Differential regulation of agouti-related protein and neuropeptide Y in hypothalamic neurons following a stressful event

Martien J H Kas, Adrie W Bruijnzeel[1], Jurgen R Haanstra[2], Victor M Wiegant and **Roger A H Adan**

Department of Pharmacology and Anatomy, Rudolf Magnus Institute of Neuroscience, University Medical Centre Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

[1]Department of Psychiatry, University of Florida, Gainesville, Florida, USA

[2]Department of Molecular Cell Physiology, Free University, Amsterdam, The Netherlands

(Requests for offprints should be addressed to M J H Kas; Email: m.j.h.kas@med.uu.nl)

Abstract

Stress affects eating behaviour in rodents and humans, suggesting that the regulation of energy balance and the stress response are coupled physiological processes. Neuropeptide Y (NPY) and agouti-related protein (AgRP) are potent food-stimulating neuropeptides that are highly co-localised in arcuate nucleus neurons of the hypothalamus. Recent studies have shown that NPY and AgRP mRNA levels in these neurons respond similarly to fasting and leptin, indicating functional redundancy of the neuropeptide systems in these orexigenic neurons. However, we have found that NPY and AgRP mRNA expression in arcuate nucleus neurons are dissociated immediately following a stressful event. Two hours following a brief session of inescapable foot shocks, AgRP mRNA levels are *down*-regulated ($P < 0{\cdot}0001$). In contrast, NPY mRNA levels are *up*-regulated ($P < 0{\cdot}0001$). To provide physiological relevance for this acute down-regulation of AgRP, an inverse agonist of melanocortin receptors, we have shown that acute intracerebroventricular injection of a melanocortin receptor agonist, α-melanocyte-stimulating hormone (α-MSH), caused a significantly stronger activation of the hypothalamus–pituitary–adrenal-cortical (HPA) axis following a stressful event than in controls. Thus, AgRP and NPY mRNA levels in similar arcuate nucleus neurons are differentially regulated following a stressful event. This may contribute to increased sensitivity for α-MSH to activate the HPA axis following a repeated stressful experience.

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Introduction

An organism requires tightly regulated physiological processes to properly respond to stressful situations. For example, predator exposure demands inhibition of behaviours incompatible with the fight or flight reaction, such as of foraging behaviour. Indeed, a wide variety of studies have shown that stressful events markedly affect eating behaviour also in humans (Fryer *et al.* 1997). For example, rats exposed to immobilisation stress exhibit decreased food intake and body weight on the first day following the stressor (Valles *et al.* 2000). These observations suggest that systems involved in the regulation of the stress response and of energy balance are highly integrated.

Neuropeptide Y (NPY) and agouti-related protein (AgRP) are traditionally classified as food-stimulating neuropeptides and are highly co-expressed neuropeptides in arcuate nucleus neurons (Stanley & Leibowitz 1985, Morley *et al.* 1987, Hahn *et al.* 1998, Rossi *et al.* 1998, Hagan *et al.* 2000). For example, it was shown that AgRP was found in up to 99% of the arcuate NPY-containing neurons (Hahn *et al.* 1998). Recent studies have shown that NPY and AgRP respond similarly to fasting and leptin administration (Hahn *et al.* 1998, Elias *et al.* 1999, Wilson *et al.* 1999). It has therefore been proposed that these orexigenic systems are redundant in ensuring compensation during a negative energy balance (Hahn *et al.* 1998). Since stress affects eating behaviour and since disruption of arcuate nucleus connections to the paraventricular nucleus in the hypothalamus alter energy balance and stress responsiveness in rats (Bell *et al.* 2000), we predicted that the arcuate nucleus food-stimulating neuropeptides NPY and AgRP are regulated shortly after a stressful event, leading to functional implications in the stress responsiveness of the animal.

Materials and methods

Experiment 1

Adult male outbred rats (strain Wistar Utrecht:Wistar Unileve (U:WU) Central Animal Facility, University of

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Utrecht, The Netherlands, $n=8$/group) were anaesthetised with a mixture of 10 mg/ml fluanisone and 0·315 mg/ml fentanyl citrate (Hypnorm; Janssen Pharmaceutica, Beersse, Belgium; 0·1 ml/100 g body weight; i.p.) and midazolam (Dormicum; Hoffman-LaRoche, Mijdrecht, The Netherlands; 0·05 ml/100 g body weight; i.m.) and were implanted with a vena jugularis cannula, as described previously (Brakkee *et al.* 1979). Following 2 weeks of recovery with handling and baseline measurements of body weight, these rats (mean body weight of 300 ± 20 g) were exposed to a brief session of inescapable foot shocks (10 randomised foot shocks (6 s, 0·5 mA) during a 15-min session (Van Dijken *et al.* 1992, Bruijnzeel *et al.* 2001) in the third hour of the light phase (12 h light:12 h darkness cycle). Via the jugularis vena cannula, blood was sampled (0·5 ml) in freely moving animals at $t=0$ min (just before the stressful event), $t=15$ min (just after the stressful event) and $t=120$ min (just before they were killed). From these samples, plasma adrenocorticotrophin (ACTH) and corticosterone levels were determined by means of an RIA (Von Frijtag *et al.* 1998). Two hours after the beginning of the foot-shock session ($t=120$ min), brains were removed after decapitation, frozen in ice-cold iso-pentane (20 s at -30 °C) and stored at -80 °C.

In situ hybridisation

Pretreated 20 μm cryostate sections from rat hypothalamus were hybridised with ^{33}P-labelled anti-sense mRNA probes for AgRP, pro-opiomelanocortin (POMC) and NPY according to van der Kraan *et al.* (1998). mRNA expression in the arcuate nucleus (for NPY, AgRP and POMC) and reticular nucleus of the thalamus (for NPY) was quantified using MCID-M5 (Imaging Research, St. Catherines, Ontario, Canada). mRNA levels are expressed in c.p.m. as calculated from a standard curve of diluted probe mix on the same film as the slides were measured on. From each animal, two measurements per probe were taken in the regions of interest (arcuate nucleus and reticular nucleus of the thalamus) and subsequently averaged to calculate the mean c.p.m. per probe for that region.

Experiment 2

Adult male outbred rats (strain Wistar (U:WU), $n=8$/group) were anaesthetised with a mixture of 10 mg/ml fluanisone and 0·315 mg/ml fentanyl citrate (0·1 ml/100 g body weight; i.p.) and midazolam (0·05 ml/100 g body weight; i.m.). An intracerebroventricular (i.c.v.) cannula was surgically implanted (co-ordinates, 0·8 mm posterior and 1·0 mm lateral from the bregma). After 2 weeks of recovery and daily handling, corticosterone levels were compared in stressed rats (following the same foot-shock protocol as in experiment 1) and non-stressed home-cage controls which received an i.c.v. injection of either saline (3 μl) or α-melanocyte-stimulating hormone (α-MSH; 3 μg/3 μl; Bachem, Bubendorf, Switzerland) 2 h following the foot-shock session. Fifteen minutes after the injection, animals were decapitated and trunk blood was collected. Methylene blue dye (2 μl) was injected in the i.c.v. cannula to confirm correct placement of the cannula. Plasma levels of corticosterone were determined using RIA (Von Frijtag *et al.* 1998).

Experiment 3

Adult male outbred rats (strain Wistar (U:WU), $n=8$) were, upon arrival from the vendor, adapted for 2 weeks to our laboratory conditions (as described in experiment 1). One group of animals was then exposed to a foot-shock session (as in experiment 1) while the other group was left untreated. Two hours following the foot-shock session, all rats were subjected to a 15-min open field session (a circular, black arena with a diameter of 130 cm). Following these 15 min, animals were decapitated and trunk blood was collected. Plasma levels of corticosterone were determined using RIA (Von Frijtag *et al.* 1998). All the procedures described were approved by the ethical committee on the use and care of animals of the University of Utrecht, The Netherlands.

Data analysis

All data are presented as means ± S.E.M. Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA) for Windows version 11·5 software. Time-dependent changes of plasma ACTH and corticosterone levels were analysed using repeated measure ANOVA. In the presence of a main effect, Tukey contrast statistics were applied ($\alpha=0·05$). Neuropeptide mRNA expression levels and corticosterone levels following i.c.v. injections and open field tests were compared by using an independent sample *t*-test. Differences were considered significant at $P<0·05$.

Results

Different regulation of NPY and AgRP following a stressful event

The foot-shock session induced a strong activation of the hypothalamus–pituitary–adrenal-cortical (HPA) axis. Plasma levels of ACTH and corticosterone significantly increased following this session ($t=15$ min) and returned to baseline levels at $t=120$ min (Fig. 1; $P<0·0001$). Two hours following the foot-shock session (at $t=120$ min), AgRP, POMC and NPY mRNA levels in the arcuate

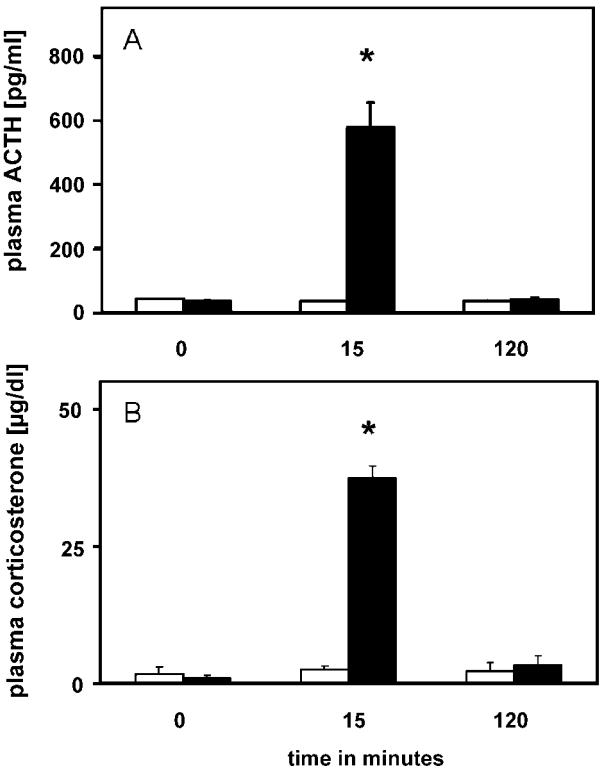

Figure 1 A brief foot-shock session induced a strong activation of the HPA axis. Plasma corticosterone levels in the stressed animals (solid bars) were significantly increased at t=15 min, immediately following the foot-shock session. At the end of the experiment (t=120 min), these hormone levels were returned to baseline and control levels (open bars). There were eight rats in each group. *P=0·0001.

nucleus were determined by means of *in situ* hybridisation and were compared with levels in home-cage controls. AgRP mRNA levels in the arcuate nucleus were markedly reduced 2 h after the foot-shock session (P<0·0001). AgRP levels exhibited a 75% reduction when compared with controls (Fig. 2A–C). In neighbouring sections from the same brains we studied mRNA levels of NPY in the arcuate nucleus. In contrast to the reduction in AgRP, NPY gene levels were strikingly increased (Fig. 2D–F; P<0·0001). To demonstrate that NPY up-regulation within the arcuate nucleus did not reflect a general up-regulation of brain NPY gene expression, we showed that NPY mRNA levels were not changed in the reticular nucleus of the thalamus as a function of the stressful event (254 ± 51 c.p.m. (control) versus 257 ± 26 c.p.m. (stressed); t= − 0·4, P=0·97). Adjacent to AgRP/NPY expression neurons, distinct subpopulations of arcuate nucleus neurons have been identified that express mRNA encoding the melanocortin precursor, POMC. POMC is the precursor hormone of, for example, the anorexigenic neuropeptide α-MSH. Two hours following the foot-shock session, POMC gene expression in arcuate nucleus neurons was not different from controls (555 ± 70 c.p.m. (control) vs 672 ± 151 c.p.m. (stressed); t= − 0·768, P=0·457). Thus, orexigenic neuropeptide systems in the arcuate nucleus of the hypothalamus that respond similarly to fasting are differently regulated following a brief stressful event.

α-MSH-induced HPA axis activation following a stressful event

While a brief stressful event reduced AgRP mRNA levels, we wondered whether the responsiveness of the

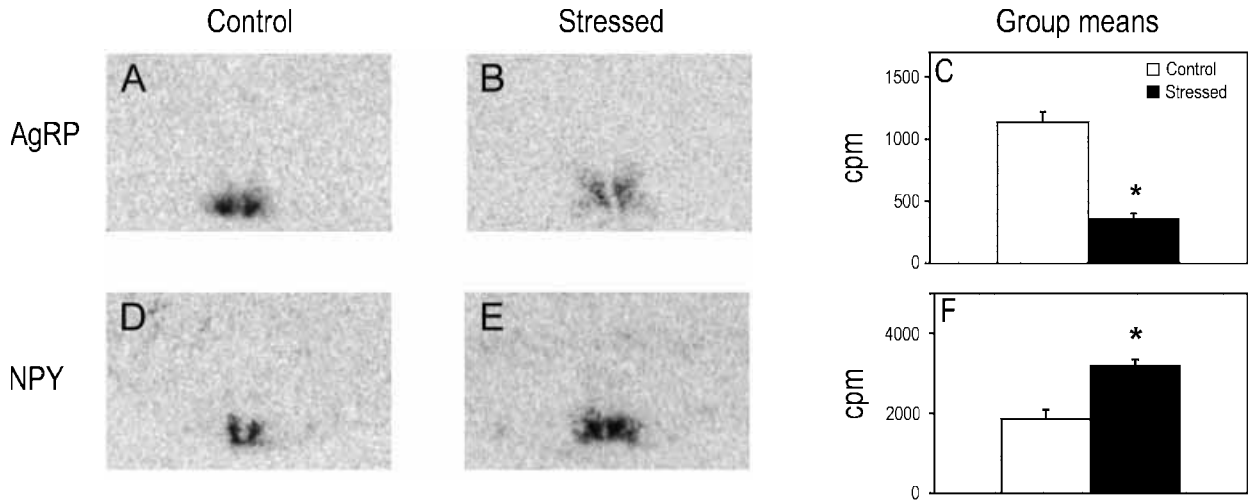

Figure 2 (A–C) AgRP and (D–F) NPY mRNA expression in the arcuate nucleus are dissociated 2 h after a brief stressful event. Group means showed that this stressful experience (C) significantly reduced AgRP mRNA levels, but (F) increased NPY mRNA levels in the arcuate nucleus of the hypothalamus. There were eight rats in each group. *P=0·0001 as compared with controls.

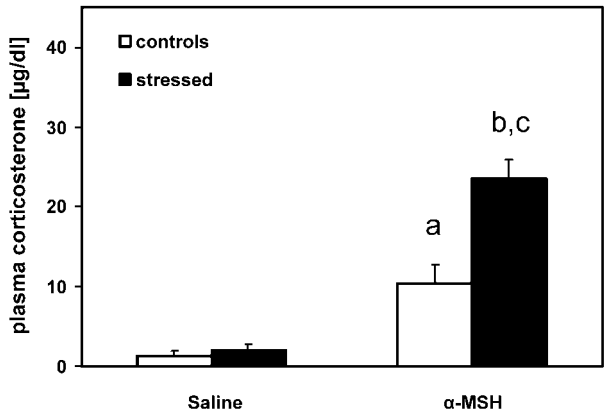

Figure 3 Corticosterone release following central administration of α-MSH is further increased following a brief stressful event. Four experimental groups of rats were injected i.c.v with either saline or α-MSH 2 h after a short session of inescapable foot shocks (or in home-cage controls). In stressed rats, the same dose of α-MSH induced a larger corticosterone release than in home-cage controls. There were eight rats in each group. a, P=0·002 compared with saline control; b, P=0·0001 compared with foot-shock saline; c, P=0·002 compared with α-MSH control.

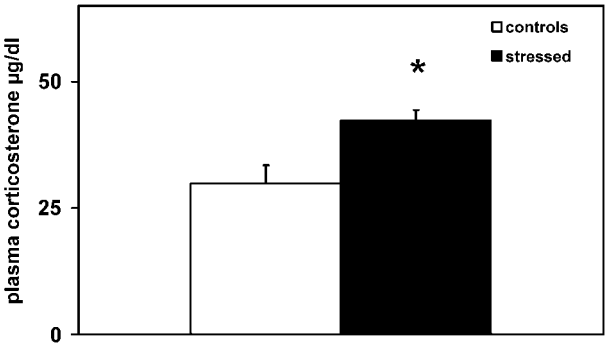

Figure 4 Exposure to a novel environment, shortly (2 h) after a foot-shock session induced a potentiated corticosterone response. Corticosterone release following a 15-min open field session is significantly higher in animals that experienced a brief stressful event 2 h prior to the open field session when compared with home-cage controls. There were eight rats in each group. *P=0·008.

brain to melanocortin activation was also changed at this corresponding time of day. Rats were therefore exposed to the foot-shock protocol and received an i.c.v. injection of α-MSH (or saline) 2 h after this brief stressful event (a time-point at which AgRP mRNA levels are down). Fifteen minutes following this injection, plasma corticosterone levels were measured. This study showed that central administration of the same dose of α-MSH activated corticosterone release in both stressed and home-cage control rats. However, stressed rats showed a significantly stronger corticosterone response to the same dose of α-MSH (Fig. 3; control saline vs control α-MSH, t= − 4·071, P=0·002; foot-shock saline vs foot-shock α-MSH, t= − 7·888, P=0·0001; control α-MSH vs foot-shock α-MSH, t= − 4·029, P=0·002).

Increased sensitivity of the HPA axis following a brief stressful event

To provide physiological relevance for increased responsiveness of the HPA axis 2 h following exposure to an acute stressful event, HPA axis activity was studied following exposure to a novel environment in previously stressed and non-stressed rats. Two hours after the foot-shock session, rats were placed in an open field arena. After 15 min, plasma corticosterone levels were measured and compared with that of animals that were not pre-exposed to the foot-shock session. When compared with normal baseline corticosterone levels (e.g. see Figs 1B and 3), the 15-min open field session increased corticosterone levels in both groups; however, corticosterone levels were significantly higher in previously stressed rats when compared with controls (Fig. 4; t= − 3·068, P=0·008).

Discussion

Thus far, it has been assumed that NPY and AgRP are redundant food-stimulating neuropeptides (Hahn *et al.* 1998). Indeed, food restriction results in up-regulation of both AgRP and NPY mRNA levels in arcuate nucleus neurons of the hypothalamus, indicating that these highly co-localised neuropeptide systems are also highly co-regulated following fasting. However, we have now provided evidence that NPY and AgRP gene regulation in arcuate neurons can be dissociated and that they may serve separate physiological functions following a stressful event. Since stressed rats have reduced AgRP mRNA levels and also have increased HPA axis responsiveness following central α-MSH administration, the present data have shown that the regulation of the stress response and the regulation of energy metabolism are highly integrated at the level of melanocortin receptor signalling. To provide physiological relevance for a sensitised HPA axis activity briefly after a stressful event, we showed that 2 h after a foot-shock session rats have a potentiated corticosterone response when subjected to a novel environment.

Our data have confirmed previous observations on the regulation of NPY mRNA levels in the hypothalamus following stressful events (Conrad & McEwen 2000). Others have reported on increased NPY mRNA levels in the arcuate nucleus following restrained stress. Our finding that AgRP mRNA levels in similar neurons of the arcuate nucleus are strongly down-regulated following a stressful event is novel and shows that highly

co-localised neuropeptide systems can be dissociated. It remains to be investigated whether similar changes will be observed at the level of protein translation and neuropeptide release. To provide further evidence that down-regulation of AgRP, the endogenous inverse agonist of melanocortin receptors (Nijenhuis *et al.* 2001), has physiological consequences, we reasoned that, due to the reduced inhibition of melanocortin receptors, the sensitivity for α-MSH to activate the HPA axis was increased. Rats were therefore centrally administered with α-MSH, the agonist of the melanocortin system, following the foot-shock procedure. Indeed, these rats had a stronger activation of corticosterone release by α-MSH than home-cage controls. Although these findings cannot, at this moment, be directly related, the melanocortin system seems more sensitive for its endogenous agonists shortly following a brief stressful event, possible due to reduced blockade of its receptors by AgRP. Altered sensitivity of the melanocortin system following intervening with the stress–hormone system is consistent with recent findings that show that adrenalectomy alters the sensitivity of the central melanocortin system (Drazen *et al.* 2003).

While the responsiveness of AgRP and NPY neurons was measured at the level of the arcuate nucleus of the hypothalamus, these neurons have projections throughout the hypothalamus. For example, AgRP/NPY neurons highly innervate neurons in a wide variety of hypothalamic regions, such as the dorsal medial hypothalamus and the paraventricular nucleus of the hypothalamus (Haskell-Luevano *et al.* 1999). Recent findings suggest that the latter is a very likely candidate region for the integration of feeding regulation and stress responsiveness (Bell *et al.* 2000, Lu *et al.* 2003). For example, neurons of the paraventricular nucleus containing corticotrophin-releasing factor (CRF), a neuropeptide that is highly involved in the regulation of both the HPA axis and feeding behaviour, have melanocortin-4 receptors on their surface. In addition, both the food-inhibitory effects and corticosterone-stimulating effects of melanocortin agonists can be blocked with a CRF antagonist (Lu *et al.* 2003). However, since CRF mRNA and protein levels are regulated after chronic but not after acute foot-shock sessions (Imaki *et al.* 1991, Bruijnzeel *et al.* 2001), further studies are necessary to determine whether CRF in the paraventricular nucleus of the hypothalamus is a good candidate acting as a downstream mediator of the acute responses observed in melanocortin-induced eating behaviour and HPA axis activity.

Loss or gain of AgRP and NPY gene function may result in inadequate adaptive behavioural responses to environmental events, such as stress, and may potentially contribute to the development of eating disorders. For example, recent studies have shown that polymorphisms in the AgRP gene are associated with anorexia nervosa (Vink *et al.* 2001) and obesity (Mayfield *et al.* 2001). Recent studies suggest that inadequate AgRP signalling during stress may result in binge eating. Binge eating, an eating disorder characterised by episodes of excessive overeating, is associated with stressful events (Pinaquy *et al.* 2003) and with individuals who have mutations in the melanocortin-4 receptor (Branson *et al.* 2003).

It has also recently been hypothesised that anorexia nervosa may result from an excess of both orexigenic and anorexigenic signalling (Inui 2001); interactions between increased signalling of NPY and of stress-related anorexigenic factors may produce a conflicting signal regarding satiety and desire for food following a stressful event. Indeed, activation of the stress–hormone axis and increased levels of NPY in cerebrospinal fluid have been observed in anorexia nervosa patients (Kaye *et al.* 1990, Licinio *et al.* 1996). We therefore propose that inaccurate regulation or integration of NPY and/or AgRP signalling following a stressful event may increase the risk for eating disorders such as anorexia nervosa and obesity.

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