuate (Arc) with different parts. The marking for CL on the left side and SL at right is only for better differentiation. The mammilothalamic tract (mt), the fornix (f) and the 3rd ventricle (3V) were regarded as landmarks for introduction of the electrode.

Results

Action of CART on neuronal firing of controls and of overweight SL rats

Neurons of the VMHDM and DMH were studied in brains of 28 control and 27 small litter rats. Rats of both groups were fed before the experiment. SL rats gained significantly more weight until weaning. At day 21, mean body mass was 63 ± 10.3 g (SL) compared to 46 ± 4.1 g (CL, $p < 0.001$, SNK). The rats remained overweight until day 60, i.e. prior to the beginning of electrophysiological studies (363 ± 31.0 g SL compared to 336 ± 19.2 g CL, $p < 0.01$, SNK). The mean body weight at the experimental day during the 9th to 14th week of age did not significantly differ (428 g SL compared to 424 g CL, ANOVA, $F = 0.125$). This can be due to an insignificant difference in the mean age of the animals (76 ± 11 days SL; 79 ± 11 days, CL; ANOVA, $F = 1.59$, $p = 0.21$). The location of the neurons studied in CL and SL rats is shown in Figure 1. The mean discharge rate of VMHDM neurons was in controls 3.51 ± 2.2 spikes/s, $n = 35$, and in SL 3.47 ± 2.2 spikes/s, $n = 22$, that of DMD/V neurons was 2.43 ± 2.3 spikes/s, CL, $n = 33$, and 2.84 ± 2.5 spikes/s, SL, $n = 51$. CART (mean concentration 11.5 nM) more often activated than inhibited VMHDM neurons ($n = 35$) in controls. A dose-dependent activation of a neuron is shown in Figure 2. Neurons of the DMD/V were significantly activated by CART ($p < 0.01$, paired t-test, $n = 33$, mean 9.2 nM CART). In brains of SL rats, VMHDM neurons were significantly inhibited ($p < 0.05$, paired t-test, $n = 22$, 8.8 nM CART). The difference to controls in the effect was significant ($p < 0.05$, $n = 57$, ANOVA, SNK). The difference in the proportion of responsive neurons did not reach the range of statistical significance. An example of a neuronal response to CART in a SL rat is shown in Figure 3 A. Similarly,

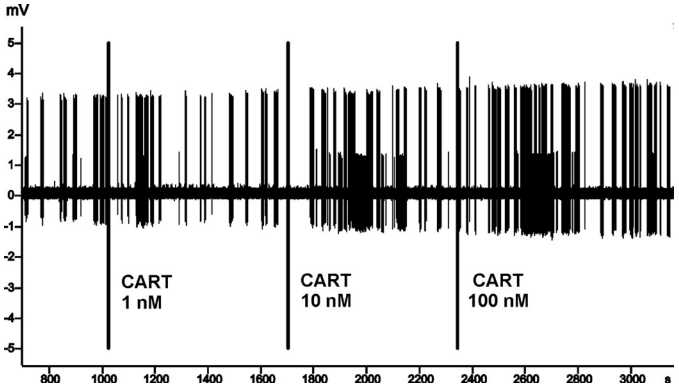

Fig. 2. Part of an original record of action potentials of VMHDM neurons in the brain of a CL rat. In this case potentials of different value discharged by two neurons are shown that were differentiated by means of a window-discriminator. Especially the neuron with the small potential is activated by CART with 10 and 100 nM (in the drop).

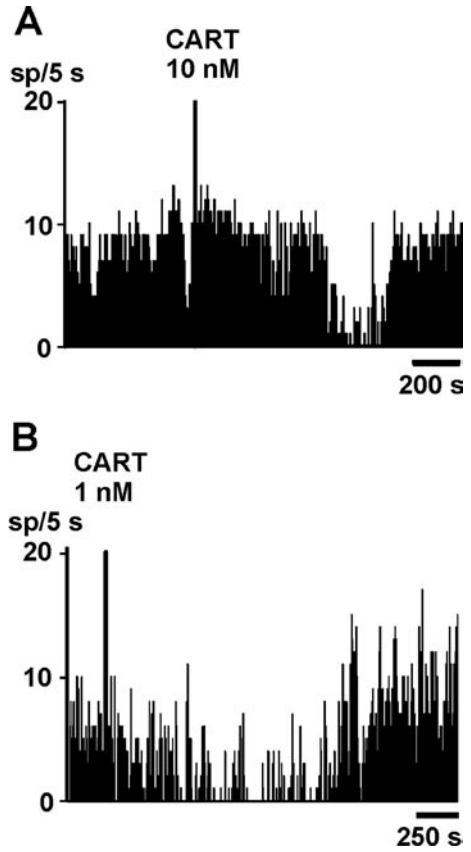

Fig. 3. Frequency-time histograms of the discharge rates of two neurons. Administration of CART is marked by a long vertical line. A: firing rate (spikes/5s) of a VMHDM neuron of a SL rat. CART induces an inhibition. B: firing rate of a DMD neuron of a hungry rat.

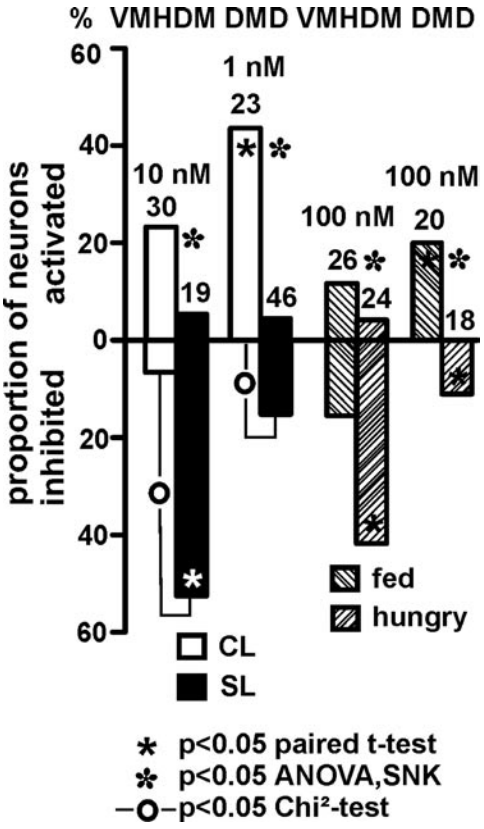

Fig. 4. Proportion of neurons activated and of neurons inhibited by CART shown for the two nuclei studied and for the different groups (CL controls, SL small litter; fed and hungry). The numbers above the columns show the number of neurons investigated with CART in the concentration given above. The asterisks within a column mark the significance of the difference of CART-induced firing to background firing (paired t-test), the asterisks besides two columns show the significant difference between the neuronal groups in the effect of CART (ANOVA, SNK). The sign at the connecting line shows a significant difference between neuronal responsive groups in the number of activated and inhibited responses.

there was a change to reduced activation and increased inhibition by CART for DMD/V neurons (n = 51, 3.7 nM). The difference between controls and SL rats in the effect of CART was significant ($p < 0.01$, ANOVA, SNK, n = 84), also the difference in general responsiveness ($p < 0.05$, Chi_2-test). Fewer DMD neurons of SL than CL rats responded to CART. Figure 1 shows the distribution of the responsive neurons in sections of the nuclei. Figure 4 summarizes the results obtained from neuronal populations tested with comparable concentrations of CART. It shows that CART-responsive neurons of SL rats differ significantly in their respose types – increase or decrease of firing rate – from controls ($p < 0.05$, Chi_2-test). The statistical results are the same as for the whole populations.

Action of CART on neurons of fed and of food-deprived rats

The neurons were studied in brain slices of 35 fed and 30 deprived male rats. The mean body weight of the rats did not significantly differ at the day of the experiment after age of six weeks (269 ± 65.8 g fed compared to 257 ± 73.6 g deprived, ANOVA, F = 0.494, P = 0.48). There was a significant difference in the weight compared to the controls of the first series ($P < 0.01$, SNK, n = 63). The mean spontaneous firing rates in brain slices of fed rats were 3.32 ± 3.0 spikes/s, VMHDM, n = 46, and 3.29 ± 3.6 spikes/s, DMD/V, n = 44. Neuronal responses to CART in fed rats were similar to those of older CL rats, although the proportion of responsive DMD/V neurons was significantly reduced in the former ($p < 0.05$, Chi_2-test). VMHDM neurons (n = 46) were activated or inhibited (mean 59.1 nM CART). CART significantly increased firing of DMD/V neurons ($p < 0.05$, paired t-test, n = 44, 7.8 nM CART). Neurons of hungry rats were significantly inhibited (VMHDM, $p < 0.01$, paired t-test, n = 32, mean 75.8 nM CART, DMD/V, $p < 0.05$, n = 27, 24.3 nM). The differences between fed and hungry rats in the neuronal effects of CART are significant ($p < 0.01$, ANOVA, SNK, VMHDM n = 78, DMD/V n = 71). An example of a neuronal inhibition is shown in Figure 3 B. Figure 4 summarizes the data of neuronal populations tested with comparable concentrations of CART. The statistical significance of the results is the same as for the whole populations.

Discussion

The main result of the study was a difference between normal rats and overweight SL rats as well as fed and food-deprived rats in activating compared to inhibitory effects of CART. Generally, activating effects were reduced and inhibitory effects increased in hungry rats as well as in fed SL rats. Weight and age were not decisive for this difference, because neuronal responses were similar in brains of rats with body mass around 270 g and those around 420 g. CART significantly excited DMD neurons in normal fed rats of both series, the proportion of activating and suppressing effects on VMHDM neurons of these rats was also similar. The reduced responsiveness to CART and thus reduced proportion of activated DMH neurons of younger rats could be regarded as the only sign of differences depending on development. Suppressing effects on neuronal activity in the VMHDM could lead in vivo to increased food intake and decreased energy expenditure. Inhibition by administration of GABA (γ-aminobutyric acid) or muscimol into the ventromedial hypothalamic nucleus (VMH) was reported to induce feeding [29]. Activation by leptin shown with electrophysiological methods [17, 30] or the occurrence of the immediate early gene c-fos [31] increases the sympathetic tone [32]. The dorsomedial hypothalamic nucleus is also involved in the regulation of body weight and serves a wide range of further function [33]. It has connections to other feeding-relevant hypothalamic nuclei such as the VMH and the lateral hypothalamic area, the arcuate and the paraventricular nucleus [34–36]. The DMH contains different sub-nuclei [28]. Leptin receptors are especially found in the dorsal and ventral parts where we recorded from, but not the central compact zone [37]. Leptin seems to activate DMH neurons, since c-fos increases after its administration [31]. Activation of DMH induces an increased corticotrophic hormonal and sympathetic tone and increased energy expenditure [38, 39]. Intracerebroventricular administration of CART suppresses feeding and induces in the DMH c-fos that is regarded as sign of activation [9]. Electrophysiological effects of CART were studied in the hippocampus, where CART reduced the calcium channel activity [40]. How far such different effects can be due to different receptors or signaling pathways [16] or the involvement of interneurons is not known until now. CART receptors are not yet identified. The DMH itself contains CART neurons that have GABA inducing enzymes co-localized [6]. The VMH does not contain CART mRNA neurons [6]. The next question concerns the observed changes in the effect of CART in our study, the increased inhibition in hungry and in fed SL rats. Such an increased inhibition after fasting could explain the orexigenic effects of CART induced by direct administration into the VMH or DMH [11]. Recently reported effects of leptin on synaptic wiring within the hypothalamic arcuate nucleus including a change in the expression of inhibitory and excitatory synaptic events during a short period [26] make a change in the response types with fasting and altered leptin levels understandable. Leptin has a trophic action on projections from the arcuate nucleus in developing animals [25]. Increased levels of leptin in overweight SL rats during the critical postnatal differentiation period could induce persistent changes in neuronal connections and synaptic wiring. A similar increase of suppressing and decrease of activating effects in the VMHDM of SL rats was observed for the anorectic leptin [17], insulin [21] and CRH [18]. Also the orexigenic peptides neuropeptide Y [41], agouti-related peptide [42] and melaninconcentrating hormone [43] had a changed effect in this range. These rather unspecific changes could also be explained by a general change in the expression of inhibitory and excitatory synapses [26]. In contrast, cholecystokinin and orexin A did not have a modulated action on VMHDM neurons of SL rats [41]. Thus, there must be also a specific, peptide-depending component. A possibility consists in the location of receptors. The last mentioned peptides have possibly only a direct action on VMH neurons, whereas in other cases interneurons or presynaptic receptors located at axon terminals could be involved. In conclusion, the increase in inhibition of ventromedial and dorsomedial neuronal firing induced by CART in brains of overweight SL rats might in vivo contribute to increased food intake and reduced energy expenditure of these neonatally overfed rats as occurring in hungry rats. The increased expression of inhibitory responses to CART may therefore reflect a more general mechanism in adaptation of neuronal regulatory systems to the nutritional state, in fed adult small-litter rats acquired during the postnatal critical differentiation period, thus leading to permanently altered function.

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