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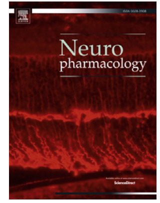

High doses of the histone deacetylase inhibitor sodium butyrate trigger a stress-like response

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The hypothalamic–pituitary–adrenal (HPA) axis is activated by a wide range of stimuli, including drugs. Here we report that in male rats, a dose of sodium butyrate (NaBu) that is typically used to inhibit histone deacetylation (1200 mg/kg) increased the peripheral levels of HPA hormones and glucose. In a further experiment, we compared the effects of two different doses of NaBu (200 and 1200 mg/kg) and equimolar saline solutions on peripheral neuroendocrine markers and brain c-Fos expression to demonstrate a specific stress-like effect of NaBu that is not related to hypertonicity and to localise putatively involved brain areas. Only the high dose of NaBu increased the plasma levels of stress markers. The equimolar (hypertonic) saline solution also activated the HPA axis and the c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN), a key area for the control of the HPA axis, but the effects were of a lower magnitude than those of NaBu. Regarding other brain areas, group differences in c-Fos expression were not observed in the medial prefrontal cortex or the medial amygdala, but they were observed in the central amygdala and the lateral ventral septum. However, only the latter area of the NaBu group showed enhanced c-Fos expression that was significantly higher than that after hypertonic saline. The present data indicate that high doses of NaBu appear to act as a pharmacological stressor, and this fact should be taken into account when using this drug to study the role of epigenetic processes in learning and emotional behaviour.

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1. Introduction

The activation of the hypothalamic–pituitary–adrenal (HPA) axis is one of the prototypical responses to stressors. Signals from different types of stressors reach the hypophysiotropic (medial parvocellular dorsal) region of the paraventricular nucleus of the hypothalamus (PVNmpd) to activate the release of corticotrophin-releasing hormone (CRH) and other ACTH secretagogues into the pituitary portal blood. These stimulatory factors act on the corticotrope cells of the anterior pituitary to induce the synthesis and release of ACTH. Finally, ACTH acts on the adrenal cortex to promote the synthesis and release of glucocorticoids (corticosterone in rats and mice).

The HPA axis is activated by both systemic and emotional stressors, but the brain pathways greatly differ among the different types of systemic stressors and between all of them and the emotional stressors (Pacak and Palkovits, 2001; Armario, 2006a). In addition to stimuli that are well-characterised as stressors, the HPA axis is also activated by a wide range of psychotropic drugs (including drugs of abuse, anxiolytics, antipsychotics and antidepressants) (Keim and Sigg, 1977; Armario and Garcia-Marquez, 1987; Meltzer et al., 1989; Gudelsky et al., 1989; Armario, 2010) and also by other drugs that have markedly different effects on the organism (see Armario, 2006b for overall discussion). The reason for this shared property of drugs acting at distinct biochemical/cellular targets is unknown, but it might be because the drugs cause some homoeostatic alterations that are similar to those induced by stressors.

During the course of an experiment studying the possible role of histone modifications on emotional learning, we observed that the administration of NaBu at the frequently used dose of 1200 mg/kg (Levenson et al., 2004; Lattal et al., 2007; Fischer et al., 2007; Stafford et al., 2012) markedly increased plasma levels of ACTH and corticosterone. These preliminary results suggested that NaBu, like some other drugs, was acting as a pharmacological stressor. However, although we diluted the NaBu solution more than in previous reports, at the concentration used, the solution was still

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List of abbreviations

ACTH	adrenocorticotrophin hormone
A1/C1	ventrolateral medulla
A2/C2	nucleus tractus solitarius
CeA	central amigdala
Cg1	medial prefrontal cortex cingulated
CRH	corticotrophin releasing hormone
GEE	generalised estimating equations model
GzLM	generalised linear model
HPA	hypothalamic–pituitary–adrenal
IHC	immunohistochemistry
IL	medial prefrontal cortex infralimbic
i.p.	intraperitoneal injection
LSV	lateral septum ventral
MeA	medial amigdala
NaBu	sodium butyrate
Prl	medial prefrontal cortex prelimbic
PVN	paraventricular nucleus of the hypothalamus
PVNmpd	paraventricular nucleus of the hypothalamus medial parvocellular dorsal region
PVNm	paraventricular nucleus of the hypothalamus magnocellular region
RIA	radioimmunoassay
SMA	sympathetic-medullary-adrenal
SON	supraoptic hypothalamic nucleus
SPSS	statistical package for social science

hypertonic (1.09 M). As hypertonic solutions are also able to activate the HPA axis (Veitia et al., 1982; Irvine et al., 1989; Watts, 1992; Kiss and Aguilera, 1993; Garcia et al., 2000), we reasoned that in order to demonstrate the possible stressor-like properties of NaBu, we should compare the response to NaBu with that of an equimolar solution of NaCl. Thus, in the present work, we report the preliminary data and the results of a second experiment that aimed to characterise the effects of two different doses of NaBu (200 and 1200 mg/kg) and a dose of NaCl equimolar to the higher NaBu dose on plasma levels of HPA hormones, glucose and brain c-fos induction. Plasma glucose levels were measured because stress-induced hyperglycaemia is a reflection of the activation of the sympathetic-medullary-adrenal (SMA) axis (Armario, 2006b). The expression of immediate early genes, particularly c-fos, has been used extensively to identify neuronal populations that are activated by stress and other stimuli (Pacak and Palkovits, 2001; Armario, 2006a).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats obtained from the breeding centre of the Universitat Autònoma de Barcelona were used. They were 2–3 months old at the beginning of the experiments. The animals were housed in pairs in polypropylene opaque wire-topped cages with solid bottoms (21.5 × 46.5 × 14.5 cm; Type "1000 cm^2", Panlab S.L.U., Barcelona, Spain) containing bedding of wood shavings (Lignocel 3/4, Harlan Interfauna Ibérica, Barcelona, Spain) under standard temperature conditions (21 ± 1 °C) and on a 12:12 h light/dark schedule (lights on at 08:00 h). Food (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and water were available ad libitum. The experimental protocol was approved by the Committee of Ethics of the Universitat Autònoma de Barcelona, following the "Principles of Laboratory Animal Care," and was carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2. General procedure

Starting at least five days after their arrival, all animals were handled at least three times for approximately 2 min a day on different days. In addition, one blood sample was taken under basal conditions to habituate animals to the procedure. Blood samples were taken by tail-nick, consisting of gently wrapping the animals with a cloth, making a 2-mm incision at the end of one of the tail veins and then massaging the tail while collecting (within 2 min) 300 μl of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain). This procedure is extensively used in our lab and by others because very low resting levels of hormones are obtained under appropriate conditions (Belda et al., 2004; Vahl et al., 2005). Cage-mates were sampled simultaneously (two experimenters were sampling at the same time and a third was gently holding the two rats). The blood was centrifuged at 4930 × g (15 min, 4 °C), and plasma was frozen (−20 °C) until assay. Animals were assigned at random to the different experimental groups as a function of their date of birth and body weight. The experimental treatments were always carried out in the morning.

2.3. Experimental designs

In Exp. 1, the animals were assigned to two groups: Vehicle ($n = 8$), injected with ClNa 0.9% (0.15 M); and NaBu1200 ($n = 8$), injected with sodium butyrate (Sigma-Aldrich, Madrid, Spain) 1200 mg/kg (1.09 M). The injection volume was 10 ml/kg. Sixty minutes after injection, a blood sample was taken. This time point was chosen because activation of the HPA axis typically reaches a maximum between 30 and 60 min (Armario, 2006b).

In Exp. 2, the animals were assigned to five groups: Control ($n = 4$), undisturbed animals; Vehicle ($n = 6$), injected with ClNa 0.9%; NaBu200 ($n = 8$), injected with sodium butyrate 200 mg/kg (0.18 M); NaBu1200 ($n = 8$), injected with sodium butyrate 1200 mg/kg (1.09 M); and HT ($n = 8$), injected with ClNa 1.09 M (hypertonic saline). The injection volume was 10 ml/kg. Blood samples were taken in all groups (except controls) at 30 and 120 min after injection. Two hours after injection or under resting conditions (controls), the rats were anesthetised with isoflurane and transcardially perfused with saline solution (0.9% NaCl, 4 °C) for 2 min and with 4% paraformaldehyde (PFA) and sodium tetraborate 3.8% (borax) (4 °C) for 12 min. After perfusion, the brains were removed, submerged in PFA and stored at 4 °C for 24 h. Then, PFA was replaced by a solution containing 30% sucrose potassium phosphate-buffered saline and the brain was maintained in this condition for 48 h at 4 °C. Subsequently, the brains were frozen using dry-ice-chilled isopentane at −50 °C. They were conserved at −80 °C before slicing into 20-μm coronal sections with a microtome. Then, sections were preserved at −20 °C in antifreeze solution (sodium phosphate 0.05 M, pH 7.3; 30% ethylene glycol; 20% glycerol) until analysis. The 2 h post-injection time was chosen because maximum levels of Fos protein are reached at this time, even after a short exposure to stressors (Armario, 2006a).

An additional experiment (Exp. 3) was designed to compare the effects of the 1200 mg/kg dose of NaBu on histone acetylation in the hypothalamus as compared with a reference area such as the hippocampal formation. To this end we studied monoacetylation of histone H3 at lysine 9 (monoAcH3). The animals were distributed into 4 groups: Veh-30 min ($n = 4$), NaBu-30 min ($n = 6$), Veh-1h ($n = 4$) and NaBu-1h ($n = 6$). Animals were injected with vehicle (saline 0.9%) or NaBu and sacrificed 30 min or 1 h after injection. Their brains were immediately removed and the appropriate areas dissected on ice and frozen in liquid nitrogen.

2.4. Biochemical analysis

Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassay (RIA). In brief, ACTH RIA used ^{125}I-ACTH (PerkinElmer Life Science, Boston, USA) as the tracer, rat synthetic ACTH 1–39 (Sigma, Barcelona, Spain) as the standard and an antibody raised against rat ACTH (rb7), kindly provided by Dr. W.C. Engeland (Department of Surgery, University of Minnesota, Minneapolis, USA). The characteristics of the antibody have been described previously (Engeland et al., 1989), and we followed a non-equilibrium procedure. The first antibody was added on day 1, and ^{125}I-ACTH was added on day 2. After one additional 18-h incubation period, the second antibody was added. Corticosterone RIA used ^{125}I-corticosterone–carboxymethyloxime–tyrosine–methylester (ICN-Biolink 2000, Barcelona, Spain) as the tracer, synthetic corticosterone (Sigma, Barcelona, Spain) as the standard, and an antibody raised in rabbits against corticosterone–carboxymethyloxime-BSA, which were kindly provided by Dr. G. Makara (Inst. Exp. Med., Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003). Plasma glucose levels were measured by the glucose-oxidase method using a commercial kit (Glucose RTU, Biomerieux, Barcelona, Spain). Plasma sodium levels were measured by an ion-selective electrode method with an Olympus AU400 analyser. The linearity range was 50–200 mmol/L. All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 3.8% for ACTH, 7.8% for corticosterone and less than 2.5% for glucose. The sensitivity of the assays was 12.5 pg/ml for ACTH, 1 ng/ml for corticosterone and 5 mg/dl for glucose.

2.5. Immunohistochemistry (IHC)

c-Fos activation in response to the different injections was used as a marker of brain activation. For c-Fos IHC (Rotllant et al., 2007; Ons et al., 2010), potassium phosphate buffer (0.05 M, pH = 7.4) containing 0.15 M NaCl (KPBS) was always used.

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