Neuropeptide Y mediates the initial hypothalamic–pituitary–adrenal response to maternal separation in the neonatal mouse

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Abstract

The function of the hypothalamic–pituitary–adrenal (HPA) axis of the neonatal mouse or rat is characterized by a period of quiescence, where mild stimuli are unable to elicit a pronounced increase in circulating corticosterone. A disruption of this period by maternal separation has been shown to result in a variety of long-term consequences, including neuroendocrine and behavioral disturbances. We have recently shown that peripheral metabolic markers like glucose or ghrelin are altered by maternal separation and that these changes precede the effects on corticosterone secretion. In the current study, we investigated whether the initial activation of the HPA axis is mediated via neuropeptide Y (NPY). To test this hypothesis, we studied the effects of an 8 h maternal

separation in NPY-deficient mice. In addition, we compared the effect of the genotype with the previously described pharmacological effect of a ghrelin receptor antagonist. We could show that the peripheral response to maternal separation is decreased in NPY heterozygous and homozygous animals. In addition, maternal separation effects on corticotropin releasing hormone and glucocorticoid receptor expression in the brain were prevented in NPY-deficient pups. These effects were similar to a pharmacological ghrelin receptor blockade. We conclude that metabolic signals via an NPY-mediated pathway play a crucial role in activating the stress system of the neonatal mouse.

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Introduction

The neonatal period in the mouse or rat has been shown to be characterized by a high degree of vulnerability to disruptive stimuli (Sanchez *et al.* 2001). Dozens of studies over the last 60 years have demonstrated robust long-term consequences of different postnatal manipulation paradigms on neuroendocrine function, memory and behavior (Levine 1957, 1959, Zarrow *et al.* 1972, Plotsky & Meaney 1993, Liu *et al.* 1997). This high degree of vulnerability is generally related to the so-called stress hypo-responsive period (SHRP) that lasts from about postnatal day 1 (P1) to P12 in the mouse (Schmidt *et al.* 2003a). During this period, basal circulating corticosterone levels are very low and mild stressors are unable to induce a peripheral corticosterone response (Levine *et al.* 2000). However, a variety of severe or prolonged stressors disrupt the quiescence of the hypothalamic–pituitary–adrenal (HPA) axis during this time of development (Schoenfeld *et al.* 1980, Walker *et al.* 1991, Yi & Baram 1994). The high levels of circulating corticosterone induced by these treatments are usually seen as the cause for a consequently altered developmental trajectory and finally persistent consequences in the adult animal.

One of the most common paradigms used to disrupt HPA axis function in the neonate is a separation of the pup from the

mother for various time intervals (Stanton *et al.* 1988, Plotsky & Meaney 1993, Schmidt *et al.* 2002). During the absence of the mother from the nest, two main parameters have been identified as being crucial for the effects of maternal separation: the lack of maternal care and the lack of food. While a replacement of maternal care behavior (anogenital stroking) could only reverse maternal separation-induced changes in central HPA axis parameters, feeding in combination with stroking could also prevent the initial peripheral activation of the HPA axis (Rosenfeld *et al.* 1993, Suchecki *et al.* 1993, van Oers *et al.* 1998). In a previous study by our group, we could show that a number of peripheral metabolic markers such as glucose, leptin, and ghrelin are markedly altered following an 8 h maternal separation (Schmidt *et al.* 2006). In the same study, we were able to ameliorate the initial HPA axis activation due to maternal separation by glucose replacement or by antagonizing the growth hormone (GH) secretagogue receptor (i.e. ghrelin receptor). Based on these data we hypothesized that neuropeptide Y (NPY) neurons projecting from the arcuate nucleus to the paraventricular nucleus of the hypothalamus (PVN) may be one final common pathway that is responsible for the activation of the HPA axis following maternal separation.

This hypothesis was supported by a number of studies on NPY in adult animals. It has been shown that

NPY-containing neurons form a prominent afferent pathway from the arcuate nucleus to the PVN (Liposits *et al.* 1988, Li *et al.* 2000). In addition, the activity of these NPY neurons is increased by food deprivation in adult animals (Dube *et al.* 1992, Hanson *et al.* 1997) and a paraventricular release of NPY is known to stimulate food intake, an effect that is suppressed by corticotropin releasing hormone (CRH; Heinrichs *et al.* 1993). Further, NPY release in the PVN modulates the activity of parvocellular neurons and increases adrenocorticotrophin (ACTH) and corticosterone release (Inoue *et al.* 1989, Albers *et al.* 1990). CRH neurons in the PVN contain NPY1 (Y1) receptors that seem to mediate the activation of these neurons (Dimitrov *et al.* 2007). In addition, a recent study by Kakui & Kitamura (2007) also provided direct evidence for an involvement of Y5 receptors in regulating HPA axis function in the adult rat. Taken together, these data strongly support our hypothesis that NPY could mediate the initial activation of the HPA axis during prolonged maternal absence.

To test this hypothesis, we investigated the effects of an 8 h maternal separation in NPY-deficient mice. In addition, we compared the effect of genetic inactivation of NPY with the previously described pharmacological effect of a ghrelin receptor antagonist.

Materials and Methods

Animals

The offspring of heterozygous NPY knockout mice (Erickson *et al.* 1996; obtained from The Jackson Laboratory, Bar Harbor, ME, USA) was used in this study. Always one female was mated with one male in polycarbonate boxes containing sawdust bedding. Pregnant females were transferred to clean polycarbonate cages containing sawdust and two sheets of paper towels for nest material during the last week of gestation. Pregnant females were checked for litters daily between 0900 and 1000 h. If litters were found, the day of birth was defined as postnatal day 0 (P0) for that litter. The day of testing for all experiments was P8. At this age, the animals are in the middle of the stress hyporesponsive period (Schmidt *et al.* 2003a). Litters remained undisturbed from the day of birth until the day of testing. All animals were housed under a 12 h light:12 h darkness cycle (lights on at 0600 h) and constant temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) conditions. Food and water were made available *ad libitum*.

The experiments were carried out in accordance with European Communities Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

Genotyping of the offspring was performed by PCR analysis. Homozygous, heterozygous, and wild-type animals

were obtained from the same litters, thereby excluding inter-litter effects.

Maternal separation

Litters were randomly assigned to either a maternally non-separated (NSEP) or a maternally separated (SEP) condition. Maternal separation took place in a separate room in the animal facility under similar light and temperature conditions as mentioned previously. If a nest was assigned to maternal separation, mothers were removed from their home cages between 0800 and 1000 h. The home cage containing the litter was then placed on a heating pad, which maintained the nest temperature at $30\text{--}33^\circ\text{C}$, for 8 h. Neither food nor water was available during the separation period. The non-separated controls remained undisturbed with their mothers.

Experimental design

All mice were tested at P8. Maternally separated litters were randomly assigned to injections with either vehicle (physiological saline; SEP-veh) or the GH secretagogue receptor antagonist [D-Lys3]-GHRP-6 (SEP-GA) in a dose of 12 mg/kg body weight (dose based on Beck *et al.* (2004) and Schmidt *et al.* (2006)). The injection volume was always adjusted to 30 μl . The GH secretagogue receptor antagonist [D-Lys3]-GHRP-6, which is a potent ghrelin receptor antagonist, was obtained from Biotrend Chemicals GmbH (Cologne, Germany). The non-separated pups were all injected with vehicle. We did not include a non-separated group treated with [D-Lys3]-GHRP-6, as we could previously show that this treatment has no effect under basal conditions during the SHRP (Schmidt *et al.* 2006). All pups were injected subcutaneously 6 h before testing (for separated pups this was 2 h after the onset of the separation period).

Testing procedure

At the time of testing, all pups were killed by decapitation. For non-separated litters, the mother was removed from the home cage immediately before testing. Trunk blood from all pups was collected individually in labeled 1.5 ml EDTA-coated microcentrifuge tubes. All blood samples were kept on ice and later centrifuged for 15 min at 3300 g at 4°C . Plasma was transferred to clean, labeled 1.5 ml microcentrifuge tubes. All plasma samples were kept frozen at -20°C until corticosterone measurement (determined by radio immune assay; MP Biomedicals Inc., (Solon, OH, USA), sensitivity 3 ng/ml, intra-assay variation 4.4%, inter-assay variation 6.5%). Blood glucose was measured with a glucometer (Elite, Bayer). Whole heads (without skin and jaw) were removed, frozen in isopentane at -40°C , and stored at -80°C for *in situ* hybridization. Tail tips were removed and frozen for determination of the genotype of the pups.

In situ hybridization

The brains of eight animals per group were used for *in situ* hybridization. Only homozygous NPY knockouts and wild-type animals were used. Frozen brains were sectioned at -20°C in a cryostat microtome at $16\text{ }\mu\text{m}$ in the coronal plane through the level of the hypothalamic PVN and the dorsal hippocampus. The sections were thaw-mounted on superfrost slides, dried, and kept at -80°C . Every eighth section was mounted on the same slide so that each slide contained sections covering the whole anatomical region of interest.

In situ hybridization using 35S UTP labeled ribonucleotide probes (CRH, glucocorticoid receptor (GR)) were performed as described previously (Schmidt *et al.* 2002). Briefly, sections were fixed in 4% paraformaldehyde/0.5% glutaraldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes for CRH (full length) and the GR (1250 bps) were transcribed from a linearized plasmid. Tissue sections were saturated with 100 μl of hybridization buffer containing $\sim 1.5 \times 10^6$ cpm 35S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55°C . The following day the sections were rinsed in $2 \times \text{SSC}$ (SSC), treated with RNase A (20 mg/l), and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in $0.1 \times \text{SSC}$ for 1 h at 65°C and dehydrated through increasing concentrations of alcohol.

For arginine vasopressin (AVP), an oligo *in situ* hybridization was performed. The oligonucleotides (sequence: 5'-gggcttggcagaatccacggactctgtgtccagcgcgtgtaccag-3') were labeled with 35S dATP using terminal transferase (TdT, Boehringer) and added to the hybridization mix. Brain sections were coverslipped and incubated overnight at 45°C . The following day the sections were washed in $1 \times \text{SSC}$ at 55°C and dehydrated through increasing concentrations of alcohol.

The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co.; CRH: 6 days, GR: 3 days) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion Corporation, Frederick, MD, USA). The mean of four measurements of two slices with the strongest hybridization signal was calculated from each animal. For AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co) and exposed at 4°C for 3 days. Slides were developed, counterstained with cresyl violet, and examined under a light microscope with both bright and dark field condensers. For the quantitative analysis of AVP, dark field pictures of the PVN were inverted and made binary (black/white) at a specific gray value threshold. Subsequently, a circle of fixed size was superimposed on the parvocellular PVN area and the average grey value was obtained.

Data analysis

The commercially available program SPSS 12.1 was used for statistical analysis. Comparisons were made by a two-way ANOVA with genotype (wild-type, heterozygous, or homozygous) and condition (NSEP-vehicle, SEP-vehicle or SEP-GA) as independent factors. When appropriate, tests of simple main effects were made with the student's *t*-test or Tukey's *post hoc* analysis. The initial analysis included sex as a factor; once it was determined that sex was not a significant factor, the data were collapsed across this variable. In the PVN, the parvocellular part of the PVN was analyzed for CRH and AVP expression. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements. The level of significance was set at $P < 0.05$.

Results

Endocrine parameters

Plasma levels of corticosterone and glucose were determined in all three genotypes under basal conditions (NSEP-veh) as well as following 8 h of maternal separation. Animals from the maternally separated group were either injected with the ghrelin receptor antagonist [D-Lys3]-GHRP-6 or vehicle.

For corticosterone (Fig. 1A), ANOVA revealed a significant effect of genotype ($F(2,89) = 5.463$ ($P < 0.006$)) and condition ($F(2,89) = 47.42$ ($P < 0.001$)) as well as a genotype $\times$ condition interaction ($F(2,89) = 5.883$ ($P < 0.001$)). Under non-separated conditions, corticosterone levels were very low, with no differences between the three genotypes. Maternal separation resulted in a significant increase of plasma corticosterone. This increase was significantly attenuated in NPY heterozygous and knockout animals. Treatment with the ghrelin receptor antagonist also significantly decreased the corticosterone response to 8 h of maternal separation in wild-type animals. Treatment of NPY heterozygous or knockout pups with the ghrelin receptor antagonist did not result in a further reduction of corticosterone levels.

For glucose (Fig. 1B), ANOVA revealed a significant effect of genotype ($F(2,89) = 6.073$ ($P < 0.003$)) and condition ($F(2,89) = 407.38$ ($P < 0.001$)), but no significant interaction of the two factors. Wild-type, heterozygous, and knockout animals had similar glucose levels under non-separated conditions. Maternal separation resulted in a dramatic reduction of peripheral glucose levels in all groups. However, ghrelin antagonist-treated NPY knockout pups showed significantly lower glucose levels compared with wild-type animals from the same treatment group. Vehicle-treated and maternally separated NPY knockouts also had lower glucose levels than wild-types, but this effect did not reach statistical significance ($P < 0.07$).

Figure 1 (A) Plasma corticosterone (B) and glucose concentration of NPY wild-type, heterozygous or homozygous knockout pups at P8 under non-separated conditions (NSEP) or following 8 h of maternal separation (SEP). Maternally separated animals were either injected with the ghrelin receptor antagonist [D-Lys3]-GHRP-6 (GA) or vehicle (saline) 6 h before testing. Data represent mean $\pm$ s.e.m., *significant from SEP-veh of the same genotype, #significant from wild-type (+/+) of the same condition, $P < 0.05$.

Neuronal expression of HPA parameters

In the PVN, we determined the expression level of CRH, AVP, and the GR. For CRH (Fig. 2A and B), we found a significant effect of condition ($F(2,40) = 4.33$ ($P < 0.021$)) as well as a genotype $\times$ condition interaction ($F(2,40) = 4.162$ ($P < 0.024$)). There were no differences in CRH mRNA expression between both genotypes under non-separated conditions. Following maternal separation, wild-type pups showed a significant reduction of CRH expression in the PVN. This effect was not observed in NPY knockout animals. Ghrelin antagonist treatment in wild-type animals also prevented the maternal separation-induced reduction of CRH expression, but had no further effect in separated NPY knockout mice.

For the GR (Fig. 2C and D), ANOVA revealed a significant effect of condition ($F(2,42) = 4.896$ ($P < 0.013$)) as well as a genotype $\times$ condition interaction ($F(2,42) = 8.424$ ($P < 0.001$)). The results are very similar to the ones observed for CRH mRNA expression. We did not observe a significant difference in GR expression under non-separated conditions between the wild-type and knockout animals. Maternal separation significantly reduced GR mRNA levels in wild-type, but not in NPY knockout animals. Furthermore,

treatment with the ghrelin receptor antagonist also prevented the separation-induced reduction in GR mRNA expression in wild-type pups. The same treatment in separated knockout animals had no additional effect.

We also analyzed the expression levels of AVP in the parvocellular part of the PVN and GR in the Cornu Ammonis (CA)1 region of the hippocampus. The data are summarized in Table 1. For both transcripts, no significant effects of genotype or condition could be found (AVP: genotype: $F(2,38) = 1.278$ ($P < 0.266$), condition: $F(2,38) = 0.549$ ($P < 0.583$); GR: genotype: $F(2,45) = 0.062$ ($P < 0.804$), condition: $F(2,45) = 2.492$ ($P < 0.095$)).

Discussion

In the current study, we tested the hypothesis that NPY is directly involved in activating the HPA axis of neonatal mice following 8 h of maternal absence. We could show that NPY-deficient pups show a significantly attenuated maternal separation response compared with wild-type pups.

Maternal separation has been shown to activate the HPA axis during the SHRP in neonatal mice and rats. We have previously shown that 8 h of separation are sufficient for a dramatic increase in plasma corticosterone as well as associated gene expression changes in the brain (Schmidt *et al.* 2004). Further, these changes were accompanied (or even preceded) by alterations in peripheral metabolic signals as glucose or ghrelin. A suppression of these metabolic signals in the periphery resulted in an attenuation of corticosterone release and prevented separation-induced gene expression changes (Schmidt *et al.* 2006). Here, we demonstrate the same effect in NPY-deficient mice, suggesting a direct involvement of this neuropeptide in the activation of the HPA axis during maternal separation. This finding is supported by the fact that treatment with a ghrelin receptor antagonist resulted in a similar physiological outcome and that there were no additive effects of ghrelin antagonism and NPY deficiency, suggesting a similar route of action.

Our data are also in line with the literature on NPY function in adult animals, where arcuate nucleus NPY neurons are activated by food deprivation and increase corticosterone release from the adrenal glands (Dube *et al.* 1992, Heinrichs *et al.* 1993). Interestingly, adult NPY knockout mice show no alterations in neuroendocrine function, suggesting adaptational or compensatory processes during postnatal and adolescent development (Erickson *et al.* 1997). Similar findings have also been reported for NPY/agouti-related protein double knockouts, giving further support for this line of reasoning (Qian *et al.* 2002). So far it is still unclear which NPY receptor is responsible for the modulatory role on the HPA axis, as both Y1 and Y5 receptors have been implicated (Dimitrov *et al.* 2007, Kakui & Kitamura 2007).

In contrast to the adult, an activation of the neonatal HPA axis by maternal separation is accompanied by a decrease in

Figure 2 Expression levels of (A) CRH mRNA and (C) GR mRNA in the PVN of NPY wild-type or homozygous knockout pups at P8 under non-separated conditions (NSEP) or following 8 h of maternal separation (SEP). Maternally separated animals were either injected with the ghrelin receptor antagonist [D-Lys3]-GHRP-6 (GA) or vehicle (saline) 6 h before testing. (B and D) Representative expression autoradiograms of CRH and GR expression in the PVN respectively. Data represent mean $\pm$ S.E.M. *significant from other conditions of the same genotype, #significant from wild-type (+/+) of the same condition, $P < 0.05$.

CRH mRNA expression in the PVN rather than an expected increase. However, also in the adult high levels of corticosterone are capable of decreasing CRH mRNA expression (Kretz *et al.* 1999, Viau *et al.* 1999). Further, CRH expression decreases at the end of the SHRP when basal corticosterone levels increase, indicating a very sensitive negative feedback regulation of CRH gene expression in the

neonate (Schmidt *et al.* 2003a). In mice with a genetic disruption of the CRH receptor 1 throughout the body, which are unable to induce a corticosterone response to maternal separation, CRH expression is unaltered following prolonged maternal absence (Schmidt *et al.* 2003b). It has therefore been suggested that the decrease of CRH expression following maternal separation is a direct consequence of the

Table 1 Expression levels of arginine vasopressin (AVP) mRNA in the parvocellular part of the paraventricular nucleus (PVN) and glucocorticoid receptor (GR) mRNA in the CA1 region of the hippocampus of neuropeptide Y (NPY) wild-type or homozygous knockout pups at P8 under non-separated conditions (NSEP) or following 8 h of maternal separation (SEP)

		AVP mRNA in the PVN						GR mRNA in the CA1					
		NSEP		SEP				NSEP		SEP			
		Veh		Veh		GA		Veh		Veh		GA	
Genotype		+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-
n		5	6	6	8	7	7	7	7	8	8	8	8
Mean		23.8	26.9	25.1	28.7	27.4	31.2	45.3	44.4	40.1	40.9	38.5	36.5
S.D.		6.8	14.1	8.3	9.9	9.3	6.6	7.2	8.2	10.2	11.6	9.0	6.8

Maternally separated animals were either injected with the ghrelin receptor antagonist [D-Lys3]-GHRP-6 (GA) or vehicle (saline) 6 h before testing.

enhanced levels of circulating corticosterone. The observed decrease in GR expression in the PVN following maternal separation, which could be prevented by ghrelin antagonism and NPY deficiency, might therefore reflect an adaptational mechanism to lower the feedback pressure on CRH expression. In contrast, we observed no effects of 8 h maternal separation on AVP expression in the PVN or GR expression in the CA1 region of the hippocampus. These data are in line with our previous findings, indicating that both transcripts change only in response to longer separation periods (Schmidt *et al.* 2004). Taken together, it seems likely that the NPY-mediated activation of the HPA axis in the neonate mainly acts on CRH expressing neurons in the PVN.

The effects of maternal deprivation on corticosterone secretion were not fully prevented in NPY-deficient animals, as we still observed significant increases in circulating corticosterone in separated NPY knockout pups. Interestingly, these changes were not accompanied by central effects on gene expression of HPA axis parameters. It can thus be concluded that the remaining effect is likely to be independent of a hypothalamic regulation of the HPA axis. One possibility is a direct effect of metabolic signals on the pituitary or the adrenal gland. Numerous studies have indicated an increase in adrenal sensitivity to ACTH following prolonged maternal separation (Okimoto *et al.* 2002). In our previous paper we have already suggested a role of leptin in such processes, as Salzmänn *et al.* (2004) have demonstrated a leptin-mediated suppression of the corticosterone response to an ACTH challenge in maternally separated pups. Thus, a marked decrease of circulating leptin levels, as observed during maternal absence, could increase the adrenal sensitivity to ACTH, thereby resulting in an increased corticosterone secretion. Alternatively, a direct modulation of the adrenal sensitivity by the sympathetic nervous system has been demonstrated in adult animals and could also contribute to the observed phenotype in neonates (Raff *et al.* 2004).

The findings presented in this paper also raise the question of the involvement of NPY in transmitting persistent effects of early traumatic experiences to adulthood. The hypothalamic NPY system undergoes highly dynamic changes during postnatal ontogeny in rats, which makes this period potentially highly critical for metabolic disruptions such as fasting (Grove *et al.* 2003). These findings concur with data from Enthoven (personal communication), where already a second separation period of 8 h results in a greatly decreased corticosterone response, while there is no adaptation in the peripheral metabolic response. Thus, already one period of 8 h maternal separation might induce adaptive changes in the hypothalamic arcuate nucleus — PVN system, which might be long lasting and could contribute to the later involvement of NPY in disorders with metabolic phenomena (Weber-Hamann *et al.* 2002, Kishi & Elmquist 2005, Mathe *et al.* 2007).

In summary, we could demonstrate that the initial HPA axis response to maternal separation is critically dependent on the

NPY system. The lack of NPY or a pharmacological intervention in this pathway attenuates the corticosterone response to maternal separation and prevents separation-induced changes in gene expression. Thus, as in the adult animal the stress system of the neonate is regulated by metabolic signals via a NPY-mediated pathway.

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