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Kinin B1 receptor gene ablation affects hypothalamic CART production[b]

Abstract: A role for the kinin B1 receptor in energy-homeostatic processes was implicated in previous studies; notably, the studies where kinin B1 receptor knockout mice ($B1^{-/-}$) were shown to have impaired adiposity, impaired leptin and insulin production, lower feed efficiency, protection from liver steatosis and diet-induced obesity when fed a high fat diet (HFD). In particular, in a model where the B1 receptor is expressed exclusively in the adipose tissue, it rescues the plasma insulin concentration and the weight gain seen in wild type mice. Taking into consideration that leptin participates in the formation of hypothalamic nuclei, which modulate energy expenditure, and feeding behavior, we hypothesized that these brain regions could also be altered in $B1^{-/-}$ mice. We observed for the first time a difference in the gene expression pattern of cocaine and amphetamine related transcript (CART) in the (lateral hypothalamic area (LHA) resulting from the deletion of the kinin B1 receptor gene. The correlation between CART expression in the LHA and the thwarting of diet-induced obesity corroborates independent correlations between CART and obesity. Furthermore, it seems to indicate that the mechanism underlying the 'lean' phenotype of $B1^{-/-}$ mice does not stem solely from changes in peripheral tissues but may also receive contributions from changes in the hypothalamic machinery involved in energy homeostasis processes.

Keywords: hypothalamus; kinin B1 receptor; knockout mice; lateral hypothalamic area.

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Introduction

The kallikrein-kinin system is a complex molecular signaling network in which kinin peptides are cleaved from kininogen precursors by the kallikrein serine proteases and then activate a set of two G-protein coupled receptors denominated B1 and B2. These receptors in turn trigger distinct intracellular signal transduction cascades, which are classically correlated to effects on blood pressure, nociception and inflammation systems (for a comprehensive review see Moreau et al., 2005). Contrary to the B2 kinin receptor, whose expression is constitutive, the B1 receptor is mostly expressed in inflammatory conditions in response to tissue injury (Prado et al., 2002).

In addition to these more classically associated physiological functions, the kallikrein-kinin system has also been implicated in processes of homeostasis of energy, such as glucose uptake and insulin responsiveness (Beard et al., 2006). A role of the B1 receptor in metabolic processes was suggested by observations that animal models of insulin-dependent diabetes mellitus were protected

from hyperglycemia, insulitis and renal damage when treated with a B1 antagonist (Zuccollo et al., 1999). While these early findings implicate a contribution for the B1 receptor in energy balance mechanisms, the exact metabolic processes involved could be shoown in more detail with further exploration of the B1 knockout (B1$^{-/-}$) phenotype. Araújo et al. (2006) verified that B1$^{-/-}$ mice have pancreatic abnormalities, such as fewer islets of Langerhans and lower glucose-triggered insulin release. They also observed that the endogenous B1 agonist des-Arg9-bradykinin (DBK) stimulates insulin release *in vivo* in wild type mice by increasing pancreatic vascular permeability (Araújo et al., 2006).

Notably, Mori et al. (2008b) verified that B1$^{-/-}$ mice are completely resistant to obesity induced by a high fat diet (HFD) (e.g., a 'lean' phenotype). A set of experiments were designed to investigate a putative influence of the B1 receptor in the homeostasis of leptin (Mori et al., 2008a), a 16 kDa cytokine produced by adipocytes that has a major role in the induction of satiety. The *in vivo* results showed a clear positive effect of DBK on serum leptin levels in wild type mice, but no effect whatsoever was seen in leptin secretion by wild type adipocytes cultured *in vitro* (Mori et al., 2008a). Taken together these observations suggest that the DBK-triggered increased leptin production cannot be accounted for by a direct mechanism alone, where DBK would bind B1 receptors on adipocytes and initiate a signal transduction cascade, culminating in leptin secretion. Recently our group has shown that leptin also plays an important role in the liver lipid metabolism in B1$^{-/-}$ animals. These animals showed a reduction in hepatic lipogenesis and steatosis after HFD, possibly through a SCD-1 dependent mechanism (Fonseca et al., 2013).

The task of coordinating metabolic processes across the entire body is fulfilled by the central nervous system. In particularl, signals conveying information from different sources of currently available and stored fuels converge in the hypothalamus. At that portion of the brain metabolic status signals are integrated and may further affect leptin secretion by adipocytes (Cota et al., 2007).

In that regard, the arcuate nucleus (ARC) plays an important role, as it has a high density of leptin receptors (Ob-Rb) and the modified blood-brain-barrier (e.g., no tight-junctions at the median eminence; Norsted et al., 2008), which allows the passage of leptin and insulin. The ARC neurons can be categorized into orexigenic and anorexigenic. The orexigenic population synthesizes the neuroactive compounds neuropeptide Y (NPY) and agouti-related peptide (AgRP) and promotes the gain of body mass. The anorexigenic population synthesizes pro-opiomelanocortin (POMC) and cocaine and amphetamine

related transcript (CART) and promotes weight loss when stimulated by leptin (Williams et al., 2001). In fact, many of the anorexigenic effects of leptin are related to neuron projections stemming from the ARC to other hypothalamic regions, such as the paraventricular nucleus (PVH) and the lateral hypothalamic area (LHA), each of which participates in distinct ways in the control of feeding behavior and energy expenditure (Bouret and Simerly, 2007). In the ARC, leptin increases the frequency of action potentials in CART/POMC neurons (Cowley et al., 2001) and also inhibits NPY/AgRP neurons, reduces appetite and increases metabolism.

It was recently shown that kinin B1 receptor ablation affects adipogenesis, resulting in reduced number and volume of adipocytes (Mori et al., 2008b), which have a major role in leptin secretion in the body. As a consequence, serum leptin levels are diminished in B1$^{-/-}$ mice. As we know that leptin has a role in modulating the maturation of the hypothalamus (Simerly, 2008), we could expect that lowered serum leptin levels would lead to an anomalous hypothalamus. Evidence for the neurotrophic role of leptin was reported as abnormal wiring of hypothalamic circuits in mice models of severe obesity (ob/ob) in which leptin signaling is absent. Transient restoration of fiber density (Bouret et al., 2004) and number of synapses (Pinto et al., 2004) is achieved with leptin treatment.

In this work we hypothesized that the B1$^{-/-}$ 'lean' phenotype might be related to changes in gene expression patterns in fuel-state-sensing hypothalamic nuclei and tried to verify the hypothesis by semi-quantitatively comparing the local expression levels of NPY and CART in the hypothalamus of B1$^{-/-}$ and wild type (WT) mice.

Results

There is no difference in NPY mRNA expression level in B1$^{-/-}$

In the hypothalamus NPY is expressed in neurons in the ARC. These neurons project their axons mainly to the PVH and adjacent perifornical area of the LHA, there promoting a positive state of energy balance by increasing energy intake and decreasing energy expenditure as well as stimulating lipogenesis (as reviewed by Woods et al., 1998). In order to investigate a possible NPY-dependent mechanism that contributes to the lean phenotype seen in the B1$^{-/-}$, the NPY mRNA expression was elected for measurement in the ARC by *in situ* hybridization (ISH). The NPY mRNA hybridization signal was intense at a portion of the ARC

nucleus closer to the median eminence and the third ventricle (Figure 1A). The NPY mRNA expression level at this region in the ARC was not different between the groups of B1$^{-/-}$ and WT mice (Figure 1B).

Increased CART expression in B1$^{-/-}$ LHA

It has been reported that the CART-expressing neuron population in the ARC has projections adjacent to those of the NPY-expressing neurons and seem to exercise the contrary physiological function, i.e., to quench the appetite and promote energy expenditure (Vrang, 2006). Once the NPY neurons (Figure 1) are not altered in B1$^{-/-}$, we hypothesized that the CART neurons may be acting to promote the lean phenotype of B1$^{-/-}$. The CART mRNA expression level in the ARC nucleus seems to be the same in both groups, as revealed by ISH (Figure 2A and B). The PVH, in addition to containing CART-expressing neurons, receives input from anorexigenic populations such as those in the ARC. The neuron population of this nucleus is quite heterogeneous, with distinct co-expression of several other molecules also implicated in the modulation of the energy balance (Vrang, 2006) and is thus a good candidate site for differential CART expression. Nevertheless, no significant difference was observed between B1$^{-/-}$ and WT mice
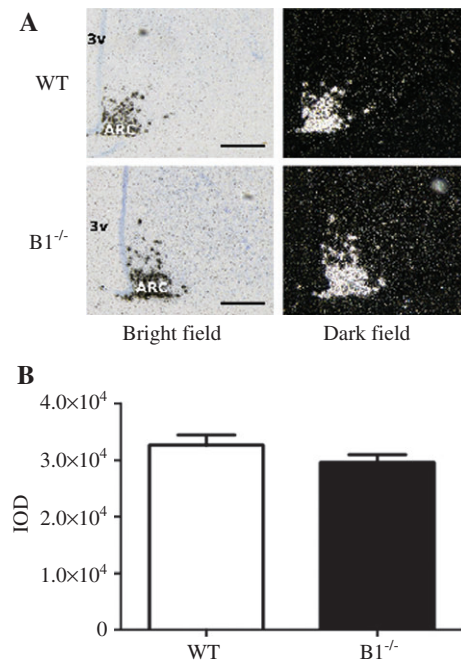

Figure 1 NPY mRNA expression in the ARC.
(A) Representative photomicrographs showing the NPY mRNA hybridization probe distribution across the ARC. Scale bars=200 μm. 3v, third ventricle; ARC, arcuate nucleus. (B) Semiquantitative comparison of NPY mRNA expression. Data are expressed as mean±SEM (n=5 WT and 5 B1$^{-/-}$). IOD, integrated optical density.
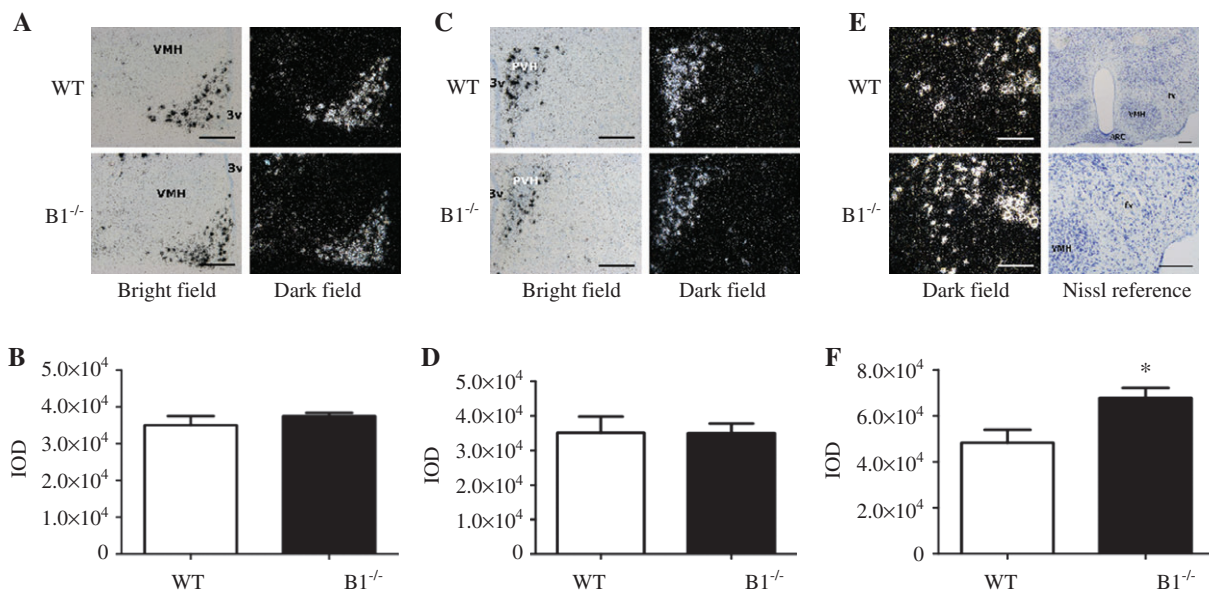

Figure 2 CART mRNA expression in the ARC, PVH and LHA.
(A, C and E) Representative photomicrographs showing the CART mRNA hybridization probe distribution across the ARC, PVH and LHA respectively. Scale bars=200 μm. 3v, third ventricle; ARC, arcuate nucleus; fx, fornix; PVH, paraventricular nucleus; VMH, ventromedial nucleus. (B, D and F) Semiquantitative comparison of CART mRNA expression in the ARC, PVH and LHA respectively. Data are expressed as mean±SEM (n=5 WT and 5 B1$^{-/-}$). *$p < 0.05$. IOD, integrated optical density.

CART mRNA expression in the PVH (Figure 2C and D). Numerous CART-expressing neurons are found dispersed across the LHA with a distinctly higher density in the perifornical area (PeF) (Elias et al., 2001; Vrang, 2006). This region was elected for investigation in B1^{-/-} mice and a higher CART mRNA level was present in this group when compared to the CART expression levels of the control group (Figure 2E and F). The quantification of the CART peptide was also performed by computer-assisted counting of CART-immunoreactive cells inside a rectangular region of interest (see Methods) that contained the fornix (Figure 3A). A greater mean number of CART-expressing cell-bodies was counted in the knockout group than in the WT group (Figure 3B).

Unchanged spontaneous locomotor activity in B1^{-/-} mice

Spontaneous locomotor activity was measured to verify whether the lean phenotype of B1^{-/-} mice was caused by or related to behavioral change. In this model, the amount of movement was similar in B1^{-/-} and WT mice in the light and in the dark cycle (Figure 4).
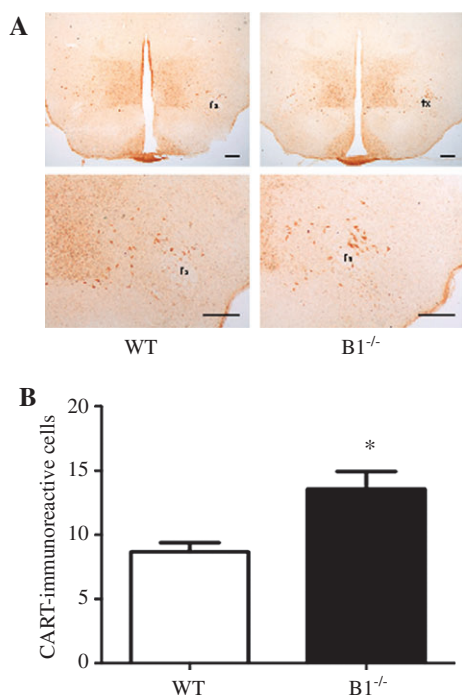

A

WT   B1^{-/-}

B

Figure 3 CART peptide expression in the LHA.
(A) Representative photomicrographs showing the CART immunoreactivity across the LHA. Scale bars =200 μm. fx, fornix. (B) Quantitative comparison of CART-expressing cells. Data are expressed as mean±SEM (n=5 WT and 5 B1^{-/-}). *$p < 0.05$.
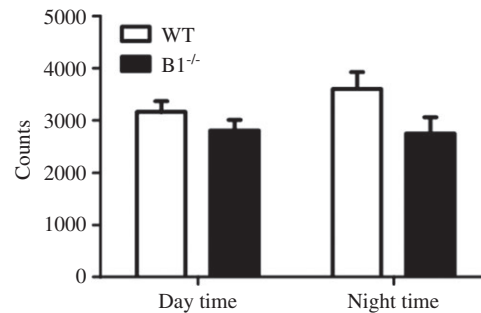

Figure 4 Spontaneous locomotor activity in WT and B1 knockout mice (n=10 WT and B1^{-/-}).

Discussion

As shown previously (Mori et al., 2008b), the mean body weight of B1^{-/-} mice and WT mice are not significantly different when fed a regular chow diet. Surprisingly, when fed a HFD B1^{-/-} diet mice remain lean while the WT mice become obese (Mori et al., 2008b). When the kinin B1 receptor is expressed exclusively in the adipose tissue, the plasma insulin level is restored, as is the weight gain after HFD, but no change is seen in leptin levels (Mori et al., 2012). Kinin B1 receptor antagonism in rats is able to reduce plasma levels of insulin, glucose, and reverses the enhancing effect of glucose feeding on whole body and epididymal fat mass and on the expression of inflammatory markers in retroperitoneal adipose tissue and aorta (Dias and Couture, 2012a,b). In those same models, the B1 antagonist also reversed B1R overexpression in the spinal cord, aorta, liver and gastrocnemius muscle and the high blood pressure in a dose-dependent manner, acting through a NADPH depend pathway (Dias et al., 2010). Interestingly, these authors did not observe changes in the circulating levels of leptin after treatment with kinin B1 receptor antagonist.

It has been shown that deletion of one kinin receptor could lead to the modulation of the other kinin receptor expression (Duka et al., 2008; Marcon et al., 2013). Therefore, the theory could be profered that the kinin B2 receptor could be compensating for the lack of the kinin B1 receptor. However, in our model, we measured the mRNA expression of kinin B2 receptor in total hypothalamus and it was not different in comparison to the control animal (data not shown).

In this sense, the genetic or the pharmacological models for targeting the kinin B1 receptor have shown the importance of this receptor in energy-metabolism; however the mechanisms involved are still largely unknown. Our group has already demonstrated that the mechanism might be linked to the regulation of Ob-Rb

receptors involved in the signaling of the weight controlling hormone leptin (Mori et al., 2008b). We hypothesized that hypothalamic wiring abnormalities caused by the differential expression of energy-metabolism neuromodulators in the hypothalamus of B1^{-/-} mice that could contribute to their 'lean' phenotype might exist. Therefore we chose to evaluate the expression of metabolism-related neuromodulators locally, in specific hypothalamic nuclei. Our measurements show a higher density of CART mRNA and CART-immunoreactive cells in the PeF area of the LHA, which could contribute to the 'lean' phenotype.

At the LHA, CART is known to be co-expressed with melanin concentrating hormone (MCH), a neuropeptide that has an orexigenic role at this area – a paradox that might be resolved by the hypothesis raised by Vrang and collaborators that CART is not anorexigenic at this site and may be involved in other physiological processes (Vrang, 2006).

The sending of inputs from CART-expressing neurons to the ventral tegmental area (VTA) – a component of the mesolimbic system – suggests a role in the modulation of reward and reinforcement systems that control motivational behavior (Philpot et al., 2005). Furthermore, most drugs causing drug addiction act by increasing dopamine release in the mesolimbic circuit, interfering with the normal functioning of this ancient reward system; the wiring of which has evolved to control – among other things – food-seeking behavior, which in turn impacts every other behavior involving energy expenditure (for a review focusing on the role of reward in feeding and appetite the reader is referred to Fulton, 2010).

In the VTA in particular, the CART peptide seems to act as a potent stimulator of locomotor activity and conditioned place preference (Jaworski et al., 2003, 2007), a form of Pavlovian conditioning also elicited by addictive drugs (Tzschentke, 1998). It is thought that the behavioral effects of CART activity in the VTA occur by CART directly stimulating dopaminergic neurons or indirectly un-inhibiting gamma-amino-butyric-acid-ergic interneurons (Kuhar et al., 2005). A facilitated capacity to reduce dopamine availability could counter the feeding reward effect, and contribute to diet-induced-obesity resistance in B1^{-/-} mice.

It seemed plausible that the increased mean number of CART-producing cells in the PeF of B1^{-/-} mice would presumably cause increased CART stimulation in the VTA and consequent increased basal locomotor activity in comparison to WT counterparts. Higher locomotor activity in turn could lead to less energy accumulation in fat stores and contribute to the 'leaner' B1 phenotype. It was observed, however, with the method we applied that the B1^{-/-} mice do not move significantly more than control mice in normal circumstances of chow diet. As the 'lean' phenotype is observed only when these mice are challenged with a HFD, it could be the case that differences in locomotor activity also only take place under HFD, a hypothesis that remains untested.

In B2^{-/-} animals, it was seen during aging that they did not increase the consolidation and retention of memory after a learning challenge, but that was not the case in B1^{-/-} (Lemos et al., 2010). This suggests that kinin B2 receptor activity might contribute to efficient learning behavior and an increase in locomotor activity. A second important point is that it could be expected because the 'lean' phenotype is observed only when these mice are challenged with a HFD. HFD seems to promote increased energy expenditure and lower feed efficiency (body weight gain/energy consumed) in B1^{-/-} in comparison to WT mice as revealed per calorimetric observations (Mori et al., 2008b). In fact, it is known that HFD increases 30% the CART expression in hypothalamic tissue extracts (Lee et al., 2010), presumably as a compensatory mechanism to maintain body weight. One hypothesis is that in B1^{-/-} model the machinery could be prepared with higher amount of CART neurons in the LHA but its effects are not required under basal conditions, probably because of its compensatory increased respiratory coefficient (Mori et al., 2008b).

The finding that genetic manipulation of B1 receptors affects CART neurons comes as a surprise and seems to indicate there is a gene-regulatory coupling between these gene systems. We believe that the B1 gene ablation disturbs the CART-mediated energy expenditure adaptation mechanism that exists in WT mice, allowing them to gain weight in conditions of HFD.

The idea that CART has a role in the B1^{-/-} 'lean' phenotype seems likely in the face of knowledge that CART expression is reduced in situations where energy expenditure is decreased, as discussed in a recent study in which a screening for genetic influences on energy expenditure of obese individuals was performed (Goossens et al., 2009).

The data presented in this work show for the first time a change in the LHA-CART expression related to a diet-induced-obesity resistance phenotype and seems to corroborate previous indications that CART is not a solely anorectic peptide in the hypothalamus. The observations here also corroborate previous indications that the B1-receptor-triggered mechanism impinging on body weight is not (solely at least) peripheral.

Future work should investigate putative gene-regulatory relationships between the kinin B1 receptors and pro-energy expenditure CART neuropeptides in the hypothalamus and mesolimbic systems. In addition, it

is important to analyze the axonal projection neurons to ensure its importance. Finally, the study furthers the possibility that B1 receptor might be useful as target for anti-obesity drug development, as supported by many previous works.

Material and methods

Animals

Data were obtained from 14-week-old male kinin B1 receptor knock-out mice produced by Pesquero et al. (2000) and C57BL/6 WT control mice bred at Universidade Federal de São Paulo, Brazil. The investigation conforms to *The Guide for the Care and Use of Laboratory Animals* (U.S. NIH, 1996). Animals were maintained on standard mouse chow at 22°C on a 12-h light-dark cycle with *ad libitum* access to food and tap water. Body weights were measured directly before the animals were sacrificed.

Brain preparation

Mice were deeply anesthetized with a solution containing ketamine (80 mg/kg) and xylazine (30 mg/kg) and perfused intracardially with a 0.9% saline solution and followed by a formaldehyde solution (4% m/v) in 0.01 M phosphate buffer (PBS). The heads were removed and kept in the formaldehyde solution for 24 h at room temperature. Later, the brains were removed from the skulls and maintained in the formaldehyde solution for an additional 12 h. Brains destined for immunohistochemistry were used from this point. Brains destined for *ISH* were postfixed in cryoprotectant solution (20% sacharose and 4% formaldehyde) at 4°C for 12 h before proceeding.

In situ hybridization

Coronal sections of formaldehyde-preserved brains were cut with a microtome at 30 µm thickness, preserved in cryoprotectant and stored at -70°C. *In situ* hybridization for CART and NPY mRNA was performed as reported by Elias et al. (1998) in a single batch. One of every third section was mounted and used for probe hybridization. The respective [35S] riboprobes were generated as described previously and kindly provided by Dr. Jackson Cioni Bittencourt's lab: CART (Douglass et al., 1995; Elias et al., 2000; Borges et al., 2007) and NPY (Higuchi et al., 1988; Borges et al., 2007). Briefly, the probes were *in vitro* transcribed with the appropriate RNA polymerases (either T3 or SP6) according to the producer's protocol (Promega).

[35S]cDNA probes for each mRNA was diluted to 106 cpm/ml in hybridization solution (50% formamide, 10 mM Tris-HCl, pH 8.0, 10 mM dithiotreitol (DTT), 10% dextran sulphate, 0.3 M NaCl, 1 mM EDTA pH 8.0 and Denhardt's solution (Invitrogen). Brain sections were incubated with a hybridization solution film applied between each glass slide and a flexible coverslip for 16 h at 57°C in a hybridization oven. Sections were then incubated in 0.002% RNAse A with 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA for 30 min. Subsequently, sec-

tions were washed in decreasing concentrations of SSC containing 0.25% DTT (2-SSC at 50°C for 1 h, 0.2-SSC at 55°C for 1 h and 0.2-SSC at 60°C for 1 h). Sections were then dehydrated in 70% ethanol containing 0.25% DTT for 10 min. Slides were air-dried and placed into X-ray film cassettes with BMR-2 film (Kodak, Rochester, NY) for 20 days. Slides were then dipped into NTB2 photographic emulsion (Kodak), dried and stored in boxes with desiccating pellets and foil-wrapped boxes at 4°C for 2 weeks. Finally, slides were developed with D-19 developer (Kodak), at 15–16°C for 1 min, counterstained with thionin, dehydrated in graded ethanol concentrations, cleared in xylene and coverslipped with Permaslip.

Immunohistochemistry

Thirty µm coronal sections of formaldehyde-preserved brains were cut in a vibration microtome (Leica VT1000 S) and kept in anti-freeze solution (90 mM saccharose, 50 mM sodium PBS, 2.5 mM poly-vinilpyrrolidone (PVP)-40 (Sigma) and 30% v/v etylene glycol) at -20°C. All further processing was carried out in one batch, i.e., every third section of mice brain was exposed to identical buffer, antibody and chromogenic solutions in order to minimize possible variables.

The protocol was established based on preliminary assays on antibody titration and incubation conditions. The anti-CART rabbit immunoglobulin (Phoenix Pharmaceuticals) optimal dilution was 1:20,000. All the steps were carried out in the free-floating mode on an orbital shaker according to the streptavidin-biotin-peroxidase method.

After rinsing, sections labeled with primary antibodies were incubated for 10 min at room temperature in secondary-antibody solution (a biotinylated anti-mouse, goat and rabbit antibody from DAKO LSAB™ Universal Kit).

Image acquisition and semiquantitative analysis

The ISH photomicrographs were captured with a SPOT RT® digital camera (Diagnostic Instruments Sterling Heights, MI, USA), adapted to a Leica DMR microscope (Leica, Wetzlar, Germany) and the Nis-Elements 3.0 program (Nikon Corporation, Melville, NY). All digital image processing was read double-blind (filenames were assigned a single random number by a Python program in order for the experimental group to be unknown to the researcher). The hybridization signal was estimated by double-blind comparison of the integrated optical density (IOD; mean pixel intensity on a gray scale ranging from 0 to 255 multiplied by area) in a constant area of three representative rostrocaudal levels of each nucleus, matched as closely as possible according to the atlas of Paxinos and Franklin (2001).

Analysis of spontaneous locomotor activity

Mice spontaneous locomotor activity was measured during the day (9 am) and at night (9 pm) using Opto-Varimex photocell cages (Columbus, OH, USA), which read motion by interpreting adjacent infrared light-bean disruption as horizontal movement. Prior to the experiments, mice were habituated to the room for 1 h and then placed into the activity chambers for 30-min measurement sessions.

Statistical analysis

Data were expressed as mean±SEM of integrated optical densities. Differences between groups were analyzed using either two-tailed unpaired Student's t-test (when analyzing two groups) and two-way ANOVA and Bonferroni's *post hoc* test in the spontaneous locomotor activity experiment. Significance was rejected when $p > 0.05$. All statistical analyses were carried out with the Graphpad Prism (La Jolla, CA, USA) software package.

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