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Adaptive Changes in Basal and Stress-Induced HPA Activity in Lactating and Post-Lactating Female Rats

Abbreviated Title: Adaptive changes in HPA activity in lactation

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Précis: Stress-induced HPA responses are suppressed in lactation but exaggerated after weaning when diurnal release is attenuated and phase-shifted. Altered glucocorticoid feedback may underlie these changes.

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Abstract
Lactation represents a period of marked adaptation of the HPA axis. We characterised basal and stress-induced HPA activity during lactation and experimental weaning using dynamic blood sampling in rats. Pulsatile and diurnal corticosterone release occurred at all reproductive stages studied (virgin; day 10 of lactation; 3 and 14 days after experimental weaning on day 10 of lactation). However, in lactating rats the diurnal peak was significantly reduced, resulting in a flattened rhythm, and three days after weaning basal HPA activity was markedly suppressed: the number of pulses and underlying basal levels of corticosterone were reduced and the diurnal rise phase delayed. Marked changes in the HPA response to 10 min noise stress also occurred at these times: being completely absent in lactating animals, but restored and highly prolonged in early weaned animals. Injection of methylprednisolone (2 mg, iv) was used to determine whether changes in fast glucocorticoid suppression correlated with these adaptive changes. Methylprednisolone induced a rapid suppression of corticosterone in virgin animals, but this effect was markedly attenuated in lactating and early weaned animals and was accompanied by significant changes in relative expression of hippocampal glucocorticoid and mineralocorticoid receptor mRNA. All effects were reversed or partially reversed 14 days after experimental weaning. Thus, the presence of the pups has an important influence on regulation of the HPA axis, and whilst post-partum adaptations are reversible, acute weaning evokes marked reorganisation of basal and stress-induced HPA activity.
The hypothalamo-pituitary-adrenal (HPA) axis regulates the level of circulating corticosteroids which, in turn, play a key role in metabolic homeostasis. The HPA axis has particular significance during pregnancy and lactation when the metabolic demands placed on the mother are increased, and specific adaptations of both basal and stress-induced HPA activity occur across the reproductive cycle to fulfil the needs of the offspring (1-4). Studies in rats have shown that lactation is associated with a flattening of the diurnal rhythm of secretion (5,6), such that there is a rise in the nadir levels of corticosterone (e.g.7,8) and a decrease in the peak evening levels. This change in HPA activity may serve several roles in the lactating rat. Firstly, it may provide a more constant level of glucocorticoids required to cope with the increased metabolic demands, such as those associated with galactopoiesis. Secondly, since glucocorticoids can freely enter the maternal milk and influence the offspring, the stabilization of levels may prevent neonatal exposure to varying glucocorticoid levels which are known to have long term programming effects (9,10).

Frequent automated sampling of plasma corticosterone levels has demonstrated that HPA activity comprises a pulsatile (ultradian) pattern, with pulses of secretory activity occurring approximately once every 60-90 min, the changing amplitude of which determines the circadian rhythm (e.g.11,12). In both rats and man this pattern of activity has been shown to alter with pathophysiological demand. For example, we have shown changes in amplitude and/or frequency of pulses in response to chronic stress of adjuvant-induced arthritis (13) and to early life exposure to infective agents (14). In the present studies we investigated whether changes in pulsatile activity contribute to alterations in basal corticosterone levels both during lactation and in the period following removal of the suckling litter.

In addition to changes in basal HPA activity, a remarkable and consistent finding is that the HPA response to stress is markedly attenuated during lactation (1-4,15). Early studies by Levine and colleagues showed that responses to footshock and ether stress were reduced in lactating rats (16,17), and subsequent studies have shown attenuated responses to a wide variety of stresses. These include both psychological stresses, such as noise stress (18), conditioned footshock (19), forced swimming (20-22), and restraint (23,24); and physical stresses, such as intraperitoneal injection of NaCl (25) or lipopolysaccharide (26), or exposure to ether vapour (23). Whilst this stress hyporesponsiveness is
consistent in rodents, the situation is less clear in primates (15). Free-ranging lactating macaque monkeys show a similar response to the stress of capture as non-lactating animals (27), while in women lactation-related stress hyporesponsiveness is seen in response to physical exercise (28) but not to breathing 35% CO₂ (29). Furthermore, whether there is an attenuated response to the Trier Social Stress Test appears to depend on parity (30,31) or whether the mother has recently breastfed (32).

In rats, stress hyporesponsiveness has its onset sometime towards the end of gestation (24,33,34), possibly coinciding with changing levels of ovarian steroids (35). Indeed, steroid treatment of ovariectomized virgin rats to simulate the changes in progesterone and estrogen following luteolysis produces a similar hyporesponsive state (36). While the onset of stress-hyporesponsiveness occurs within a defined time window in late gestation and is maintained by the presence of the pups, few studies have addressed the recovery of the stress response after experimental removal of the litter. It has been shown that the HPA response to ether remains suppressed 24h after removal of the litter (7) and that the accumulation of CRH mRNA in the PVN following intraperitoneal injection of hypertonic NaCl only becomes fully restored 48-72h after separation of the dam from the litter (25).

In the current study we characterised basal and stress-induced HPA activity at different stages of the post-partum period, particularly examining the change following experimental weaning. We also examined whether the adaptive changes relate in any way to corticosteroid receptor expression and/or fast inhibitory actions of exogenous corticosteroid administration (37, 38).

**Materials and Methods**

**Animals and cannulation**

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. Studies were performed on female Sprague-Dawley rats (Bantin and Kingman, Hull, UK) maintained on a 14h light: 10h dark illumination cycle (lights on at 05:00h). All animals were provided with wood chippings and a small amount of shredded paper for bedding. To obtain groups of lactating, weaned, and post-lactating animals, virgin rats were mated and then housed singly at the end of pregnancy in order that they could give birth in their home cage. Virgin animals remained group housed until the
time of surgery. Cannulation was performed five days prior to sampling as described previously (11), with a liquid swivel allowing access to all parts of the home cage. The timing of studies relative to lactation and weaning are given in Table 1.

**Study 1: Diurnal and stress-induced corticosterone secretion**

For collection of blood samples animals were attached to an automated blood sampling system (11,39) at 18:00h prior to commencing sampling. A simple flush cycle was initiated to keep the cannula patent. The collection of blood samples for the measurement of corticosterone concentrations commenced automatically at 06:00h on the day of study and continued every 10 min. At 08:00h on day 2 (26h after initiating sampling) a white noise generator was activated and rats exposed to 114 dB for 10 min. Sampling then continued for a further 120 min.

**Behavioral analysis**

To examine whether episodes of corticosterone secretion were associated with specific interactions with the litter, a 5h period of video recording was obtained from the lactating group between 06:00h and 11:00h during the 24h sampling period and was coded for behavior. This time period matched the observation period around noise stress on the subsequent day. The time the dam was on the nest and periods of feeding, grooming and exploratory rearing were coded, as were any milk ejection-related events. The latter comprised discrete events in which the litter displayed signs of coordinated stretching to obtain milk and/or the dam exhibited an accentuated arched back posture (kyphosis) at the same time as increased pup activity. Behavior was also examined during the period of noise stress in all groups. Each animal was video recorded for a period of 30 min, commencing 10 min before the onset of the noise. Behavior was coded every minute by recording: (i) total activity (time spent moving; maximum 60 s); and (ii) exploration (number of rearing events).

**Study 2: Fast glucocorticoid suppression**

To examine changes in fast glucocorticoid suppression of HPA, separate groups of animals were cannulated at times shown in Table 1 and acute injection of methylprednisolone performed as
described elsewhere (38). Five days after surgery, automated sampling was initiated at 15:00h at a frequency of once every 5 min. The initial 1 h of samples was discarded to avoid any non-specific release and measurements commenced at 16:00h. Sampling during late light phase was expected to be near the peak of the diurnal rhythm of corticosterone secretion (cf. Fig.1). Injections of methylprednisolone (2 mg methylprednisolone sodium succinate; Solu-Medrone, Pharmacia and Upjohn Ltd., Milton Keynes, UK) or 0.9% NaCl (a group of virgin animals only) were performed at 17:00h by briefly disconnecting the iv cannula from the swivel and injecting through the sampling cannula. This procedure was completed between two samples and caused minimal disturbance to the animals. Sampling continued for a further 60 min after injection.

**Corticosterone measurements and analysis**

Total plasma corticosterone concentrations were measured directly in plasma by radioimmunoassay using a citrate buffer at pH 3.0 to denature the binding globulin (4 µl of diluted plasma fraction diluted in 100 µl buffer), antiserum kindly supplied by Professor G. Makara (Institute of Experimental Medicine, Budapest, Hungary) and [¹²⁵I]-corticosterone (ICN, High Wycombe, UK) with a specific activity of 2-3 mCi/µg. The assay had a limit of detection of 5±1 ng/ml (n = 20) and cross-reactivity of the assay with methylprednisolone was less than 0.4% (38).

**Study 3: Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) gene expression**

Parallel groups of non-cannulated animals were killed by decapitation between 08:00-10:00h on the comparable days to those shown for noise stress in Table 1. The brains were rapidly dissected, frozen on dry ice and stored at -80°C prior to analysis of gene expression. Cryostat sections (12 µm) were cut to include the dorsal hippocampus and hypothalamic paraventricular nucleus (PVN). Three sections separated by 50 µm were collected for each animal. MR and GR mRNA expression levels were analysed using antisense ³⁵S-labelled riboprobes. The probes were kindly donated by J. Seckl (University of Edinburgh) and the method used was as previously described (40). All sections for a given probe were hybridized in the same reaction and exposed to autoradiographic film together with a series of ³⁵S standards for 6 days. The films were subject to densitometric analysis using public access
software ‘Image’ (http://rsb.info.nih.gov/nih-image). The integrated optical density (area detected above threshold x mean optical density within this area) was measured for each area of the hippocampus and the PVN; the mean value for a given animal being determined from all sections analyzed. Reproductive status was unknown to those performing the in situ analysis.

**Data analysis**

The Pulsar programme (41) was used to analyse number of pulses, pulse amplitude, pulse length, and mean baseline (i.e. the mean value of samples which were not considered to contribute to a pulse) as previously described (11). For determination of diurnal variations in pulse characteristics the 24h cycle was divided into four 6h periods commencing at 06:00h and analysed separately for each animal. ANOVA and post-hoc Tukey’s test were used to compare endocrine and behavioral data between the different experimental groups. Hourly diurnal hormone levels were calculated from the mean of samples falling within that time period. The data for mRNA were expressed as the percentage difference from the virgin group. ANOVA and post hoc Fischer’s test were used to compare all values (hormone levels, pulse characteristics, behavioral measures and gene expression) between the experimental groups.

**Results**

**Study 1: Ultradian and diurnal rhythms of corticosterone**

A significant diurnal rhythm of corticosterone levels was detected across all groups (P<0.001, F=9.560, 23, 541) with highest values during the period spanning the end of the light phase and the beginning of the dark phase (Fig.1). This rhythm was produced from an underlying variation in baseline secretion and from an ultradian pattern of corticosterone release, with hormone release occurring as a series of pulses which could be detected both during lactation and after experimental weaning (Fig.2). Overall analysis of the characteristics of this pulsatile corticosterone release indicated that the diurnal pattern was principally derived from changes in the number of pulses at different times of the day (Fig.3A; P<0.001, F=12.224, 3, 92) and from a change in the underlying baseline hormone
levels (Fig.3C; P<0.001, F=20.15, 3, 92), but not from differences in either the amplitude (Fig.3B) or length of pulses (data not shown).

Comparison between the groups showed significant differences in the diurnal patterns of corticosterone release (Fig.1; P<0.01, F=45.88, 3, 541). Within the virgin group, post hoc comparisons showed that hourly average corticosterone varied significantly over the 24h period: levels rose between 11:00h to a peak at 16:00h before falling again over the remainder of the 24h period (Fig.1A), with the greatest decline occurring between 22:00h and 03:00h. Analysis of the number of pulses in the four 6h time periods (Fig.3A) showed a significantly greater number were detected during both 12:00-18:00h and 18:00-24:00h than 24:00-06:00h or 06:00-12:00h (P<0.001, F=2.95, 3, 28). Similarly the baseline levels of corticosterone varied significantly over the four 6-hour time blocks, being highest at 18:00-24:00h and lowest at 06:00-12:00h (P<0.02, F=4.04, 3, 28). Although pulse amplitude also showed some tendency to vary across the day (Fig.3B) this did not reach statistical significance, even using one-way analysis of variance within this group (P=0.12, F=2.08, 3, 28).

Whilst a significant diurnal variation in corticosterone release was also seen during lactation, this was associated with a flattening of the rhythm (Fig.1B). Although early morning values were comparable with those seen in the virgin group and a rise in corticosterone levels commenced in parallel in the two groups (Fig.1B), the peak values at 16.00 h were significantly lower in lactating rats (P<0.05). This flattened rhythm was also reflected in the underlying pulse characteristics: in contrast to the virgin group, the number of detected pulses did not vary significantly over the 24h cycle (Fig.3A). However, the baseline hormone release continued to show a diurnal variation, being significantly greater at 18:00-24:00h than at any of the other time periods measured (Fig.3C) (P<0.001, F=12.20, 3, 23). The ultradian pattern of hormone release seen in the lactating group did not appear to be related to any one aspect of specific maternal activity (Fig.4): there being no clear association between any of the measured behaviors (time on the nest, milk ejection activity, feeding, or exploration (rearing)) and the timing or amplitude of pulses.

Very marked changes in corticosterone release were seen after experimental weaning. Post hoc tests revealed that corticosterone levels were significantly suppressed throughout the 24h period in these animals when compared with any of the other groups (Fig.1C). Although these animals did
show a pulsatile pattern of hormone release (Fig.2C), \textit{post hoc} tests showed that overall the number of pulses was significantly lower than any other group (Fig.3A), pulse amplitude was significantly lower compared with the virgin controls (Fig.3B), and baseline levels were significantly lower than those seen in both the lactating and post-lactating groups (Fig.3C). Despite these overall lower levels, analysis within this group revealed the presence of significant diurnal variations in the number of pulses (Fig.3A; $P<0.01$, $F=5.08$, 3, 27) and baseline hormone levels (Fig.3C; $P<0.01$, $F=5.49$, 3, 27), suggesting that the diurnal drive to pulsatility was quickly re-established following removal of the suckling litter. However, there was a marked delay in the onset of the diurnal rise in corticosterone levels: in the virgin group this began between 11:00-12:00h while in the weaned animals the rise was not evident until 15:00-16:00h (Fig.1C).

By 2 weeks following removal of the litter, the diurnal and ultradian rhythms of corticosterone were comparable with those seen in the virgin controls in terms of mean hormone levels (Fig.1D) and pulse characteristics (Fig.2D; Fig.3). However, \textit{post hoc} tests on the diurnal profile of hormone release did show a significant difference between the two groups, mainly due to the continued presence of a phase-shift in the diurnal rise which occurred between 13:00-14:00h, earlier than in the weaned animals but later than in the virgin controls (Fig.1).

\textbf{Study 1: Stress-induced HPA activity and behavior}

In virgin animals noise stress caused a rapid and significant increase in plasma corticosterone concentrations (Fig.5A; $P<0.001$, $F=17.665$, 17, 161): levels were significantly elevated above all basal measurements within 10 min of the onset of the stress, reached a peak of $177\pm21$ ng/ml after 20 min, before rapidly returning to baseline (Fig.5A). In contrast, no significant change in plasma corticosterone levels was seen in the lactating group following the noise stress (Fig.5B). In the experimentally weaned group the initial response to noise was similar to the virgin group – overall a significant elevation of plasma corticosterone levels was seen ($P<0.01$ $F=3.824$, 17, 155), being significantly raised within 10 min of stress onset and peaking at 20 min with a value of $160\pm32$ ng/ml. However, in this group levels remained significantly elevated above all basal measurements for a further 60 min and only slowly declined towards baseline (Fig.5C). In contrast, the response to noise
stress in the post-lactating group had returned to a pattern that was indistinguishable from that seen in the virgin controls (Fig.5D).

Noise stress also evoked behavioral responses from the animals. Overall, there was a significant increase in total activity levels in response to the noise (Fig.6A-D; P<0.005, F=6.288, 3, 68), and this response did not differ significantly between the different stages of lactation or after weaning. Most groups displayed an immediate increase in activity following onset of the noise, which was maintained in the 10 min period after the noise (Fig.6A,C,D). However, even though not statistically different from the other groups, the pattern in the lactating group (Fig 6B) had a different profile - the level of activity in the pre-noise period tended to be greater than the other groups, so the increase evoked by noise was smaller, and in the post-noise period activity levels fell as the dams directed their attention to the pups. Analysis of stress-induced exploratory behavior, showed an immediate and significant increase in the number of rearing events in all groups when compared with the control period (Fig.6E-H; P<0.01, F=13.848, 3, 68) which, after the end of the noise, declined to low levels in most groups or to complete cessation of rearing in the lactating group (Fig.6F). Noise-induced rearing did not vary significantly with the stage of lactation, indicating that lactating animals were able to react to the stimulus despite the lack of an HPA response.

**Study 2: Fast glucocorticoid suppression**

When sampled during the late light phase, iv injection of methylprednisolone caused a rapid decrease in basal plasma corticosterone levels, becoming significantly lower than the average pre-injection level by 40 min after treatment and continuing to be suppressed throughout the remaining 2h sampling period (Fig.7A – closed symbols). The time taken for corticosterone levels to fall to half of pre-injection levels (t½) was calculated at 9.5±4.0 min. Corticosterone levels were not significantly affected by injection of saline vehicle (Fig.7A – open symbols). The response to methylprednisolone was markedly attenuated in the lactating group, with no significant decline below pre-injection levels during the 120 min of sampling (Fig.7B). Animals which had been weaned from their litter showed a similar pattern of response to lactating animals: i.e. no significant suppression. However, by 2 weeks after removing the litter the suppressive effect of methylprednisolone was again evident (Fig.7D),
although a significant effect was not detected until 60 min after injection and the t½ was slower (17±4 min), suggesting that the loss of inhibition seen in the lactating and weaned groups had not been entirely reversed.

**Study 3: MR and GR mRNA expression**

Animals in all groups displayed high levels of MR mRNA in all sub-fields of the dorsal hippocampus but in all areas expression varied significantly between the different stages of lactation and after weaning (CA1, P<0.007; CA2, P<0.001; CA3, P<0.001; dentate gyrus (DG), P<0.001). *Post hoc* tests showed that MR levels were significantly reduced during lactation and remained lower in the post-lactating animals (Fig.8A,B). GR mRNA expression was detected in the CA1 and DG (but not in CA2 and CA3) and in both of these subfields expression also varied significantly with the phase of lactation and weaning (CA1 P<0.011; DG P<0.012). However, in both sub-fields expression was highest in the experimentally weaned group, although this difference only reached significance in the DG where levels were significantly higher than in the post-lactating group (Fig.8C,D). When the relative ratio of MR:GR was calculated (Figure 8E,F), a significant variation was seen over the stages of lactation and weaning within the DG (P<0.003), being significantly lower in both the lactating and early weaned groups compared to virgin animals. A similar variation in CA1 did not reach significance. GR mRNA levels in the PVN did not vary significantly across any groups (P=0.34, data not shown).

**Discussion**

These data demonstrate the marked changes in HPA axis activity during the course of lactation and following experimental weaning, and build on work demonstrating that the dynamic patterns of HPA activity (diurnal, pulsatile, and stress-induced) show reversible changes in response to physiological stimuli which may have adaptive significance. Lactating animals displayed a flattening of the diurnal pattern of basal corticosterone secretion and marked suppression of stress reactivity, which results in more stable (unvarying) corticosteroid levels that may be important for maintaining metabolic activity and minimizing the programming effects on the suckling young. Whilst these adaptive changes were largely reversed within 2 weeks of removal of the young, in the early weaning period HPA axis
activity showed a marked suppression of basal corticosterone secretion and the re-emergence of stress responsiveness which was characterised by a highly prolonged pattern of activation. These adaptive changes may, in part, arise from a change in the relative expression of central corticosteroid receptor mRNA and the attenuation of fast glucocorticoid feedback.

**Changes in pulsatile and diurnal HPA activity**

There has been considerable interest in the regulation of the HPA axis during the reproductive cycle, both because of the importance of corticosteroids in programming of the neonatal nervous system (9,10) and because of the remarkable attenuation of stress-induced HPA activation (1-4,18-26). The present data extend earlier observations that lactation is associated with a flattening of the diurnal rhythm of basal corticosterone secretion. Previously single point sampling has shown a rise in the nadir levels of corticosterone (e.g.7,8) and/or a decrease in the peak evening levels (5,6). The current detailed characterisation of the underlying dynamics of pulsatile secretion show that this flattening is not due to a loss of the underlying pulsatile pattern, but rather the number of detected pulses fails to show significant variation across the day, as it does in virgin animals. One reason for this might be that during lactation the drive to the HPA axis may have a greater dependency on the persistent sensory cues from the litter, and this may override the drive from diurnal inputs. Studies using a variety of paradigms in which the pups are separated and re-united with the dams have shown that the onset of suckling is stimulatory to HPA activity (e.g.5), a response that is contingent upon pup generated cues (43). However, large increases in pup-induced HPA activity do not occur naturally when the mother regulates the frequency and duration of the suckling bouts. Consistent with this we found that neither milk-ejection events nor the onset of individual suckling episodes show any direct relationship with the individual pulses of corticosterone secretion. Nevertheless, the presence of the litter is essential for maintaining HPA activity, as shown by the changes following experimental weaning.

The dependence of basal HPA activity on the presence of the litter is demonstrated by the fact that ACTH and corticosterone levels begin to decrease within 3.5h of removal of the suckling litter (5), and it has been assumed that basal HPA function reverts to normal within a rapid time frame (24-
48h) after weaning (5,8,15). However, the present data show that during this early post-lactating period basal corticosterone secretion is far from normal. Whilst a clear diurnal pattern of secretion was restored within 3 days of weaning, the overall level of corticosterone is markedly suppressed in the absence of the litter. Interestingly, the greatest effect occurs during the late light phase leading to a marked phase delay in the diurnal rise in corticosterone (cf. Fig. 1A,C) and a residual effect is still seen during this time period 2 weeks after weaning (Fig. 1D). Whilst the underlying mechanisms of these adaptive changes in afferent input remain to be determined, it is likely that these changes in diurnal patterns of HPA activity are a reflection of the modulation of circadian systems that occur during lactation (44) and could involve modulation of the circadian generator in the suprachiasmatic nucleus. Furthermore, a potential contribution may come from alteration of the noradrenergic drive which underlies diurnal HPA activity, since there has been shown to be both a reduction of noradrenergic input, that appears in late gestation (45) and persists through lactation (46), and a reduction in HPA sensitivity to alpha-1 adrenergic activation in the lactating rat (47).

**Adaptive changes in stress responsiveness**

It has been consistently observed that, in rodents, HPA responses to stress are highly attenuated during lactation (1-4,18-26), although this hyporesponsiveness may be reversed for certain stressors which represent a threat to the offspring (48). The present data support our previous report (18) that the corticosterone response to noise stress is completely abolished in lactation. Loud white noise is one of the few stresses that has been applied in the presence of the suckling litter (thereby overcoming the possible confound of acute withdrawal of pups) and the preservation of normal behavioral reactivity (i.e. stress-induced exploration) suggests that the HPA hyporesponsiveness is not the result of insensitivity to the sensory stimulus. Nevertheless, measures of stress-induced immediate-early gene (24,26,49,50) or CRH (25) mRNA expression in the PVN have indicated that the strength of afferent stimulation of the HPA axis is attenuated during lactation. Indeed, since stress-induced release of oxytocin (51,52) and prolactin (53) are also markedly reduced in lactation, it is likely that attenuation of a common stress network underlies the hyporesponsive state. Furthermore, since the present data
show that basal pulsatile corticosterone secretion is largely unaffected, it is unlikely that stress hyperresponsiveness arises from downstream blockade of the HPA axis.

It has been assumed that the lactation-related attenuation of neuroendocrine and behavioral responses to stress can rapidly revert to normal after removal of the pups (15). However, very few studies have directly examined recovery of neuroendocrine responses to stress after weaning (25,54,55). The current data show that, whilst restoration to a pre-pregnancy state does occur after 14 days, there are marked changes arising from acute withdrawal of the litter. Particularly notable is the marked dissociation between basal and stress-induced HPA activity, in that while basal corticosterone levels are highly suppressed the response to the relatively brief stimulus of 10 min noise stress involves highly prolonged HPA activation (significant elevation for >80 min). We have previously observed this persistent activation using a paradigm in which animals were exposed to two periods of noise stress (one on day 7-10 of lactation and the other 3 days later after removing the pups) (18). The present data using animals exposed to only a single noise stress show that this highly prolonged response is not due to any sensitization from a previous stress. This pattern of response is particularly notable as the sampling conditions and noise stress protocol have been applied to wide variety of pathophysiological states that modify HPA responses to stress (e.g. adjuvant-induced arthritis (13), chronic hypothyroidism (56), neonatal programing (14), intra-uterine growth retardation (57), gonadectomy (12)), but this is the only instance of such prolonged responses, suggesting that a unique set of factors underlie this response. Even when the noise stimulus is extended to 30 minutes the corticosterone levels does not persist and start to decline as soon as the noise stimulus ends (e.g.38). Whilst others have reported a slightly prolonged autonomic responses to stress after experimental weaning (42), this apparent HPA hyper-responsive state was not associated with a greater behavioral response (either number of rearings or overall activity) compared to virgin or post-lactating groups, suggesting that this may be directly related to the mechanisms terminating HPA axis activation. Of the various mechanisms suggested to be responsible for modulating the termination of stress-induced HPA activity the most widely cited is corticosteroid negative feedback.

Corticosteroid feedback
Variation in corticosteroid negative feedback is one mechanism that may underlie adaptive changes in HPA activity. It has been reported previously that corticosteroid negative feedback is intact (5,7,25) or even increased (54) in lactating rats. The parallel time course and magnitude of CRH mRNA accumulation following adrenalectomy of virgin and lactating rats (25), and the levels of ACTH achieved after adrenalectomy and constant corticosterone replacement (5), have been cited as evidence that the sensitivity to negative feedback is not significantly altered. However, the clamping or removal of corticosterone levels used in these studies may itself lead to a normalisation of feedback mechanisms which might be otherwise modulated during lactation. In contrast to the reports in rats, studies in humans using exogenous glucocorticoid administration suggest that negative feedback may be reduced over the reproductive cycle. Both during pregnancy (58) and lactation (59) there is a reduction in the ability of dexamethasone (a GR agonist) to suppress circulating levels of cortisol. When tested during the first two weeks post-partum, plasma cortisol levels 17h after a single oral dose of dexamethasone were found to be significantly higher than those measured in non-pregnant women (59).

We examined the rapid component of negative feedback using a protocol of injecting methylprednisolone that has been shown to cause suppression of basal HPA activity and blunting of response to noise stress (37,38). Using this approach we found that during lactation and the early post-lactating period there was a significant attenuation of this rapid HPA suppression by methylprednisolone. Importantly, this effect was reversed by 2 weeks post-lactation when HPA function is largely restored. Our characterisation of the effect of methylprednisolone has shown a component of the response is due to a central (suprapituitary) site of action (38). We considered whether this attenuation of negative feedback might arise from changes in corticosteroid receptor expression, since Meaney and colleagues (60) had reported that the level of binding of [\(^3\)H]dexamethasone in the soluble fraction prepared from the hippocampus was reduced in lactation. Although it will act on both GR and MR, methylprednisolone has a preferential selectivity for GR and recent characterisation of the rapid feedback effect suggests the involvement of both GR (38) and MR (37). Our data showed that there was no significant fall in GR mRNA in lactation while there was small increase after experimental weaning, an effect that may arise from the fact that hippocampal GR
mRNA is under negative regulation by circulating corticosteroids (e.g.61,62) and might, therefore, be triggered by the dramatic decline in circulating corticosterone. In contrast, compared to virgin animals, MR mRNA levels were reduced in the lactating and post-lactating groups, and the relative expression of the two receptor mRNAs showed a decline in MR:GR ratio in the lactating and weaned animals, particularly in the dentate gyrus. Interestingly, a similar change in relative expression of MR:GR mRNA has been reported in the DG of late pregnant (day 21) rats, although this arises from a small decline in MR mRNA and a significant increase in GR mRNA (34). This change in corticosterone receptor expression in pregnancy was also shown to be associated with a slowing of the rapid negative feedback of ACTH after chemical adrenalectomy. Although the relationship between corticosteroid receptor mRNA expression and rapid negative feedback is not yet established, it is tempting to speculate that this relative change in hippocampal corticosteroid receptor expression may underlie the attenuation of fast negative feedback observed during lactation and weaning, and indeed may explain the slower turn off of the corticosterone response to noise stress in the weaned animals.

In conclusion, we have shown that lactation and experimental weaning are associated with major changes in the dynamic regulation of HPA activity. Despite assumptions of a rapid restoration of normal patterns of regulation after weaning, our data show that in the early period following withdrawal of the litter both basal and stress-induced HPA activity show marked adaptation of regulatory mechanisms with a dissociation between these modes of secretion (i.e. basal hypoactivity and stress hyperactivity). It is proposed that the loss of stimulatory drive from the suckling stimulus combined with the persistent attenuation of rapid negative feedback may be important factors underlying this dynamic process of reorganisation.
REFERENCES


5. Walker C-D, Lightman SL, Steele MK, Dallman MF 1992 Suckling is a persistent stimulus to the adrenocortical system of the rat. Endocrinology 130:115-125


23. **Banky Z, Nagy GM, Halasz B** 1994 Analysis of pituitary prolactin and adrenocortical response to ether, formalin or restraint in lactating rats: rise in corticosterone, but no increase in plasma prolactin levels after exposure to stress. Neuroendocrinology 59:63-71


29. **Kaye J, Soothill P, Hunt M, Lightman S** 2004 Responses to the 35% CO challenge in postpartum women, Clin Endocrinol (Oxf) 61:582-588


42. Mezzacappa ES, Tu AY, Myers MM 2003 Lactation and weaning effects on physiological and behavioral response to stressors. Physiol Behav 78:1-9


46. **Toufexis DJ, Thrivikraman KV, Plotsky PM, Morilak DA, Huang N, Walker CD** 1998 Reduced noradrenergic tone to the hypothalamic paraventricular nucleus contributes to the stress hyporesponsiveness of lactation. J Neuroendocrinol 10:417-427


54. **Schlein PA, Zarrow MX, Denenberg VH** 1974 The role of prolactin in the depressed or ‘buffered’ adrenocorticosteroid response of the rat. J Endocrinol 62:93-99

55. **Banky Z, Nagy GM, Halasz B** 1994 Analysis of pituitary prolactin and adrenocortical response to ether, formalin or restraint in lactating rats: Rise in corticosterone, but no increase in plasma prolactin levels after exposure to stress. Neuroendocrinology 59:63-71


62. Chao HM, Ma LY, McEwen BS, Sakai RR 1998 Regulation of glucocorticoid receptor and mineralocorticoid receptor messenger ribonucleic acids by selective agonists in the rat hippocampus. Endocrinology 139:1810-1814
FIGURE LEGENDS

**Figure 1**
Diurnal rhythms of corticosterone secretion in groups of virgin rats (A), rats on day 9 of lactation (B), weaned dams 2 days after pup-removal (C) or post-lactating dams 13 days after pup-removal following 10 days of lactation (D). Values are mean ± SE of 6-11 animals per group and have been derived by combining successive groups of six samples to obtain an average value for every hour. In panels B-D the mean values from the virgin group have been displayed for comparison (gray line). The shaded area indicates the dark phase of the illumination cycle.

**Figure 2**
Examples of ultradian patterns of corticosterone secretion in a virgin rat (A), a rat on day 9 of lactation (B), a weaned dam 2 days after pup-removal (C), and a post-lactating dams 13 days after removing the litter following 10 days of lactation (D). The shaded area indicates the dark phase of the illumination cycle.

**Figure 3**
The characteristics of the pulsatile pattern of corticosterone release seen in virgin rats, rats on day 9 of lactation, weaned dams 2 days after pup-removal, or post-lactating dams 13 days after removing the litter following 10 days of lactation. The data are derived from Pulsar analysis following division of the 24 h cycle into four 6 h blocks; from 06:00-12:00 h (open bars), 12:00-18:00 h (light gray bars), 18:00-24:00 h (black bars) and 24:00-06:00 h (dark gray bars). The dark phase of the daily cycle ran from 19:00-05:00 h. The data shown are the mean ± SE of 6-11 animals per group for the number of pulses detected (A), mean pulse amplitude (B), and average baseline corticosterone secretion after exclusion of pulsatile release (C). See text for statistical comparisons.
Figure 4
Corticosterone levels (continuous line) and behavioral measures from two lactating rats on day 9 of lactation obtained between 06:00 and 11:00. Video tapes were examined for periods spent feeding (F), self-directed grooming (G), and rearing to explore the cage (*), as well as for milk ejection-related activity (arrows). Black bars indicate the time that the dam was present on the nest.

Figure 5
Corticosterone response to the psychological stress of white noise (110 dB x 10 min; shaded bar) in virgin rats (A), rats on day 10 of lactation (B), weaned dams 3 days after pup-removal (C), or post-lactating dams 14 days after pup-removal following 10 days of lactation (D). Values are mean ± SE of 6-11 animals per group. *P<0.05 compared to all corticosterone values prior to initiation of the noise.

Figure 6
The effect of noise stress of total activity levels (panels A-D) and rearing behavior (panels E-H) in virgin rats (A,E), rats on day 10 of lactation (B,F), weaned dams 3 days after pup-removal (C,G) or post-lactating dams 14 days after pup-removal following 10 days of lactation (D,H). The values are the mean + SE of the behavior during 1 min time periods, commencing 10 min prior to the period of white noise (110 dB) for 10 min, and continuing for 10 min after cessation of the stimulus. Dashed lines mark the start and end of the noise stress. The shaded blocks in panels A-D show the mean activity levels during the three 10 min periods.

Figure 7
HPA response to acute injection of methylprednisolone (2 mg, i.v.). A. Responses to saline (open symbols; n = 8) or methylprednisolone (solid symbols; n = 6) in virgin animals. B, C, D. Comparison of the responses on day 9 of lactation (B, n = 6), and post-lactating rats on day 2 (C, n = 6) and day 13 (D, n = 5) after removing the litter following 10 days of lactation. In order to compare response profiles and compensate for group differences in basal corticosterone levels, the hormone values have been normalised within each animal to the mean value over the 120 mins prior to injection. In each
panel the normalised baseline value of 1 is indicated as a horizontal line and the timing of the injection as the vertical dashed line. * indicates the period over which all data points in the methylprednisolone groups are significantly below pre-injection baseline (P<0.05).

**Figure 8**

Histograms showing the expression of MR (A,B) and GR (C,D) mRNA and their relative ratios (E,F) in the CA1 sub-field (A,C,E) and dentate gyrus (B,D,F) of the dorsal hippocampus of virgin rats (open columns), rats on day 10 of lactation (black columns), and post-lactating rats either 3 days (dark gray columns) or 14 days (light gray columns) after removing the litter following 10 days of lactation. Note that mRNA values are expressed in arbitrary units relative to the mean expression level in the virgin group. Values are mean + SE of 6-11 animals per group. *p<0.05 vs. virgin group; † P<0.05 vs. post-lactating group; +p<0.05 vs. control group.
Table 1. Timing of various procedures in relation to lactation and weaning.

<table>
<thead>
<tr>
<th>Group</th>
<th>Surgery</th>
<th>Pups removed (08:00 h)</th>
<th>Study 1: Start of 24 h sampling (06:00 h)</th>
<th>Study 1: Noise Stress (08:00 h)</th>
<th>Study 2: Glucocorticoid feedback (15:00 h)</th>
<th>Study 3: Tissue collection (08:00-10:00 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td>Any estrous stage</td>
<td>n/a</td>
<td>Day 5 after surgery</td>
<td>Day 6 after surgery</td>
<td>Day 5 after surgery</td>
<td>Day 6 after surgery</td>
</tr>
<tr>
<td>Lactating</td>
<td>Day 4 of lactation</td>
<td>n/a</td>
<td>Day 9 of lactation</td>
<td>Day 10 of lactation</td>
<td>Day 9 of lactation</td>
<td>Day 10 of lactation</td>
</tr>
<tr>
<td>Weaned</td>
<td>Day 7 of lactation</td>
<td>Day 10 of lactation</td>
<td>Day 2 after weaning</td>
<td>Day 3 after weaning</td>
<td>Day 2 after weaning</td>
<td>Day 3 after weaning</td>
</tr>
<tr>
<td>Post-lactation</td>
<td>Day 8 after weaning</td>
<td>Day 10 of lactation</td>
<td>Day 13 after weaning</td>
<td>Day 14 after weaning</td>
<td>Day 13 after weaning</td>
<td>Day 14 after weaning</td>
</tr>
</tbody>
</table>
Figure 1

A. Virgin

B. Lactating

C. Weaned

D. Post-lactation

Plasma corticosterone (ng/ml)

Time of day (h)
Figure 2

A. Virgin

B. Lactating

C. Weaned

D. Post-lactation

Plasma corticosterone (ng/ml) vs. Time of day (h)