Universidade do Minho
Escola de Ciências da Saúde

Alexandre Vladimirov Patchev

Programming of stress-related behavioral and neuroendocrine functions during early life

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Tese de Doutoramento em Medicina

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Abstract

Throughout ontogeny the mammalian brain shows windows of time during which there is an increased sensitivity towards the programming effects of external and endogenous signals. The general assumption is that such phases of increased sensitivity correspond to dynamic phases of brain development and maturation. Programming of neuronal pathways and functions takes place during phases of ontogeny which are characterized by high susceptibility to noxious stimuli and may comprise numerous mechanisms. With regard to the programming of neuroendocrine mechanisms of adaptation, the perinatal period in rodents offers the unique opportunity to examine the effect of stress and steroid hormones during a phase characterized by high vulnerability and underdeveloped (immature) capacity of neuroendocrine adaptation. Thus, inadequate signals, leading to disturbed homeostasis can result in mal-programming of brain functions, therefore leading to increased vulnerability to the development of disorders of the central nervous, but also peripheral systems. While both physiological and pathological programming have been associated with altered brain structure, gene expression and function, the mechanisms through which these effects are established and maintained throughout life are not well understood.

In the present work, we focused on the mechanisms of programming and mal-programming of the hypothalamus-pituitary-adrenal (HPA) axis and their structural, behavioral and neuroendocrine correlates. We have identified several mechanisms through which stress, glucocorticoids but also sex-steroids during early ontogeny can affect brain function in later life by altering HPA axis function. As dysregulation of HPA axis function has been associated with many disorders of the brain, such as depression and cognitive disorders, studying the factors contributing to the physiological and pathological programming of the HPA axis could contribute to a better understanding of the pathophysiology of CNS disorders.

Sumário

Ao longo da ontogenia o cérebro dos mamíferos apresenta janelas temporais nas quais há maior sensibilidade para os efeitos programadores de sinais externos e internos. A ideia generalizada é a de que estas fases de maior sensibilidade correspondem a fases mais dinâmicas do desenvolvimento e maturação cerebral. A programação das vias neuronais e das suas funções ocorre durante fases ontogénicas que se caracterizam por uma elevada sensibilidade a estímulos nóxicos e pode envolver diversos mecanismos. Com respeito aos efeitos programadores sobre os mecanismos neuroendócrinos de adaptação, o período perinatal nos roedores oferece uma oportunidade única para examinar os efeitos do stress e das hormonas esteroides durante uma fase caracterizada por uma elevada vulnerabilidade e imaturidade da adaptação neuroendócrina. Assim, sinais inadequados, induzem distúrbios homeostáticos que podem resultar em má programação das funções cerebrais, e consequentemente numa maior vulnerabilidade para o desenvolvimento de doenças do sistema nervoso central, mas também dos sistemas periféricos. Os efeitos programadores fisiológicos e patológicos têm sido associados com alterações da estrutura cerebral, mas os mecanismos pelos quais estes efeitos ocorrem e são mantidos ao longo da vida não são ainda bem conhecidos.

Neste trabalho, centramos a nossa atenção nos mecanismos programadores do eixo hipotalâmico-hipofisário-adrenal (HPA) e nos seus correlatos estruturais, comportamentais e neuroendócrinos. Identificamos vários mecanismos pelos quais a exposição ao stress, aos glucocorticóides mas também aos esteroides sexuais durante o processo ontogénico, podem afetar a função cerebral mais tarde na vida através da alteração da função do eixo HPA. Como a desregulação do eixo HPA tem sido associada a várias doenças cerebrais, tais como a depressão e perturbações cognitivas, o estudo dos fatores que contribuem para os efeitos programadores fisiológicos e patológicos do eixo HPA pode contribuir para um melhor entendimento da fisiopatologia das doenças do sistema nervoso central.

List of Abbreviations

11β-HSD	11β-Hydroxysteroid-Dehydrogenase
ACTH	Adrenocorticotrophic Hormone
AP1	Activating Protein 1
AVP	Arginine Vasopressin
AVPV	Anteroventral Periventricular Nucleus
BBB	Blood-Brain Barrier
BNST	Bed Nucleus of the Stria Terminalis
cAMP	cyclic Adenosine Monophosphate
CNS	Central Nervous System
CRH	Corticotrophin Releasing Hormone
DNA	Desoxyribonucleic Acid
ELS	Early-life stress
GABA	Gamma-Aminobutyric Acid
GC	Glucocorticoid(s)
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HPA Axis	Hypothalamus-Pituitary-Adrenal Axis
HPG Axis	Hypothalamus-Pituitary-Gonadal Axis
MR	Mineralocorticoid Receptor
mRNA	messenger Ribonucleic Acid
NPC	Neuronal Precursor Cell

PFC	Prefrontal Cortex
POMC	Proopiomelanocortin
PVN	Paraventricular Nucleus
ROS	Reactive Oxygen Species
SDN-POA	Sexually Dimorphic Nucleus of the Preoptic Area
SGZ	Subgranular Zone

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1. Introduction

The neuroendocrine regulation of hypothalamus-pituitary-adrenal axis function

The well-orchestrated secretion of glucocorticoids (GC) by the adrenal cortex represents one of the major adaptive responses in the mammalian organism. GC are steroid hormones, and as such derivatives of cholesterol. Their *de novo* production and secretion is induced in the fasciculate zone of the adrenal cortex upon stimulation through pituitary derived adrenocorticotrophic hormone (ACTH). There are two major ACTH secretagogues released by parvocellular neurons of the paraventricular hypothalamic nucleus (PVN) – corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) (Charmandari et al., 2005). Both peptide-hormones act on their respective receptors (CRH receptor 1, and Vasopressin receptor 1b) in corticotroph cells of the anterior pituitary lobe in a synergistic fashion to induce secretion of stored ACTH (Charmandari et al., 2005; Aguilera, 2011). In addition both hormones have been shown to induce transcription of the proopiomelanocortin (POMC) gene and the posttranslational processing of POMC to ACTH. The parvocellular division of the PVN, where CRH and AVP are co-expressed, receives regulatory inputs from many intra- and extrahypothalamic sites (Sawchenko et al., 2000; Aguilera, 2011; Cole and Sawchenko, 2002).

However the general assumption is that the regulation of CRH and AVP gene expression in the PVN and their secretion into the portal blood stream at the median eminence is governed by two major limbic structures: the hippocampal formation and the amygdala (Jankord and Herman, 2008). Both structures project to the bed nucleus of the stria terminalis (BNST) which plays the role of a functional relay integrating both signals (Jankord and Herman, 2008; Choi et al., 2007; Herman et al., 2002; Ventura-Silva et al., 2012). In general, the hippocampal formation exerts an inhibitory control on HPA axis function, while the amygdala stimulates adrenocortical secretions (Jankord and Herman, 2008). In the neural control of PVN activity, the BNST exerts the role of a functional relay which toggles the input of superimposed regulatory centers. Hippocampal glutamate and amygdaloid GABA-ergic pathways project onto the GABA-ergic efferent BNST neurons, which innervate the PVN (Choi et al., 2007; Herman et al., 2002; Jankord and Herman, 2008). The corresponding excitation and inhibition of BNST perikarya underlie the mechanisms of hippocampal suppression and amygdaloid stimulation of PVN activity.

GC regulate HPA axis activity through negative feedback loops which occur at all of the above described levels. A major role for autocrine regulation at the level of the adrenal cortex seems unlikely as glucocorticoid receptors (GR) are mostly (but not exclusively) expressed in the cells of the androgen-producing reticular zone (Paust et al., 2006; Gummow et al., 2006). GR expression has been shown in pituitary corticotroph cells, and recent studies suggest a role for the balance between pituitary and adrenocortical GR expression as one of the reasons for high inter-individual variability of HPA axis function in humans (Briassoulis et al., 2011). While pituitary GR can negatively affect POMC gene expression through a negative GR response element (GRE) (Drouin et al., 1989; Drouin et al., 1987), glucocorticoids – as small lipophilic molecules – readily pass the blood-brain barrier (BBB) and affect CRH and AVP expression in the PVN (Charmandari et al., 2005).

Numerous studies have suggested that GC regulate CRH gene expression, however, direct actions of GC at a GRE on the CRH promoter have so far only been shown *in vitro* in the AtT20 pituitary tumor cell line (Malkoski et al., 1997; Malkoski and Dorin, 1999). The current data suggest that GC signaling in CRH neurons in the PVN interacts with other (e.g. neurotransmitter) signaling cascades originating at the cell membrane (Kageyama et al., 2010; Aguilera and Liu, 2012; Kageyama and Suda, 2009). Similarly, while GC have been implicated in the direct regulation of AVP gene expression and mRNA stability (Volpi et al., 2004; Yoshida, 2008), the interaction with other signaling cascades seems crucial for GC negative feedback on AVP. Interestingly, to this date it is not clear to what extent the direct interaction of activated GR with CRH and AVP gene expression is involved in the regulation of the negative GC feedback (Yoshida, 2008). There are striking contrasts in the literature due to experimental models used (*in vitro* vs. *in vivo*, tumor cell lines vs. primary neuronal and organotypic slice cultures).

Studies in adrenalectomized and GC-supplemented rodents have suggested direct inhibitory effects of GC on CRH and AVP expression and secretion, as well as differential sensitivity of both genes to the inhibitory actions of GC (Albeck et al., 1994; Makino et al., 1995). However studies from our lab have shown, that dexamethasone, a highly potent and selective synthetic GR agonist, actually increases CRH secretion from cultured hypothalamic neurons, while at the same time AVP secretion is suppressed (Hellbach et al., 1998). In contrast, studies in the AtT20 cell line suggest a direct negative action of activated GR on CRH gene expression (Malkoski and Dorin, 1999). Experiments using organotypic slice cultures suggest that the activational state of

the parvocellular PVN neurons determines the extent (quality and quantity of) GC effects on CRH and AVP expression and secretion (Kuwahara et al., 2003; Arima et al., 2001; Aguilera and Liu, 2012).

Taken together there seems to be certain involvement of GR in parvocellular PVN neurons in the regulation of the negative GC feedback, however it is evident that neuronal signals from other GC-sensitive brain structures (i.e. the above mentioned limbic areas as well as brain stem monoaminergic neuronal populations) are the main regulators of CRH and AVP production and secretion from the PVN. The direct signals from brainstem catecholaminergic neuronal populations to the PVN are mostly driving HPA axis activity and represent an immediate response to physical stressors (such as pain, hemorrhage or metabolic challenges) (Pacák and Palkovits, 2001).

In contrast, the role of limbic structures in HPA axis activity regulation becomes manifest when psychological stressors are imposed. The perception of such psychological stressors is highly subjective and the quality and magnitude of the behavioral and endocrine response to these stressors is mostly dependent on previous experience and mnemonic processes (Jankord and Herman, 2008; Sousa and Almeida, 2012). Therefore it is not surprising that both, the hippocampal formation and the extended amygdala are implicated in learning and conditioning processes as well as the "mnemonic" and "emotional" control of HPA axis regulation (Jankord and Herman, 2008; Pacák and Palkovits, 2001).

The assumption of a major role of the hippocampal formation in regulating HPA axis function and GC negative feedback is derived from rodent studies, where subiculum lesions lead to a disinhibition of HPA axis activity (Herman and Mueller, 2006; Herman et al., 2003), however the anatomical route of the hippocampal output seems to determine its involvement in negative feedback regulation (Bradbury et al., 1993). Additionally the hippocampus is the one site in the brain which shows the highest expression of corticosteroid receptors (Reul and de Kloet, 1986) as well as the highest amount of binding of radio-labeled corticosteroids (De Kloet et al., 1975). The hippocampus therefore has been ascribed the role of the major site of GC negative feedback.

The effects of GC on hippocampal structure and function are numerous and highly dependent on the dose and duration of the GC and will be discussed below. Besides direct transcriptional

effects, the neural GC-mediated regulation of hypothalamic neuropeptide secretion is accomplished largely by the above-described hippocampal glutamate and amygdaloid GABA-ergic projections onto BNST efferent neurons (Choi et al., 2007; Herman et al., 2002). GR activation in hippocampal neurons is associated with a glutamatergic surge, which, via BNST stimulation, ultimately conveys suppression of PVN secretory output. On the contrary, GR activation in amygdaloid neurons results in enhanced GABA-ergic output to the BNST and, eventually, disinhibition of PVN neurosecretory cells.

Further amygdaloid "drive" originates from CRH neurons located in its central nucleus, which bolster the PVN activation through activation of glutamate and CRH-releasing efferents located in the anteromedial BNST (Makino et al., 1994a; Cole and Sawchenko, 2002). It is pertinent to note that, unlike in the PVN, activation of GR in amygdaloid CRH-producing neurons increases the expression of this neuropeptide (Makino et al., 1994b). The molecular mechanisms underlying this phenomenon are not well understood, however it seems plausible that also in the CeA GC interact with other signaling cascades, probably differently than in the PVN. Another possibility could be that the CRH gene in the amygdala has a functional positive GRE, while that is not the case in the PVN; one possible explanation for this disparity could be local differences in the epigenetic control of CRH gene expression, however to date this is mere speculation. As is the case for the hippocampus, the effects of GC on amygdaloid neurons highly depend on timing (onset and duration) and dose of GC exposure as well as on other (e.g. neurotransmitter) signals converging on these structures (Joëls et al., 2009; Krishnan and Nestler, 2010; Sarabdjitsingh et al., 2012).

In a very simplified cascade the events of HPA response to a psychological stressor we believe the following occurs:

Sensory information of the stressor is projected from the primary sensory cortical areas through thalamic gating mechanisms to the hippocampus and the amygdala, where we believe that the information is processed in terms of comparison with previous life experience (memory processes) and the emotional evaluation of this information.

The outcome of this "evaluation", which also involves reciprocal interactions between hippocampus, amygdala and prefrontal cortex (PFC) (Radley and Sawchenko, 2011; Radley et al., 2006) is the disinhibition of the PVN by the BNST leading to surge firing and release of CRH

and AVP. It should be noted that the duration and quality of the stress exposure determines the balance between the synergistic molecules CRH and AVP: while in acute settings CRH expression and release are strongly increased, in chronic (or repeated) stress situations CRH expression is "normalized" while AVP expression and secretion are further increased.

CRH and AVP are released into the portal blood stream of the anterior pituitary where they lead to the release of stored ACTH from corticotroph cells as well to the induction of POMC gene expression and its posttranslational processing. At this stage it should be noted that additional factors secreted from hypothalamic and brainstem neurons at the median eminence (such as dopamine, noradrenaline, serotonin but also glutamate and GABA) also affect corticotroph cell function (Labrie et al., 1987).

ACTH in turn binds to its receptor on adrenocortical cells (although GC are mainly produced in the fasciculate zone of the adrenal cortex, ACTH receptors are also expressed in the glomerular and reticular zone, where they have also been shown to govern steroidogenesis (production of mineralocorticoids and androgens respectively). As steroidogenic cells can not store their secretory product, all steroids are synthesized and secreted *de novo* on demand, which takes a considerable amount of time – up to several minutes.

GC are not primarily involved in the immediate stress response (which is mainly mediated by catecholamines), but rather in the adaptive response to a stressor, aiming to restore homeostasis (Charmandari et al., 2005). These effects include metabolic alterations aiming to mobilize energy from internal stores, reduction of energy expenditure by suppression of processes not immediately necessary for survival (e.g. immunomodulation) and alteration of brain functions (including behavioral and endocrine alterations and adaptation) (Sternberg et al., 1992; Patchev and Patchev, 2006).

The classic view is that in their nature as ligand activated transcription factors, corticosteroid receptors mediate GC actions by altering gene expression, which again requires more time (minutes to hours). While this underlines the importance of GC action to the mounting of an adaptive response to stress by governing the so called "late" phase of the response (de Kloet et al., 2005), over the last decades evidence of rapid (within milliseconds to few minutes) GC effects which are independent of gene transcription and *de novo* protein synthesis has accumulated , however we are still far away from understanding how these "rapid" GC effects

integrate into the adaptive response to stress (Riedemann et al., 2010). In addition the molecular mechanisms involved in rapid GC signaling are not well understood; for instance it still remains to be shown beyond doubt if there is a membrane receptor for corticosteroids and what the nature of this receptor might be. It is however plausible to assume, that the slowly increasing amounts of GC secreted by the adrenal cortex upon activation by ACTH act via these rapid signaling mechanisms to affect behavioral and endocrine functions (including a role in mediating negative GC feedback) before the effects of classical GC signaling cascade (i.e. altered gene expression and protein synthesis) can be observed.

For the sake of simplicity and brevity, rapid GC signaling will be neglected in this introduction, especially as there still is more discordance than consensus on its mechanisms and biological functions (see Riedemann et al., 2010 for a review on this topic).

It is important to point out that HPA axis function is different between both sexes. Females (rodents as well as higher primates) display substantially higher amounts of circulating ACTH and GCs, as well as higher expression of hypothalamic CRH and AVP (Patchev and Almeida, 1998). Interestingly however at the same time females display higher GC negative feedback sensitivity than males and higher expression of corticosteroid receptors in relevant limbic and hypothalamic brain areas (Patchev and Almeida, 1996). Previous studies from our lab have ascribed a significant role for the early ontogenetic sex-steroid milieu in the sex-specific organization of HPA axis function (Patchev et al., 1999) and part of the present work has attempted to dissect the distinct roles of estrogen receptor isoforms in this process.

The biology of corticosteroid receptor function

There are two types of corticosteroid receptors – the glucocorticoid (GR) and the mineralocorticoid receptor (MR). As steroid receptors, both share a common structure and act as ligand activated transcription factors (Gronemeyer et al., 2004). Both, GR and MR, can bind endogenous glucocorticoids (corticosterone in rodents and cortisol in higher primates and humans), however with different affinities. While MR binds corticosterone with a 5-7 times higher affinity than GR (Reul and de Kloet, 1986), GR is not capable of binding mineralocorticoids (aldosterone) in notable quantities (Spencer et al., 1990; Funder, 2012). However the amount of aldosterone that can pass the BBB is negligible (Geerling and Loewy, 2009) as are the amounts of this hormone which have been shown to be produced *in loco* by

some neurons and astrocytes (Gomez-Sanchez et al., 2005; Geerling and Loewy, 2009). Therefore, the main corticosteroid in the brain, which participates in the regulation of HPA axis function, is corticosterone (in rodents). The differential affinities of MR and GR have led to the "balance" hypothesis of corticosteroid signaling (De Kloet et al., 1998): brain MR are already occupied and activated by low amounts of corticosterone (i.e. at the circadian through of HPA axis activity), while GR are activated when GC levels increase (e.g. at the circadian zenith of HPA axis activity or upon exposure to and perception of stress). This finding suggests that shifts in the balance between MR and GR activation lead to differential transcriptomic and physiological responses, which depend on the availability of corticosterone.

While the balance hypothesis provides a suitable model to describe the differential effects of low and high doses of GC on hippocampal gene expression and function (Datson et al., 2012; De Kloet et al., 1998; Evans and Arriza, 1989), it should be noted that there are additional levels of fine-tuning GC signaling in the brain (and elsewhere). For instance, many tissues (including different limbic brain areas, as the hippocampus and the amygdala) express 11β-Hydroxysteroid-dehydrogenase (11β-HSD) type 2, an enzyme which can actively convert the active corticosterone (in humans cortisol) to its inactive 11-keto-form 11-dehydrocorticosterone (in humans cortisone) (Wyrwoll et al., 2011). The biotransformation of GC by 11β-HSD (especially type 2) represents an auxiliary mechanism of GC level adjustment *in loco* and fine tuning of GR- and MR-mediated control of HPA activity. Still, it should be noted that the expression of 11β-HSD itself is subject to regulation by stress and GC (Low et al., 1994; Walker et al., 1994; Wyrwoll et al., 2011).

In addition GR and MR, as all steroid receptors, are involved in complex interactions with the transcriptional machinery, e.g. direct and indirect interactions with transcription factors and their signaling cofactors. Studies from this lab and others have indicated that MR and GR recruit similar co-activators and co-repressors (such as SRC1, TIF-2, NCoR1) (Tirard et al., 2004; van der Laan et al., 2008; de Kloet et al., 2009) and it seems plausible that both receptors, when co-expressed (which is believed to be mostly the case) are in some degree competing for the interaction with these molecules (Meijer, 2002; Lonard and O'Malley, 2012), which would also represent a mechanism of fine tuning GC action.

Upon ligand activation, GR and MR dissociate from their interaction with chaperones (Grad and Picard, 2007) (such as hsp90, hsp70), form homodimers and translocate into the nucleus,

where they bind to their respective response element, and via their AP1 site also modulate cAMP-dependent gene transcription (Yoshida et al., 2006; Díaz-Gallardo et al., 2010). To date it is not clear, if GR and MR actually form heterodimers *in vivo* (Nishi and Kawata, 2007), which would add another level of complexity (but also finesse) to the regulation of GC signaling in the brain.

While the GR-MR balance hypothesis surely has contributed to our understanding of corticosteroid physiology in the hippocampus, it also bears the danger of misleading. For instance, the question to what extent the quantity of a protein is indicative of its functioning, remains unanswered. Several studies have disproved the original assumption that in the brain MR is exclusively expressed in the hippocampus and some subnuclei of the amygdala (Reul and de Kloet, 1986), and we now know that indeed in most brain areas MR and GR are co-expressed (Geerling and Loewy, 2009), however MR in much lower quantities than GR (and both less than in the hippocampus). This leads to the question if the MR-GR balance hypothesis is valid also in these structures, i.e. if in these structures GR is the dominating mediator of GC actions or if the low quantities of MR are still sufficient to balance and counteract excessive GR activation.

The reasons for our current lack of knowledge are numerous, however the limited molecular and pharmacological tools are probably the most prominent. To date there are no antibodies with high selectivity for MR, making chromatin immuno-praecipitation (ChIP) assays for MR at least not reliable beyond doubt, thus prohibiting the identification of MR and GR specific target genes (Geerling and Loewy, 2009; Gomez-Sanchez et al., 2006). Also, our pharmacological tool box is rather limited when it comes to highly selective corticosteroid receptor agonists and antagonists, especially when BBB penetration is a requirement. Dexamethasone, while highly selective for GR with negligible binding at MR, has been shown to not enter the murine, in contrast to the rat, brain (de Kloet et al., 2005) (Meijer et al., 1998) (De Kloet et al., 1975). The most commonly used GR antagonist mifepristone (RU 486) is actually a potent antigestagen (Heikinheimo et al., 1987) with strong antimineralocorticoid activity (Li et al., 1999), whose potential to penetrate the BBB is rather low (Heikinheimo and Kekkonen, 1993), thus making GR-selective dosing practically impossible.

Studies in animals with general or brain-specific transgenesis or mutations of corticosteroid receptors and their cofactors have shed some light on the distinct roles of GR and MR on brain

structure and function (Wintermantel et al., 2004; Charlier and Balthazart, 2005), however prompt the question of potential compensatory processes making up for the genetic manipulation. Some of these studies will be discussed in greater detail in the course of this manuscript, however many of them also report paradoxical phenomena (Tronche et al., 1999; Kaufer et al., 2004; Mitra et al., 2009; Patchev et al., 2007) and therefore have not been able to answer all questions of corticosteroid signaling in the brain.

Stress and glucocorticoid actions in the brain

As described above, GC mediate a major, but not the only response to a stressful stimulus. Therefore it is important to point out, that GC and stress might actually have differential effects on brain functions, i.e. that not all effects of stress on behavioral and endocrine brain functions as well as brain structure can be reproduced by exogenous GC application (Patchev and Patchev, 2006). This dissociation is interesting from a physiological point of view, in order to understand the mechanisms and effects of basal (non-stress-related) alterations in GC secretion (e.g. circadian and ultradian oscillations) (Lightman et al., 2008) vs. those induced by stress, where GC actions are only one (even if major) part of the plethora of well orchestrated physiological processes.

In general it should be considered that corticosteroids, while on the one hand absolutely necessary for brain function, can exert pathological effects on brain structure and function (de Kloet et al., 2005; Sousa et al., 2008; Sousa and Almeida, 2012). The main factors contributing to this outcome are: ***i)*** timing and duration of the exposure and ***ii)*** the quantity of GC. In general the same can be applied in respect to the adaptive and mal-adaptive effects of stress, however while the chronicity of stress and GC exposure can be compared, the individual (subjective) assessment of a potentially stressful situation (i.e. different individuals have different perceptions of one and the same stressor) is more difficult to assess, especially in rodents (Sousa et al., 2006; Patchev and Patchev, 2006).

Acute and chronic stress have extensively been shown to affect emotional behaviors in rodents and humans, including anxiety-related behavior and fear as well as coping style and depression-like behavior (Bessa et al., 2009; Pêgo et al., 2008; Pêgo et al., 2010). Thus chronic stress models employing psychological stressors have been ascribed the role of rodent models of human depression (Bessa et al., 2009; Lupien et al., 2009; McEwen, 2005). Indeed, stress in

humans has been identified as a major risk factor for CNS disorders such as depression and anxiety disorders (Krishnan and Nestler, 2010; Chrousos and Gold, 1992; Meyer et al., 2001), as well as cognitive disorders (Sotiropoulos et al., 2008b; Lupien et al., 2009; Koenig et al., 2011). Studies from our labs and others have shown that chronic unpredictable stress leads to cognitive impairments and even activates amyloidogenic pathways believed to be involved in the pathogenesis of Alzheimer's disease (Sotiropoulos et al., 2008b; Sotiropoulos et al., 2011; Sotiropoulos et al., 2008a; Catania et al., 2009). However the mechanistic dissection of how stress affects emotional and cognitive behaviors and brain structure is not easy, as only some, but not all of these effects can be reproduced (quali- and quantitatively) by chronic administration of high doses of GC (Patchev and Patchev, 2006; Liberzon and Young, 1997; Imaki et al., 1991). In terms of emotional and cognitive behavior as well as neuromorphology and physiology GC actions on the brain are dependent on the activation of both MR and GR and the balanced interplay between these receptors seems to determine the neuroadaptational outcome (de Kloet et al., 2005). Studies from our labs and others have shown that MR activity is crucial for maintenance of neuronal structure and function (Reul et al., 2000; Hassan et al., 1996; Hassan et al., 1999). For instance, patients suffering from adrenocortical insufficiency display hippocampal granule cell loss (Maehlen and Torvik, 1990). Correspondingly, adrenalectomized animals display strong cell death and dendritic atrophy in the dentate gyrus of the hippocampus (Sloviter et al., 1993; Andrés et al., 2006), which can be preserved by low dose application of corticosterone or a MR agonist (Sloviter et al., 1993; Hassan et al., 1996). On the other side, high doses of the selective GR agonist dexamethasone lead to neuronal apoptosis and reduced dendritic arborization in the hippocampus and the prefrontal cortex (Sousa et al., 2008; Hassan et al., 1996; Yu et al., 2008), structures crucially involved in emotional and cognitive behavioral functions (Sousa and Almeida, 2012; Bessa et al., 2009). Interestingly however, GR activation in the amygdala results in increased dendritic branching which indicates that not only the interplay between MR and GR, but also the cellular milieu (e.g. neuronal input, paracrine environment) influence the effects of GC on neuronal morphology and function (Sarabdjitsingh et al., 2012; Pêgo et al., 2008; Roozendaal et al., 2009).

In addition to the interplay between MR and GR and the cellular environment, the chronicity of stress/GC exposure determines their neurophysiological and –structural effects (Sousa and Almeida, 2012). Interestingly different behavioral domains seem to be affected differentially by acute vs. chronic stress/GC exposure. For instance, while acute stress or GC exposure seems

to enhance certain domains of cognition (Yuen et al., 2011), chronic settings lead to impairment of cognitive functions (Sousa and Almeida, 2012; Cerqueira et al., 2007; Joëls et al., 2006). Similarly stress and GC affect emotional behaviors differentially depending on the duration and intensity/dose: while acute, as well as chronic stress/GC application increase anxiety-related behaviors (Mitra and Sapolsky, 2008; McEwen et al., 2012), only chronic stress/GC exposure have negative effects on depression-like behaviors (Bessa et al., 2009). Taken together it seems important to point out, that GC (either fluctuating under basal circadian conditions or as an adaptive response to a stressor) are essential for the integrity of brain function and structure. However exposure to high doses of GC over longer periods (e.g. exogenous or by mal-adaptive response to a stressful situation) lead to alterations in neuronal structure and function with a distinct spatio-termporal pattern.

Programming and mal-programming of the HPA axis and the consequences for mental health

Programming of the brain is a very broad term referring to those physiological processes which determine the long-term functioning of neuronal networks (i.e. the "hard wiring" of brain circuits). The programming stimuli are both endogenous (e.g. developmental changes in hormone secretion) and environmental (e.g. alimentary state, stress) (Dörner, 1983; Hanson et al., 2011a). Timing seems to play the most important role for physiological programming and brain organization. The general concept is that programming and organization processes take place mainly during early ontogeny (embryonal, fetal, neonatal) (Andersen, 2003). However recently adolescence and puberty have also been shown to be critical time windows during which the brain is not only undergoing maturation, but also organization of neuronal networks and therefore seems highly susceptible for programming and mal-programming by endogenous and exogenous signals (Schulz et al., 2009; Vigil et al., 2011; Koenig et al., 2011). These findings indicate that brain programming is an ongoing process (or a plethora of ongoing processes) well beyond early ontogeny and that exogenous impacts can interfere with these (possibly throughout the entire lifespan) depending on their timing (onset and duration), quality and quantity (strength). Such "mal-programming" refers to the long-term consequences of disrupted programming mechanisms through exogenous or endogenous stimuli which have occurred "at the wrong time, at the wrong place" and/or with the wrong intensity. Studying the programming and mal-programming of the HPA axis is medically relevant due to the

pathophysiological involvement of GCs in many brain (e.g. depression, Alzheimer's disease) (de Kloet et al., 2005; Sotiropoulos et al., 2008b), metabolic (e.g. Diabetes mellitus, obesity, osteoporosis), immune and hematological disorders (Charmandari et al., 2005; Sternberg et al., 1992)

The objective of the present work was to assess the mechanisms of neonatal programming of neuronal circuits governing HPA axis function.

In detail we scrutinized:

- the epigenetic mechanisms involved in mal-programming effects of neonatal stress on HPA axis function and their long-term consequences ***(Chapter 1, publication by Murgatroyd, Patchev et al. 2010)***.

- the neuro-morphological, endocrine and behavioral long-term effects of selective neonatal GR activation leading to depletion of the neuronal precursor pool ***(Chapter 2, publication by Yu, Patchev et al., 2010)***.

- the involvement of estrogen receptor isoforms (ERα and ERβ) in the process of sex-specific organization of HPA axis function ***(Chapter 3, publication by Patchev et al., 2011)***.

In addition, in ongoing studies we have assessed the long-term consequences of peripubertal exposure to chronic stress and diet induced obesity. In these studies we show clearly that stress and metabolic challenges during puberty have sex-specific sustained (mal-programmed) effects on cognitive and emotional behaviors as well as HPA axis function, and we now aim to identify the cellular and molecular mechanisms involved in the establishment and maintenance of these mal-programmed effects. These findings however illustrate, that programming and mal-programming of the brain are possible even beyond the neonatal time window (e.g. during puberty).

2. Results

2.1 Chapter 1: Dynamic DNA methylation programs persistent adverse effects of early life stress

Murgatroyd C, **Patchev AV**, Wu Y et al., *Nature Neuroscience*, 2009

2.1.1 Rationale

This study aimed to examine the epigenetic mechanisms involved in programming of HPA axis functions through neonatal stress. The stress paradigm (maternal separation) was chosen for its well-established long-term behavioral and endocrine phenotype in rodents. Upon verification of the presence of hypercorticism and the expression of the tentative aberrant emotional behavioral phenotype, we chose a specialized, post-mitotic neuronal population, namely AVP neurons in the parvocellular division of the PVN, for the examination of epigenetic changes induced by early life stress.

2.1.2 Major findings

- early life stress (ELS) in mice has persistent endocrine and behavioral consequences for up to 1 year of age
- these changes are associated with increased expression of AVP in the parvocellular division of the PVN
- increased AVP mRNA expression in ELS mice is associated with reduced DNA methylation in the intergenic enhancer region of the AVP gene
- ELS-associated AVP enhancer hypomethylation does not occur immediately after neonatal stress exposure and is established within certain time lag, with biochemical hallmarks in post-mitotic neurons becoming manifest after 10 days of age.
- The emergence of ELS-induced AVP enhancer hypomethylation is, at least partly, dependent on activity-driven intracellular Ca^{2+}-signalling converging onto site-specific phosphorylation of MeCP2

- CamKII-dependent phosphorylation of MeCP2 at Serine residue 438 is increased in AVP neurons of pups exposed to neonatal stress, but not in adult animals with a history of ELS

- MeCP2 phosphorylation at S438 had been shown to be associated with reduced MeCP2 binding capacity to methylated CpG islands (Zhou et al., 2006; Flavell and Greenberg, 2008); accordingly, we presume that stress-induced MeCP2 phosphorylation leads to its dissociation form the AVP enhancer, thus enabling active or passive de-methylation of this gene locus. This process, however, seems to be time-dependent, with stress-induced MeCP2-phosphorylation playing a decisive role in its initiation.

2.1.3 Conclusions

Alterations in DNA methylation can be induced by exogenous noxious influence during early life, even in postmitotic, highly specialized neuronal populations. Once established, these epigenetic changes can persist throughout life, albeit displaying certain age dynamics (e.g. age-dependent decline in overall methylation levels). Activity (experience) driven epigenetic marking can occur during early ontogeny and have sustained physiological effects. Stress during early life can mal-program the HPA axis through epigenetic alterations in neurons of neuroendocrine relevance.

2.1.4 Outlook / ongoing studies

- We obtained first evidence for sex-specific differential targeting of neurochemical mechanisms by ELS. While the current study showing changes in AVP was carried out in males, we observed that in females with a history of ELS also CRH mRNA expression was increased. We also noticed that CRH and AVP gene methylation patterns differ between the sexes in both control and ELS conditions. Ongoing investigations aim to understand the basis of this sex dichotomy and the importance of adult sex hormone secretions for the manifestation of HPA axis dysregulation upon ELS exposure.

- Further work addresses the regulation of GR expression and function in limbic and neuroendocrine systems of animals with ELS history. We have first evidence for distinct alterations in the spatio-temporal expression patterns of GR mRNA in the brain of ELS

mice, which might explain their disturbed glucocorticoid negative feedback efficacy. In the PVN of adult mice with ELS history we detected increased GR expression and function (as measured by in vivo ChIP assays), despite increased CpG methylation within the GR promoter region. Currently we examine the role of the insulator protein YY as a crucial regulator of GR expression and function (*Bockmühl, Kuczynska, Patchev, et al. in preparation*).

- Striking functional alterations associated with ELS were also seen at the pituitary level. In pituitary corticotrophs ELS leads to increased expression of POMC mRNA in both sexes, with qualitative and quantitative methylation patterns within the POMC gene being differentially affected by sex. ELS has also sustained effects on corticotroph sensitivity to ACTH secretagogues and glucocorticoid suppression. Ongoing work is focusing on ELS-induced alterations in the epigenetic marking of the POMC (*Wu, Daniel, Patchev et al, in preparation*).

- It remains unclear whether the pharmacological and/or environmental reversibility of ELS phenotype (as reported elsewhere (MacQueen et al., 2003; Navailles et al., 2008; Francis et al., 2002) is accompanied by changes in epigenetic marking. Future studies in our lab will address this issue.

2.1.5 Contributions

- involvement in planning and performance of the in vivo studies, including behavioral and endocrine phenotyping

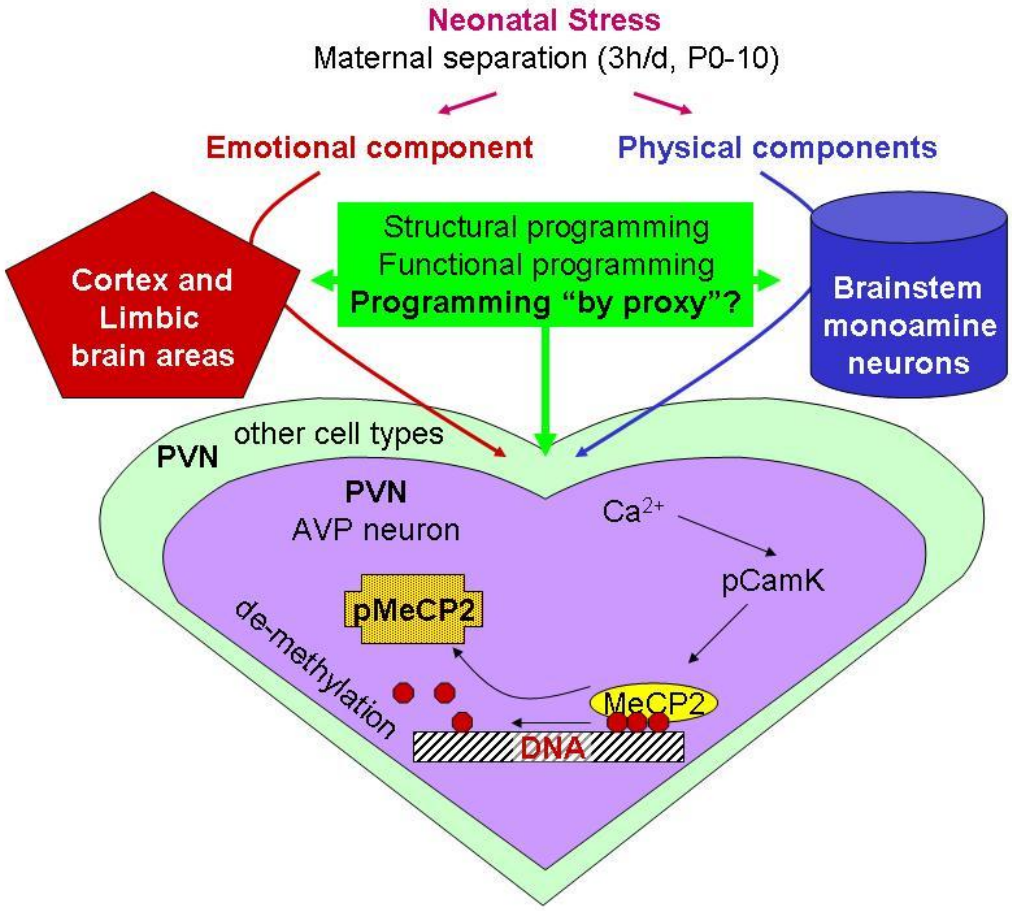
- histological analysis (ISH, ICC, confocal microscopy).

- elaboration and validation of a method for micropunching of frozen brain tissue *en block* with simultaneous collection of sections for in situ hybridization by adapting the Palkovits micro-dissection technique (Palkovits, 1986). This method allowed us to concomitantly analyze DNA methylation (in punch specimens) and RNA expression (in sections) in each animal, thus permitting individual data monitoring and cross-validation.

- Involvement in writing the publication.

Figure 1 **Summary and proposed model on the mechanisms through which neonatal stress alters DNA-methylation at the AVP enhancer region**

Neonatal stress effects on the PVN are exerted through limbic and brainstem monoaminergic signals to the PVN, where neuronal activity leads to phosphorylation of MeCP2 at S438, thus causing dissociating of MeCP2 from methylated CpG islands, which allows for demethylation. The interactions between limbic, cortical and brainstem neuronal populations under the influence of neonatal stress are not well understood, but it is assumed that neonatal stress causes structural and functional alterations in these brain areas as well; therefore the here described programming effects of neonatal stress in AVP neurons could be the "by proxy" result of these structural and functional rearrangements in limbic monoaminergic neuronal populations.



nature neuroscience

Dynamic DNA methylation programs persistent adverse effects of early-life stress

Chris Murgatroyd, Alexandre V Patchev, Yonghe Wu, Vincenzo Micale, Yvonne Bockmühl, Dieter Fischer, Florian Holsboer, Carsten T Wotjak, Osborne F X Almeida & Dietmar Spengler

Adverse early life events can induce long-lasting changes in physiology and behavior. We found that early-life stress (ELS) in mice caused enduring hypersecretion of corticosterone and alterations in passive stress coping and memory. This phenotype was accompanied by a persistent increase in arginine vasopressin (AVP) expression in neurons of the hypothalamic paraventricular nucleus and was reversed by an AVP receptor antagonist. Altered *Avp* expression was associated with sustained DNA hypomethylation of an important regulatory region that resisted age-related drifts in methylation and centered on those CpG residues that serve as DNA-binding sites for the methyl CpG–binding protein 2 (MeCP2). We found that neuronal activity controlled the ability of MeCP2 to regulate activity-dependent transcription of the *Avp* gene and induced epigenetic marking. Thus, ELS can dynamically control DNA methylation in postmitotic neurons to generate stable changes in *Avp* expression that trigger neuroendocrine and behavioral alterations that are frequent features in depression.

Epigenetic regulation of gene expression allows the integration of intrinsic and environmental signals in the genome[1]. Greater emphasis is being placed on the role of epigenetic mechanisms in facilitating the adaptation of organisms to changing environments through alterations in gene expression. Evidence that dietary or pharmacological interventions have the potential to reverse environment-induced modification of epigenetic states[2–5] has provided an additional impetus for understanding the epigenetic basis of disease, including disorders of the brain. It has been suggested that epigenetic mechanisms underlie brain plasticity, a process requiring stable modulation of gene expression[6,7]. DNA methylation is one of the most intensely studied epigenetic mechanisms[8] and recent work has suggested that this form of gene regulation may determine risk for psychiatric disorders[3,9,10].

Exposure to stress during neurodevelopment has an effect on the quality of physical and mental health[11,12]. Periodic infant-mother separation during early postnatal life is one of the most commonly used procedures for inducing ELS in rodents. It is characterized by lifelong elevated glucocorticoid secretion, heightened endocrine responsiveness to subsequent stressors and disruption of the homeostatic mechanisms that regulate the activity of the hypothalamo-pituitary-adrenal (HPA) axis, all of which are considered to be pathogenetic factors in disorders of mood and cognition[13–15].

Here we examined the coupling of experience-driven neuronal activity with DNA methylation and gene expression[6]. We focused on the expression of the two hypothalamic secretagogues that regulate HPA axis activity by increasing the synthesis and release of pituitary adrenocorticotropin, namely, AVP and corticotropin-releasing hormone (CRH). Abundant evidence links AVP and CRH to mood and cognitive behaviors[16,17], making their receptors the targets of psychopharmacological agents[18,19]. In addition to being important in the postnatal development and functional maturation of the pituitary-adrenal axis, AVP potentiates the actions of CRH under circumstances that demand sustained activation of the pituitary and adrenal glands[20]. We found that ELS induces persistent hypomethylation of the *Avp* enhancer, accompanied by sustained upregulation of *Avp* expression, increased HPA axis activity and behavioral alterations. In the course of exploring the molecular mechanisms underlying these changes, we found that MeCP2 is important in the epigenetic programming of neuroendocrine and behavioral functions.

RESULTS

ELS-induced phenotypes

Consistent with previous studies[15,21], ELS during the first 10 d of life led to sustained hyperactivity of the HPA axis, characterized by corticosterone hypersecretion under basal conditions, hyperresponsiveness to acute stressors applied later in life and escape from the inhibitory constraints of dexamethasone (**Fig. 1a**). Although it had no effect on body mass, ELS induced involution of the thymus, hypertrophy of the adrenals (**Supplementary Fig. 1**) and increased expression of pituitary *pro-opiomelanocortin* (*Pomc*) mRNA, which encodes the adrenocorticotropin pro-hormone (**Fig. 1b**). *Pomc* expression is induced by the hypothalamic neuropeptides AVP and CRH, and all of them are under negative feedback control by the glucocorticoid receptor. Conspicuously, levels of *Nr3c1* (the gene that encodes the glucocorticoid receptor) mRNA in the hippocampus, hypothalamic paraventricular nucleus (PVN) and pituitary were either unchanged or upregulated in ELS-treated mice (**Supplementary Fig. 1**), arguing against impaired corticosterone feedback as the primary cause of the observed increases in *Pomc*

Max Planck Institute of Psychiatry, Munich, Germany. Correspondence should be addressed to D.S. (spengler@mpipsykl.mpg.de).

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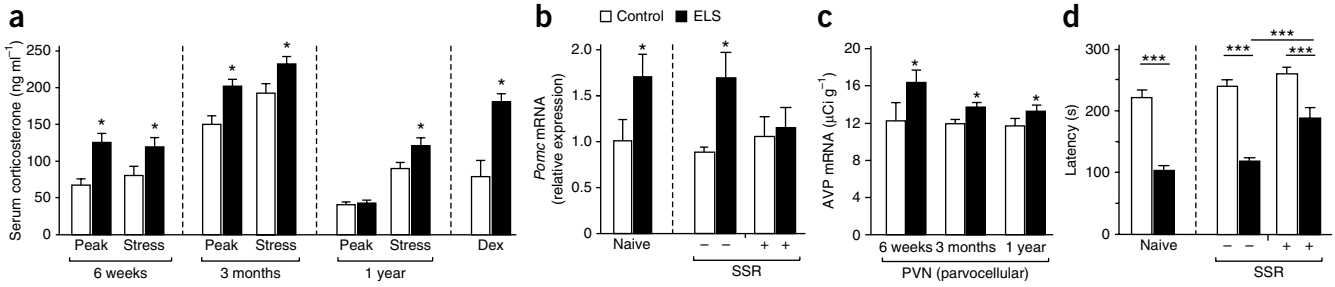

Figure 1 Endocrine and behavioral consequences of ELS depend on sustained AVP expression. (**a**) ELS mice had higher serum corticosterone levels at 6 weeks, 3 months and 1 year of age under basal conditions, at the daily nocturnal peak and after exposure to an acute stressor applied 30 min before sampling. An injection of dexamethasone (dex, 6 h before sampling) suppressed corticosterone secretion more effectively in 3-month-old control as compared with ELS mice. (**b**) *Pomc* mRNA (measured by qualitative PCR, qPCR) was significantly higher in the pituitaries of 3-month-old ELS mice. Treatment of 3-month-old control and ELS mice with the AVP V1b receptor antagonist SSR149415 (SSR) reduced *Pomc* mRNA levels (measured by qPCR) in ELS mice. (**c**) *Avp* mRNA expression (detected by ISH) was significantly higher in parvocellular PVN neurons of 6-week-old (+31%), 3-month-old (+13%) and 1-year-old (+15%) ELS mice. (**d**) ELS mice showed shorter step-down latencies 24 h after learning but not during training (control (19 ± 2 s) versus ELS (21 ± 2 s), $P > 0.05$). Treatment with the AVP V1b receptor antagonist partially reversed memory deficits by increasing step-down latencies from 47 ± 3% to 78 ± 7% of vehicle-treated controls, without affecting the behavioral performance of control mice (101 ± 6%). Data are presented as mean ± s.e.m. (n = 8–16 mice per group). *$P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (t test or ANOVA followed by Newman-Keuls *post hoc* test).

expression and glucocorticoid secretion. Although ELS did not influence hypothalamic *Crh* mRNA expression (**Supplementary Fig. 1**), the procedure resulted in a significant upregulation of *Avp* mRNA ($P < 0.05$; **Fig. 1c**). The changes in *Avp* expression persisted for at least 1 year and were restricted to the parvocellular subpopulation of neurons in the PVN, that is, in those neurons that drive the pituitary-adrenal axis (**Supplementary Fig. 2**).

AVP exerted its regulatory role on *Pomc* expression levels via activation of pituitary AVP V1b receptors. Application of SSR149415, a selective V1b receptor antagonist, normalized the elevated *Pomc* mRNA levels (**Fig. 1b**) and corticosterone secretion (data not shown), verifying the critical role of AVP in driving the disturbed endocrine phenotype in ELS mice.

ELS also produced long-lasting behavioral changes. Adult ELS-exposed mice showed memory deficits in an inhibitory avoidance task (**Fig. 1d**). In addition, they had increased immobility in the forced swim test (**Supplementary Fig. 3**). In contrast, anxiety-like behavior was unaffected by ELS in the elevated plus-maze, novelty-induced hypophagia and light-dark avoidance tests (data not shown). The ELS-induced behavioral phenotypes were reproduced in two further independent replications. Treatment with the SSR149415 partially reversed the impaired memory in ELS mice (**Fig. 1d**) and abolished the changes in behavioral stress coping (**Supplementary Fig. 3**) without influencing the behavioral performance of control mice.

Differential methylation of the *Avp* gene

Methylation of cytosine residues in CpG dinucleotides can result in epigenetic gene silencing; such CpGs are conspicuously under-represented in mammalian genomes and typically cluster in glucocorticoid-rich regions called CpG islands (CGIs)[22]. Computational analysis and a recent genome-wide classification of promoter CGIs[23] predicted 4 CGIs in *Avp*: CGI1 (intermediate CpG frequency in the promoter region), CGI2 (high CpG frequency covering the second and third exons), CGI3 and CGI4 (intermediate CpG frequency in the ~3.6-kb downstream region) (**Fig. 2a** and **Supplementary Fig. 4**). The latter region, also referred to as the intergenic region (IGR), separates the neighboring, tail-to-tail–orientated *Avp* and *oxytocin* genes and includes a composite enhancer region in the first 2.1 kb proximal to *Avp* that is important for expression[24].

Sequence analysis of bisulfite-converted DNA isolated from the PVN of naive C57BL/6N mice showed sparse methylation in the promoter CGI1 and exonic CGI2. In contrast, we found high levels of CpG methylation clustered at the more distal enhancer encompassing CpG7 to CpG32 and spanning CGI3 (**Fig. 2b**). The latter region is highly conserved between species and is important for *Avp* regulation[24]. Less-dense methylation was observed in CGI4 and the adjacent oxytocin tissue-specific enhancer region had only a few, irregularly spaced and highly methylated CpG residues (**Fig. 2b**). A similar methylation of CpG residues at the *Avp* locus was found in unrelated CD1 mice, supporting the idea that this pattern is unlikely to be strain specific (data not shown). Together, these results support the idea that CGIs in intergenic regions are more likely to be methylated than those at gene promoters and that CGIs with intermediate CpG densities are methylated more frequently[25].

Persistent hypomethylation of CGI3 after ELS

We compared PVN tissue from ELS and control mice aged 6 weeks, 3 months and 1 year and found hypomethylation of multiple CpG residues throughout the downstream *Avp* enhancer region in ELS mice (**Fig. 3a–c**). Analysis of overall methylation of the enhancer revealed substantial reductions in methylation in ELS mice of all ages (**Fig. 3d**). Significantly marked ($P < 0.05$) CpG residues largely mapped to CGI3 of the enhancer. For many of these, the degree of ELS-induced hypomethylation was consistently greater than that observed for overall CGI3 hypomethylation; for example, CpG10 showed uniformly strong reductions in methylation (by 37% at 6 weeks, ($P < 0.005$), 21% at 3 months ($P < 0.05$) and 66% at 1 year ($P < 0.005$)). This finding reveals that ELS triggers a heterogeneous response in CpG hypomethylation and indicates a functional role for marked changes.

To obtain a functional measure of those CpG residues that are likely to control *Avp* expression, we sought correlations between *Avp* mRNA levels and the degree of methylation of individual CpG residues that were significantly hypomethylated ($P < 0.05$) in ELS mice. Therefore, *in situ* hybridization (ISH) and DNA methylation analyses were performed on tissues from the same individual mice. Of the 11 CpGs that were significantly hypomethylated ($P < 0.05$) in 6-week-old ELS mice (**Fig. 3a**), only seven (CpGs 7, 10, 12, 13, 14, 15 and 17) had methylation patterns that were strongly correlated with *Avp* mRNA levels (**Fig. 3e**). For example,

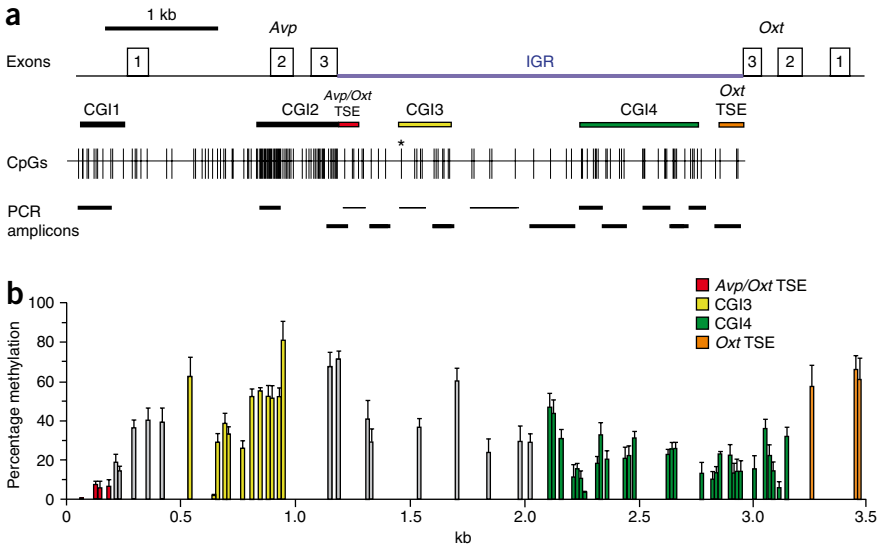

Figure 2 Selective methylation of the intergenic region of the *Avp* gene. (**a**) Schematic diagram of the *Avp* and *oxytocin* genes orientated tail-to-tail and separated by the IGR. Exons are indicated by open (numbered) boxes and CGIs by numbered bars, and the distribution of CpG residues and size and position of the respective PCR amplicons used for their analyses are shown. CpG10 is marked with an asterisk. (**b**) CpG methylation profile in the IGR of 6-week-old naive mice. Residues belonging to the *Avp-Oxt* tissue-specific enhancer (*Avp/Oxt* TSE), CpG islands 3 or 4 (CGI3 or CGI4) or the *Oxt* tissue-specific enhancer (*Oxt* TSE) are color coded (inset). CGI3, lying ~0.5 kb downstream of the *Avp* gene, had the highest level of methylation. Data are presented as mean ± s.e.m. ($n = 8$ mice per group).

although residues CpG10 and CpG22 had similar levels of methylation (methylation at CpG10 in controls and ELS was 60.3 ± 5.9% and 37.6%, respectively; methylation at CpG22 in controls and ELS was 69.5 ± 8.6% and 39 ± 9.8%, respectively; **Fig. 3a**), the methylation status of CpG10, but not of CpG22, correlated strongly with differences in *Avp* expression (CpG10, $r^2 = 0.44$, $P < 0.05$; CpG22, $r^2 = 0.07$, $P > 0.1$). Thus, ELS-induced alterations in CpG methylation appear to be important for *Avp* mRNA levels, although individual CpG residues located in CGI3 seem to contribute, in different degrees, to altered expression. Those CpGs that failed to show a priori significant differences ($P > 0.05$) in their methylation status in response to ELS correlated poorly with *Avp* mRNA levels (data not shown). Notably, differential methylation of CGI3 was not evident when DNA from the hypothalamic supraoptic nucleus of 6-week-old control and ELS mice were compared (**Supplementary Fig. 5**); the

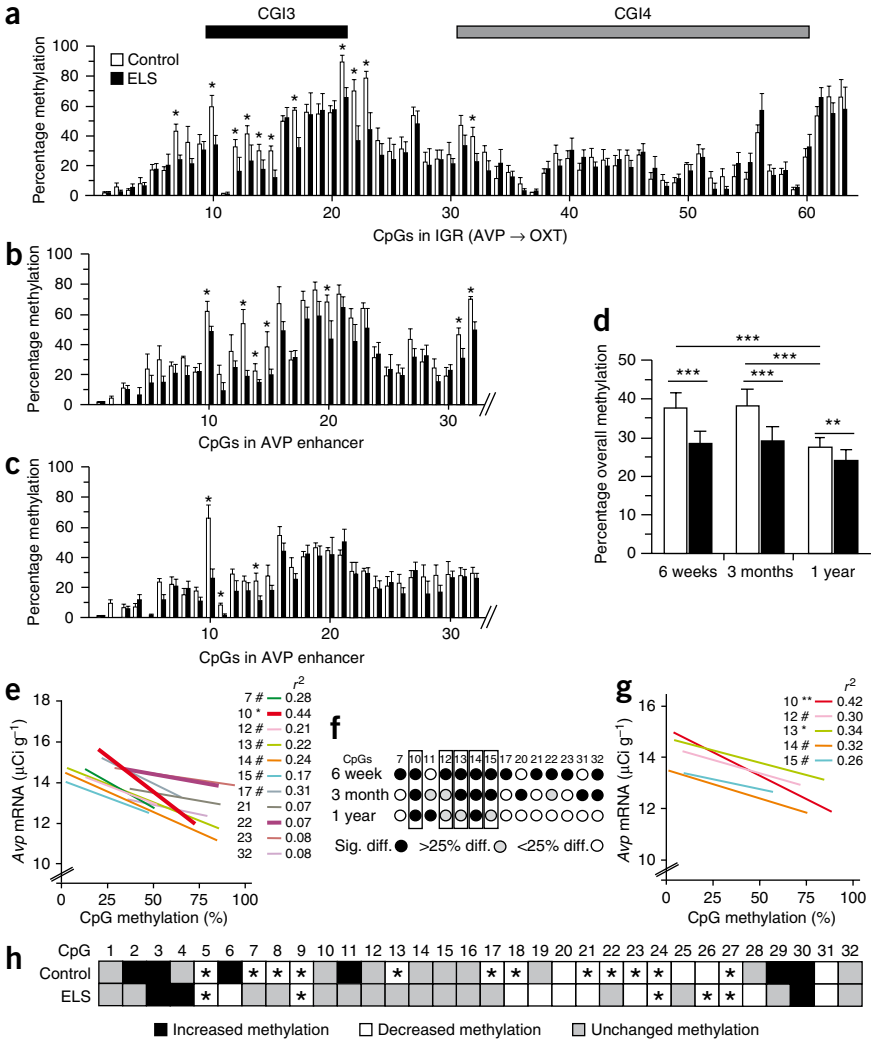

Figure 3 ELS induces hypomethylation of the *Avp* enhancer. (**a**–**c**) CpG methylation profiles of 6-week-old (**a**), 3-month-old (**b**) and 1-year-old (**c**) control and ELS mice. The entire IGR is shown in **a**, whereas **b** and **c** focus on the enhancer. (**d**) Overall methylation of the enhancer decreased significantly in ELS mice at all ages. Note the significant age-related hypomethylation in control, but not ELS, mice. (**e**) CpG methylation inversely correlated with *Avp* expression, revealed by correlating all significantly marked CpGs between 6-week-old control and ELS mice (shown in **a**) with respective *Avp* mRNA levels. (**f**) All CpG residues (shown in **a**–**c**) that differed significantly in methylation at least once (●) and by more than 25% (●) at the other two ages were defined as methylation landmarks (boxed CpGs 10, 12, 13, 14 and 15). (**g**) The composite methylation status of the methylation landmarks, in particular CpG10, correlated negatively with *Avp* expression over all ages. (**h**) Differences in methylation (>10%) between 6-week-old and 1-year-old control and ELS mice for each CpG in the enhancer revealed significant changes in both control and ELS mice. Methylation landmarks were not influenced by age in either group. Data are presented as means ± s.e.m. ($n = 8$–10 mice per group). # $P < 0.01$, * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0001$ (*t* test, ANOVA followed by Newman-Keuls *post hoc* test or Pearson's correlation coefficient).

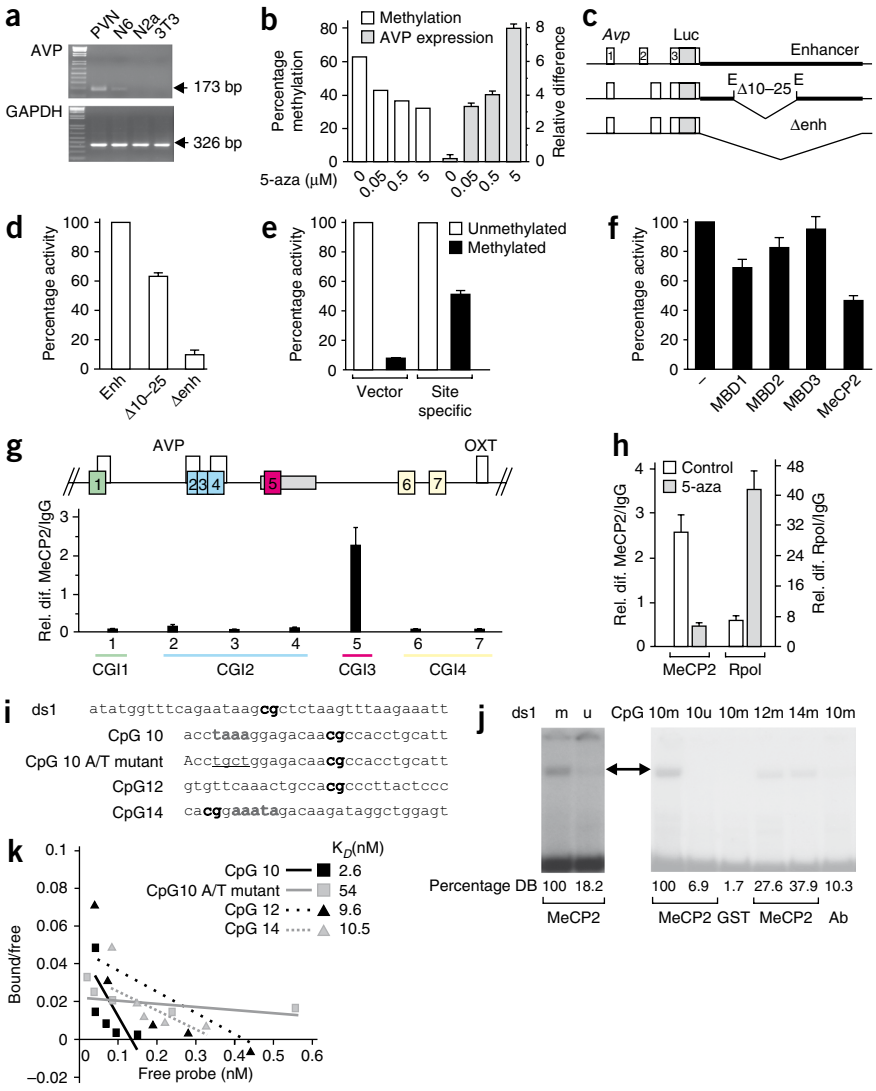

Figure 4 Enhancer methylation represses *Avp* expression as a result of MeCP2 occupancy. (**a**) Reverse-transcription PCR (RT-PCR) analysis of *Avp* expression in mouse PVN, hypothalamic N6, neuroblastoma Neuro2a and fibroblast 3T3 cells. (**b**) Treatment of N6 cells with 5-azacytidine (5-aza, 5 d) prevents CpG methylation and induces *Avp* expression. (**c**) The parent *Avp-Gaussia* construct contains the entire 2.1-kb enhancer, Δ10–25 is devoid of CpGs 10 to 25 and Δenh lacks the entire enhancer. (**d**) Deletion of either CpGs 10–25 or the entire enhancer reduced reporter activity by 37% and 90%, respectively, in N6 cells. (**e**) Entire vector or site-specific enhancer (CpGs 1–25) methylation reduced reporter activity by 90% and 50%, respectively. (**f**) MeCP2 strongly repressed the site-specific methylated CGI3 vector; transfection with MBD1, MBD2 and MBD3 resulted in weaker repression. (**g**) ChIP analysis revealed that MeCP2 selectively occupied CGI3 at the *Avp* locus in N6 cells. (**h**) Treatment with 5-azacytidine (5 μM, 5 d) relieved MeCP2 binding and enhanced activated RNA polymerase II (RpolI) occupancy at the promoter. ChIP data (**g**,**h**) are presented as means ± s.d. (four independent experiments). (**i**) The oligonucleotides used in EMSAs encoded CpG 10, 12 and 14, a mutant form of CpG10, and the high-affinity MeCP2-binding site ds1. (**j**) MeCP2 bound strongly to methylated (m), but not to unmethylated (u), CpG10 and ds1. Compared with methylated CpG10, MeCP2 bound less to methylated CpG12 (12m) and CpG14 (14m). A representative autoradiogram and mean DNA-binding values (percentage DB, four independent experiments) are shown. (**k**) Dissociation constants (K_D) for MeCP2 binding.

latter is consistent with the observation that *Avp* transcript levels in this nucleus were not influenced by ELS (**Supplementary Fig. 2**).

Methylation landmarks correlate with *Avp* expression

These data led us to hypothesize that ELS-induced changes in the methylation status of relevant CpG residues are persistent and sustain elevated *Avp* expression, whereas changes in functionally less-significant CpGs ($P > 0.05$) wane over time. To identify CpGs predictive of persistently increased *Avp* expression, we examined each CpG residue in detail. Residues considered to be of predictive value were those that were significantly methylation ($P < 0.05$) marked by ELS at one age at least and nominally altered by more than 25% at two other ages. By these criteria, CpG residues 10, 12, 13, 14 and 15 were revealed as methylation landmarks in the *Avp* enhancer (**Fig. 3f**). The number of CpG residues that were significantly marked by ELS decreased with age (11 in 6-week-old, 7 in 3-month-old and 3 in 1-year-old mice; **Fig. 3f**). With the exception of CpG10, which localized to the upstream boundary (**Fig. 2a**), all of the emerging methylation landmarks mapped to the center of CGI3 (CpGs 12, 13, 14 and 15).

We corroborated the functional role of these residues by correlating their individual methylation status with *Avp* mRNA levels in control and ELS 6-week-old, 3-month-old and 1-year-old mice (**Fig. 3g**). This revealed that the composite methylation status of these residues faithfully reflected longitudinal *Avp* expression. Poor, if any, correlations were found between *Avp* mRNA expression (at any age) and those CpG residues that showed either initial (CpGs 7, 17, 21, 22, 23 and 32) or otherwise transient (CpGs 11, 20 and 31) differences in methylation status (data not shown). This set of findings highlights the functional importance of CpG residues 10, 12, 13, 14 and 15 in the regulation of *Avp* expression.

Although significant hypomethylation ($P < 0.0001$) of the *Avp* enhancer occurred with age in control mice (**Fig. 3d**), we did not observe age-dependent changes in *Avp* mRNA levels (**Fig. 1c**). In contrast, ELS mice did not show age-related hypomethylation of the *Avp* enhancer (**Fig. 3d**), but nevertheless maintained higher levels of *Avp* mRNA, as compared to controls (**Fig. 1c**). This raised the question of whether single CpG residues might be differentially sensitive to age- versus ELS-induced hypomethylation. Analysis of the effects of aging on hypomethylation of all 32 CpGs of the *Avp* enhancer in 6-week-old and 1-year-old control and ELS mice showed that age-associated hypomethylation only occurred in 16% of the CpGs in the *Avp* enhancer region of ELS mice, as compared with 38% in control mice (**Fig. 3h**). Notably, those CpG residues with an assigned regulatory role (methylation landmarks 10, 12, 14 and 15) did not show significant hypomethylation ($P > 0.05$) with aging (**Fig. 3h**).

Enhancer methylation directs *Avp* expression

An AVP-expressing N6 mouse hypothalamic cell line[26] was used to examine whether *Avp* enhancer methylation modulates *Avp* expression

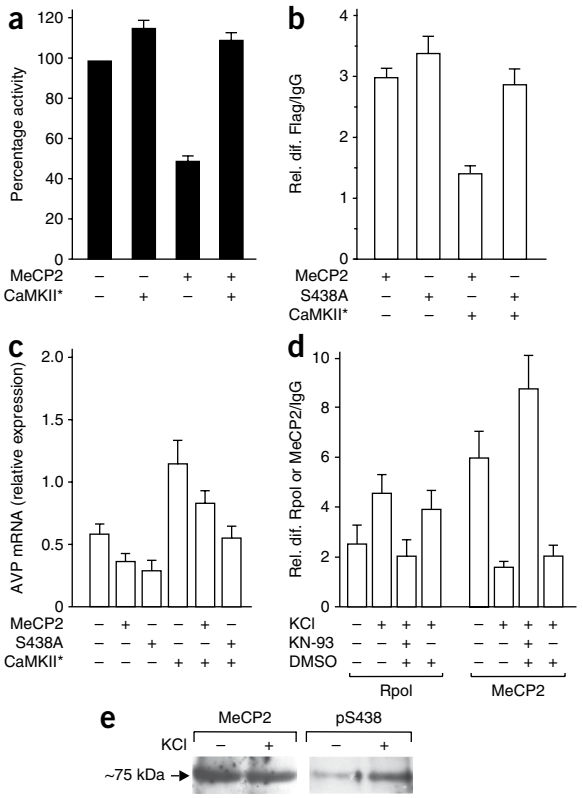

Figure 5 CaMKII relieves MeCP2 occupancy and repression of the *Avp* enhancer. (**a**) CaMKII precludes repression by MeCP2. Transfection of CaMKII* and the site-specific methylated CGI3 vector stimulated *Avp* expression and completely reversed repression by transfected MeCP2 in N6 cells. (**b**) CaMKII abolished MeCP2 occupancy at CGI3. Flag-tagged forms of MeCP2 or S438A (S438 nonphosphorylatable MeCP2) were transfected singly or together with CaMKII* in N6 cells. ChIP experiments with an antibody to Flag showed that CaMKII* mediated the release of MeCP2 from the *Avp* enhancer and that enhancer occupancy was maintained when MeCP2 (S438A) was transfected. (**c**) MeCP2 repressed endogenous *Avp* expression in a CaMKII-regulated manner. Flag-tagged forms of MeCP2 or S438A (S438A) and CaMKII* were cotransfected in N6 cells. RT-PCR analysis of *Avp* expression revealed that CaMKII* attenuated MeCP2-mediated repression; CaMKII* had only minor effects in the presence of MeCP2 S438A. (**d**) Membrane depolarization relieved MeCP2 occupancy at the *Avp* enhancer. N6 cells were depolarized with 55 mM KCl (30 min). ChIP experiments showed reduced MeCP2 occupancy at CGI3, paralleled by increased activated RpoI occupancy at the promoter (CGI1). Pretreatment of N6 cells with a CaMKII inhibitor (KN-93) reversed these effects. (**e**) Immunoblot analysis of MeCP2-S438 phosphorylation. Compared with controls, depolarized N6 cells showed increased MeCP2-pS438 immunoreactivity; depolarization did not influence MeCP2 immunoreactivity. Data (**a**–**d**) are presented as means ± s.d. (four independent experiments).

(**Fig. 4a**). We analyzed of the methylation profile in the CGI3 region that spans CpG10–14 and found a pattern that was similar to the one that we observed in the mouse PVN (**Supplementary Fig. 6**). Treatment of N6 cells with 5-azacytidine, a potent inhibitor of DNA methylation, reduced the level of methylation of the *Avp* enhancer and, concomitantly, increased *Avp* expression (**Fig. 4b** and **Supplementary Fig. 6**). In transfection assays (**Fig. 4c**), deletion of the CGI3 region reduced reporter activity by 37% and deletion of the entire enhancer resulted in almost complete abolition of reporter activity (**Fig. 4d**).

We examined *Avp* gene reporter activity after *in vitro* methylation of the entire *Avp* vector, including the promoter and transcribed regions. Methylation led to a tenfold decrease in reporter activity (**Fig. 4e**). Moreover, reporter activity was reduced by 50% when methylation was targeted specifically to CGI3 (**Fig. 4e**). Together with the results obtained in hypothalamic tissue, this finding suggests that CGI3-specific methylation is critical for the control of *Avp* expression.

MeCP2 selectively binds CGI3 and represses *Avp* expression

DNA methylation is interpreted by a family of methyl CpG–binding domain (MBD) proteins comprising MeCP2, MBD1, MBD2, MBD3 and MBD4, the first three of which couple DNA methylation to transcriptional repression. Although hypothalamic N6 cells expressed MeCP2, MBD1 and MBD2 (**Supplementary Fig. 7**), we found MeCP2 to be the most potent repressor of the CGI3 methylated vector in co-transfection experiments (**Fig. 4f**).

We directly assessed the binding of MeCP2 at the *Avp* locus by immunoprecipitation of cross-linked chromatin from N6 cells with antibodies to MeCP2 or control IgG, followed by PCR analysis of the recovered DNA using seven primer pairs bracketing the *Avp* locus (**Fig. 4g**). As expected, MeCP2 did not occupy the poorly methylated *Avp* promoter and exonic CpG islands (CGI1 and 2); moreover, MeCP2 was also absent at CGI4, which is methylated to a relatively high extent. In contrast, MeCP2 was strongly enriched at the CGI3 region (**Fig. 4g**). Notably, CGI3, but not CGI4, was poorly recovered when the same chromatin samples were immunoprecipitated with antibodies to MBD1 and MBD2 (**Supplementary Fig. 7**). Thus, MeCP2 preferentially and selectively occupies CGI3 of the *Avp* enhancer.

Pre-treatment of N6 cells with 5-azacytidine robustly decreased MeCP2 occupancy at CGI3 and increased promoter binding of activated (pSer5) RNA polymerase II and *Avp* transcription in parallel (**Fig. 4b**,**h**). Therefore, MeCP2 occupancy at the *Avp* locus is DNA methylation-dependent and, once bound, MeCP2 acts to repress transcription. High-affinity binding of MeCP2 to methylated DNA requires a local sequence context, namely a symmetrical methyl-CpG dinucleotide that localizes close to a run of four or more A/T bases that facilitate DNA binding[27]. We identified four CpG dinucleotides (CpGs 13, 14 and 21, as well as the highly relevant CpG10) that matched the latter criterion in the CGI3 sequence (**Fig. 4i**). Their function in the context of MeCP2 binding was tested by *in vitro* DNA-binding electrophoretic mobility shift assays (EMSAs), using recombinant MeCP2 and oligonucleotides that spanned CpG10, CpG12 or CpG14. As anticipated, methylation of these motifs proved to be essential for MeCP2 binding and effective self-competition (**Fig. 4j** and **Supplementary Fig. 8**). MeCP2 specifically bound to the key motif CpG10 with a K_D of ≈2.6 nM (comparable to that previously reported[27]) and DNA binding was strongly impaired (K_D > 50 nM) after mutation of the A/T run adjacent to the CpG10 dinucleotide (CpG10A/Tmut) (**Fig. 4k**). Compared with CpG10, the neighboring motifs CpG12 and CpG14 bound MeCP2 with lower affinity (K_D ≈ 9.6 and 10.5 nM, respectively) and competed poorly with CpG10 for forming a complex with MeCP2 (**Supplementary Fig. 8**). Together, these results indicate that the *Avp* enhancer contains context-specific, high-affinity MeCP2 DNA-binding sites that are important for the regulation of *Avp*.

Phosphorylation of MeCP2 prevents *Avp* enhancer occupancy

Neuronal depolarization has been shown to trigger Ca^{2+}-dependent phosphorylation of MeCP2, causing dissociation of MeCP2 from the *Bdnf* promoter and increased *Bdnf* transcription[28,29]. Recently, *de novo* Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was shown to mediate phosphorylation of rat MeCP2 at serine 421 (S438 in mouse)[30].

To explore whether this mechanism might be responsible for regulating MeCP2 occupancy at the *Avp* enhancer, we transfected N6 cells with either MeCP2 and/or a constitutively active form of CaMKII (CaMKII*) together with the CGI3-methlyated *Avp* vector. Transfection of CaMKII* increased *Avp* reporter activity slightly, and completely reversed MeCP2-mediated repression (**Fig. 5a**). Furthermore, we observed that CaMKII* markedly reduced the occupancy of Flag-tagged MeCP2 at the *Avp* enhancer, but failed to release DNA-bound nonphosphorylatable MeCP2 (S438A; **Fig. 5b**). Subsequently, MeCP2-S438A and (to a lesser degree) MeCP2 prevented CaMKII* activity–dependent increases in *Avp* expression (**Fig. 5c**); this result is consistent with a repressive role of MeCP2.

Additional experiments showed that K^+-induced depolarization of N6 cells faithfully reproduced the effects of CaMKII* transfection, that is, relieved MeCP2 occupancy at the *Avp* enhancer. A role of CaMKII in mediating MeCP2-S438 phosphorylation was confirmed by the complete reversal of this regulation after pretreatment with the CaMKII inhibitor KN-93. Moreover, K^+-induced depolarization increased the presence of activated RNA polymerase II at the *Avp* promoter (**Fig. 5d**), verifying the ability of MeCP2 to repress activity-dependent gene expression[6]. Lastly, an antibody to the regulatory MeCP2-S438 phosphorylation site (MeCP2-pS438; **Supplementary Fig. 9**) reacted strongly with extracts from membrane-depolarized N6 cells, but only weakly with extracts from nonstimulated N6 cells (**Fig. 5e**). Thus, membrane depolarization directly leads to phosphorylation of MeCP2 at S438.

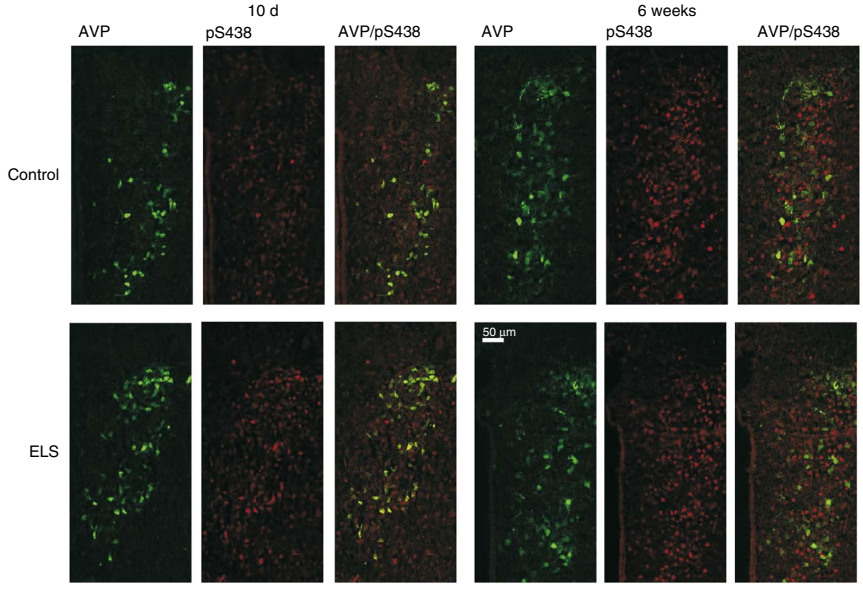

Figure 6 ELS induces phosphorylation of MeCP2 in parvocellular PVN neurons. ELS led to increased immunostaining of MeCP2-pS438 (pS438) and AVP in the PVN of 10-d-old mice. Colocalization of AVP and MeCP2-pS438 in parvocellular neurons in the PVN was apparent. Comparable levels of MeCP2-pS438 staining were detected in 6-week-old control and ELS mice. The images that are shown are representative of five mice per group and age.

ELS reduces MeCP2 occupancy at *Avp* enhancer

We next asked whether the sustained increased expression of *Avp* after ELS is triggered by MeCP2-S438 phosphorylation and subsequent relief of MeCP2 occupancy at the *Avp* enhancer. This hypothesis was supported by the observation that MeCP2-S438 phosphorylation was prominently increased in parvocellular AVP-expressing neurons in the PVN of 10-d-old ELS mice (**Fig. 6**). In addition, the PVN of 10-d-old ELS mice had increased phospho-CaMKII immunoreactivity in AVP-positive neurons, a finding that is compatible with a role for this kinase in the mediation of activity-dependent MeCP2-S438 phosphorylation (**Supplementary Fig. 10**). The extents to which CaMKII and MeCP2-S438 were phosphorylated in the parvocellular division of the PVN did not differ between 6-week-old control and ELS mice (**Fig. 6** and **Supplementary Fig. 10**). In addition, neither *Mecp2* mRNA nor total MeCP2 and CaMKII protein expression differed between the two groups (**Supplementary Fig. 10**). Lastly, MeCP2-pS438 immunoreactivity in the supraoptic nucleus did not differ between control and ELS mice at all ages, demonstrating the site-specificity of the effects (data not shown). These results indicate that there is an age- and cell type–specific role for ELS-induced MeCP2-S438 phosphorylation, prompting us to examine its relevance for enhancer occupancy.

Although RNA polymerase II occupancy at the *Avp* promoter and *Avp* expression

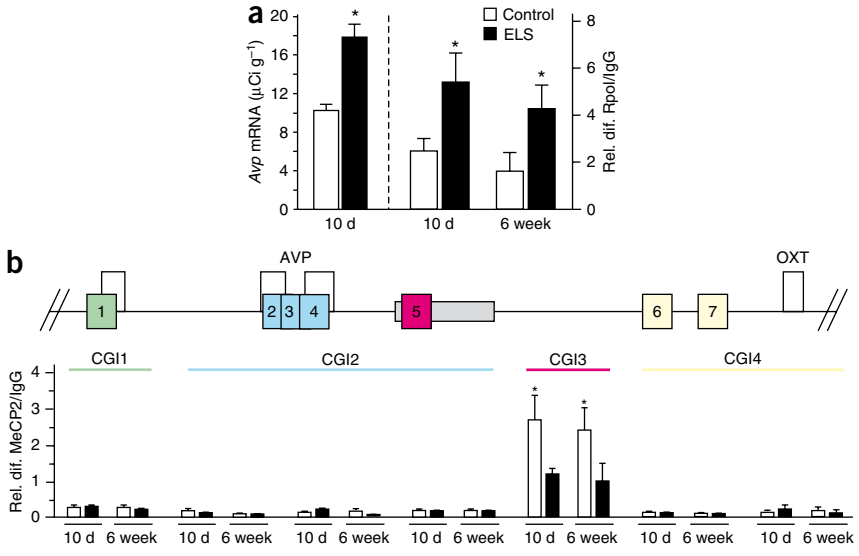

Figure 7 ELS reduces MeCP2 occupancy at the *Avp* enhancer. (**a**) ELS upregulated *Avp* expression in the parvocellular PVN (measured by ISH) of 10-d-old mice. *In vivo* ChIP analysis revealed increased activated RpoI occupancy at the *Avp* promoter (CGI1) of ELS mice, reflecting increased *Avp* transcription. (**b**) ELS reduced MeCP2 occupancy at CGI3. An *in vivo* ChIP scan of the *Avp* locus (schematized above) revealed selective MeCP2 occupancy at CGI3. ELS significantly reduced MeCP2 occupancy at this region in both 10-d-old and 6-week-old mice. Data are presented as means ± s.e.m. ($n = 8$ mice per group for ISH analysis; ChIP analysis based on five groups of pooled PVN). * $P < 0.05$ (t test).

were markedly increased in 10-d-old ELS mice (**Fig. 7a**), CGI3 methylation did not differ between control and ELS mice of this age (**Supplementary Fig. 11**). Notably, *in vivo* chromatin immunoprecipitation (ChIP) experiments on PVN tissue revealed that, of the various MBDs, MeCP2 was selectively enriched at CGI3 in 10-d-old and 6-week-old control mice (**Fig. 7b** and data not shown) and that binding of MeCP2 to the *Avp* enhancer was reduced in ELS mice of both ages (**Fig. 7b**). Given that 10-d-old control and ELS mice have identical methylation patterns, the measured differences in MeCP2 occupancy suggest that ELS-induced MeCP2-S438 phosphorylation results in relief of MeCP2 occupancy at the *Avp* enhancer. The repressive function of MeCP2 at the *Avp* enhancer was substantiated by sequential *in vivo* ChIP experiments, which revealed strong coupling of MeCP2 to transcriptionally inactive chromatin marks (data not shown), comparable with those reported for *Crh* occupancy[31].

In sum, de-repression of *Avp* transcription in 10-d-old ELS mice appears to involve increased MeCP2-S438 phosphorylation, whereas reduced enhancer occupancy in 6-week-old mice most likely reflects ELS-induced CGI3 hypomethylation (**Figs. 3a** and **7b**).

DISCUSSION

Adverse experiences during early life contribute to the etiology of psychiatric conditions in later life[14,32]. Our results suggest that ELS in mice leads to epigenetic marking (hypomethylation) of a key regulatory region of the *Avp* gene in the PVN. These epigenetic events are accompanied by persistent upregulation of *Avp* expression in the parvocellular subdivision of the PVN and, consequently, sustained hyperactivity of the HPA axis. Notably, the ELS-induced endocrine phenotype lasted for at least 1 year following the initial adverse event and could be normalized through administration of an AVP V1b receptor antagonist.

Studies in humans and in animal models suggest that stress or elevated glucocorticoid secretion are important for the function of interdependently regulated behavioral domains[33,34]. Here, ELS-treated mice showed increased immobility in the forced-swim test, which assesses stress-coping ability[35], and had deficits in step-down avoidance learning. Although acute rises in glucocorticoid secretion can facilitate inhibitory avoidance learning[13], our data support the notion that sustained elevated glucocorticoid levels impair memory performance in ELS mice. Notably, the behavioral phenotypes induced by ELS were shown to be partly reversible after antagonism of AVP V1b receptors, thus highlighting AVP as an important mediator of these processes, but not necessarily the only[14].

Our results identify CpG residues in the CGI3 region of the *Avp* enhancer whose persistent hypomethylation after ELS is critical for the regulation of *Avp* expression. Recent work defined these residues as being high-affinity, context-specific MeCP2 DNA-binding sites[27]. On the basis of previous reports[28,29], we hypothesized that signaling mechanisms controlling MeCP2 occupancy are critical for gene repression and the dynamic methylation of CGI3 in response to ELS. Supporting this, we found that depolarization of hypothalamic cells can regulate MeCP2 function by inducing its site-specific phosphorylation via CaMKII activity. Taken together, our results indicate that phosphorylation of MeCP2 at S438 is critical for MeCP2 to function as a reader and interpreter of the DNA methylation signal at the *Avp* enhancer.

That experience-dependent stimuli dynamically control the methylation of CGI3 is supported by the observation that ELS induced contemporaneous increases in CaMKII activation, MeCP2-S438 phosphorylation and *Avp* expression in 10-d-old mice. On the other hand, MeCP2-S438 and CaMKII were phosphorylated to similar extents in adult control and ELS mice, indicating that ELS-induced MeCP2 phosphorylation is important for the establishment of epigenetic marks. Once established, the observed differences in *Avp* enhancer methylation centered on MeCP2 binding sites, which appeared to be actively maintained in ELS and control mice. This interpretation is compatible with the view that MeCP2 serves as an epigenetic integration platform on which synergistic cross-talk between histone deacetylation, H3K9 methylation and DNA methylation act to confer gene silencing[22].

From a physiological perspective, it is conceivable that increased methylation of the *Avp* enhancer during early postnatal life serves to restrain the HPA axis in critical periods when homeostatic thresholds are set; this would facilitate adaptation of the endocrine system to future environmental stimuli. Our data suggest that ELS tilts the balance toward persistent hypomethylation and *Avp* overexpression by inducing reductions in MeCP2 binding. Thus, phosphorylation of MeCP2 appears to be a conduit of experience-driven changes in gene expression, serving as an important mediator of the persistent effects of ELS. In this respect, certain parallels may be drawn between the mechanisms underlying ELS and Rett syndrome; the latter, caused by mutations in *Mecp2*, also presents with altered cognitive, mood and HPA axis function[36].

Together with other recent work[3,10], our results suggest that adverse events in early life can leave persistent epigenetic marks on specific genes that may prime susceptibility to neuroendocrine and behavioral dysfunction. Focusing on DNA methylation, our results provide evidence for postmitotic epigenetic modifications in neuronal function; such modifications can serve to facilitate (or disfavor) physiological and behavioral adaptations[3,37]. These marks and their initiators, mediators and readers (for example, MeCP2) provide new inroads for understanding the molecular basis of stress-related disorders of the brain.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

The study was conceived and designed by D.S. and O.F.X.A. C.M. and D.S. designed and interpreted the molecular studies that were carried out by C.M., Y.W., Y.B. and D.F., A.V.P. and O.F.X.A. were responsible for the neuroendocrine studies and A.V.P. and V.M. carried out the behavioral experiments under the guidance of C.T.W. C.M., A.V.P., F.H., O.F.X.A. and D.S. wrote the paper, with input from all of the other authors.

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ONLINE METHODS

ELS. Maternal separation stress[11,15] was used to induce ELS. Briefly, pups delivered (postnatal day 0 (P0) on day of birth) by timed-pregnant C57BL/6N mice (Charles River) were placed, as individual litters, in a clean cage (with heating pad) for 3 h each day on P1–10, having no physical contact with their mothers. Control (non-ELS) pups remained undisturbed in the maternal nest throughout. Pups remained with their mothers until weaning (P21), when they were housed in sex-matched groups (3–5 mice per cage); only males were used for analyses. Standard laboratory animal housing conditions were maintained throughout, with 12-h daily illumination (lights on at 06:00). All procedures were approved by the Regierung von Oberbayern and were in accordance with European Union Directive 86/609/EEC.

Behavioral phenotyping. At 3 months of age, control and ELS mice were housed singly and randomly assigned to the following long-term treatment groups: naive (no injections), vehicle (5% DMSO (vol/vol), 5% Chremophor EL (vol/vol), saline, intraperitoneal injection), and SSR149415 (20 mg per kg of body weight per d). Treatments started 4 weeks before behavioral phenotyping and continued for the duration of the experiments; injections were administered 1 h before behavioral testing. An investigator who was blind to the treatments carried out the behavioral analyses; an interval of 2 d was allowed between each test procedure. Anxiety-like behavior was assessed in the elevated plus-maze[38], light-dark avoidance[39] and novelty-induced hypophagia[40] tests.

The forced swim test was used to evaluate passive stress coping behavior and was performed essentially as described previously[41]. Briefly, each mouse was placed into a glass beaker (5 l × 23.5 × 16.5 cm) that was filled with water (25 ± 1 °C) up to a height of 15 cm, for 6 min. Floating (immobility) was scored during the last 4 min of the exposure. A mouse was considered to be immobile when it floated passively in an upright position, making only small movements to keep its head above water surface.

Memory was evaluated using the step-down avoidance learning test. Training sessions involved placing mice onto a platform (2.5 × 10 × 10 cm^3) and administering a scrambled electric foot shock (0.7 mA) when they stepped off the platform onto a metal grid; mice were thereafter immediately returned to their home cages. Passive avoidance memory was tested by placing mice back onto the platform 24 h later and step-down latencies (four-paws criterion) were measured in three consecutive trials. Trials were terminated after 5 min in cases of failure to step down; a step-down latency of 301 s was ascribed to the trial. The mean of the three trials served as a measure of memory performance.

Tissue preparation and hormone assays. Serum corticosterone was measured in adulthood by radioimmunoassay in blood samples. Samples were collected at 6 p.m. (peak) and 30 min following application of a previously described acute psychological stressor (9–11 a.m.)[42]. At the age of 3 months, mice received an intraperitoneal injection of dexamethasone (10 μg per 100 g) at 12 a.m. noon and blood was collected at 6 p.m. for determination of corticosterone; the latter measurements were compared to values obtained at the nocturnal sampling on the previous day. At various ages, mice were killed by cervical dislocation and tissues (brain, pituitary, thymus and adrenal) were collected. Brains for ISH and micropunching were cryosectioned (10 μm) at the level of the rostral PVN (bregma −0.75 to −0.85) and the hippocampus (bregma −1.70 to −1.90). Punches of the PVN were obtained by *in loco* microdissection under histological control.

ISH. *Avp* and *Crh* transcripts were detected with 48/50-mer ^{35}S-labeled antisense probes, complementary to the murine *Avp* (bases 1,493–1,540, accession number M88354) and *Crh* (bases 1,685–1,732, accession number AY128673) genes, respectively. *Nr3c1* and *Mecp2* transcripts were measured using ribonucleotide probes (*Nr3c1*, bases 81–528, accession number M14053; *Mecp2*, bases 612–1,604, accession number NM010788) and previously published protocols[42]. *Avp*, *Crh* and *Mecp2* transcript signal intensities were measured in the ventromedial compartment of the PVN, representing the parvocellular division. *Nr3c1* hybridization signals were measured in the PVN and hippocampal subfields CA1–3 and dentate gyrus.

Bisulfite sequencing. Genomic DNA (200–400 ng) isolated from PVN tissue punches was digested with *Eco*RI, sodium bisulfite converted (Qiagen DNA methylation kit), aliquoted and used for PCR reactions. Primers used are listed in **Supplementary Table 1**. Products were cloned into pGEM-T vector; at least 20 independent recombinant clones per PCR and mouse were analyzed on an ABI Prism 3700 capillary sequencer. Overall methylation levels (**Fig. 3d**) were calculated for the entire enhancer region from mean levels of individual CpG residues.

RT-PCR and RNA extractions. Primer sequences are listed in **Supplementary Table 2**. The expression levels of the housekeeping genes *Hprt* and *Gapdh* were used for normalization. Total RNA was extracted with Trizol (Invitrogen) and reverse-transcription reactions were performed on 1 μg of total cell culture–extracted RNA or 100 ng of tissue-derived RNA with SuperscriptII (Invitrogen) and poly-dT primer. qPCR was carried out on a LightCycler (Roche) using LightCycler FastStart DNA Master Plus SYBR Green (Roche).

***In vitro* methylation.** Vectors were methylated with SssI and S-adenosylmethionine (New England Biolabs). For site-specific methylation, an 888-bp fragment containing CpGs 10–25 in the *Avp* enhancer was excised by digestion with *Eco*81I; the vector was further digested with *Xba*I and *Bam*HI to prevent re-ligation. Methylated or control unmethylated digests were ligated into the dephosphorylated reporter construct, cleaved with *Eco*81I. Completeness of *in vitro* methylation and maintenance (until cell harvesting) was confirmed by bisulfite sequencing.

Recombinant proteins and EMSA. GST- or His-MeCP2 fusion proteins were grown in DH5α, purified and quantified as described previously[43]. For *in vitro* DNA-binding assays (**Fig. 4j** and **Supplementary Fig. 8**), recombinant MeCP2 (0.5 μg) was incubated with 20,000 cpm of double-stranded ^{32}P end-labeled naive or *in vitro* methylated oligonucleotides[27]. Reactions were fractionated on 8% polyacrylamide gels. Although GST protein itself does not recognize methylated CpG10, inclusion of a GST antibody abolished MeCP2 binding, verifying the identity of the shifted complex (**Fig. 4j**). Dissociation constants (K_D) were deduced by Scatchard analysis of saturation binding isotherms[43].

Plasmids. The AVP expression vectors (kindly gifted by H. Gainer and R.L. Fields, US National Institutes of Health) were modified by exchanging the *egfp* reporter gene in the third exon of the *Avp* gene[44], with a cDNA for *Gaussia* luciferase KDEL encoding intracellular *Gaussia* luciferase (Targeting Systems) (see **Fig. 4c**). The parent *Avp-Gaussia* construct contained 288 bp of the promoter region, all exons (numbered) and introns, and the entire 2.1-kb enhancer. The *Avp*Δenhancer construct has the entire enhancer sequence removed, whereas the *Avp*Δ10–25 was generated by deletion of an 888-bp fragment of the enhancer containing CpGs1–25 by digestion with *Eco*81I and subsequent vector religation.

The *Mecp2* expression vector and His-tagged MeCP2 1–205 (kindly provided by A. Bird, University of Edinburgh) consisted of the mouse MeCP2α variant[45] in pRL-SV40 (Promega) and of a cDNA for the first 205 amino acids of human MeCP2 with a C-terminal His-tag[46] in a pet30b vector (Novagen), respectively. For prokaryotic expression (*pGEx2tk-Mecp2*), the MeCP2α cDNA was PCR amplified (forward primer, AAG GGA TCC GTA GCT GGG ATG TTA GG; reverse primer, TCT GAT ATC CTC AGT GGT GGA GGA GGA G) and inserted into the *Bam*HI and *Sma*I sites of *pGEx2tk* (Pharmacia).

N-terminal Flag-tagged forms of different *Mecp2* constructs were obtained by PCR cloning of wild type (forward primer, AAG GGA TCC GCC GCC GCT GCC GCC ACC GC; reverse primer, TCT GAT ATC CTC AGC TAA CTC TCT CGG TC) or of a form lacking the 45 C-terminal amino acids of MeCP2 (forward primer, AAG GGA TCC GCC GCC GCT GCC GCC ACC GC; reverse primer, TCT GAT ATC CTC AGC TAA CTC TCT CGG TC) into the *Bam*HI and *Eco*RI sites of pRK7-Flag[43]. The phosphor-acceptor residue Ser 438 was replaced by Ala in MeCP2 (S438A) by site-directed mutagenesis (forward primer, CCC GAG GAG GCC GAC TGG AAA GCG ATG GC; reverse primer, GCC ATC GCT TTC CAG TCG GCC TCC TCG GG).

The MeCP2 riboprobe (nucleotides 612–1,604, accession number NM_010788) contains the conserved sequence in exons 3 and 4 of the mouse *Mecp2* gene. A corresponding PCR product (forward primer, AAA GGT GGG AGA CAC CTC CT; reverse primer, TCC ACA GGC TCC TCT CTG TT) was cloned in the pGEM-T vector (Promega) for generation of riboprobes.

Expression vectors for MBD2 and MBD3 (kindly provided by S.T. Jacob, Ohio State University) contain the mouse MBD2 or MBD3 cDNAs[47] in the pcDNA3.1 vector (Invitrogen). The MBD1 expression vector contains the full-length cDNA for mouse MBD1 (accession number NM_013594; forward primer, TAC CTC TAG

AAT GGC TGA GGA CTG GCT GGA CTG; reverse primer, TTT CTA GAA ACA ATT TGC AAA GAA TTT TCA GG) inserted in the pRK7 expression vector.

CaMKII expression vectors contained either full-length CaMKII (1–317) or CaMKII (1–290), a constitutively active form resulting from the absence of the calmodulin-binding domain (kindly provided by A.R. Means, Duke University Medical Center). The constitutively active CaMKII(T286D) contains a replacement of Thr286, which is located in the autoinhibitory domain[48], by Asp (kindly provided G. Turrigiano, Brandeis University). All constructs used in this study were entirely sequence verified.

Cell culture and transfection experiments. Mouse hypothalamic cells (N6 line)[26] were grown using standard conditions (DMEM supplemented with 10% fetal calf serum, vol/vol). Cells (10^5) were treated for 5 consecutive days with different concentrations of 5-azacytidine (Calbiochem), which was replenished in fresh growing medium every other day. N6 cells were transfected using Lipofectamine 2000 (Invitrogen). Briefly, 8×10^5 cells were seeded 24 h earlier in 6-well plates. DNA was mixed with 4 µl of Lipofectamine, incubated at 25 °C for 20 min and then added to the cells, which were grown for 18 h. Epithelial kidney cells (LLC-PK1, ATTC CL-101) were transfected by electroporation as described previously[49]. For cotransfection, we used 0.1 µg of the *Camk2a* expression constructs, 1 µg of the pRK7-Flag *Mecp2* constructs and 1 µg of the *Mbd1*, *Mbd2* and *Mbd3* expression vectors. Luciferase values were normalized against β-galactosidase values[42].

Immunohistochemistry, immunoblots and ChIP experiments. Brains were extracted from microwave-fixed heads, placed overnight in 4% paraformaldehyde (wt/vol), cryo-preserved and sectioned (20 µm) at the level of the PVN before immunostaining. Immunoblot analysis was carried out on whole-cell extracts (50 µg) after fractionation by PAGE gel electrophoresis[43]. For ChIP experiments, chromatin from N6 cells or mouse PVN punches (individual pools formed from groups of three or five mice for 6-week-old and 10-d-old mice, respectively) was cross-linked[50], disrupted by sonification (Diagenode Bioruptor, and purified with the Magna ChIP G kit (Millipore). The ChIP primers that we used for qPCR are listed in **Supplementary Table 3**.

Antibodies. The antibodies that we used are listed in **Supplementary Table 4**. The polyclonal antibody to MeCP2, which recognizes MeCP2 (accession number GI:123122664) irrespective of its phosphorylation status, was generated by injecting New Zealand White rabbits with the KLH-conjugated peptide NH2-CSMPRPNREEPVDSRTPV-CONH2, corresponding to amino acids 480–496. The antiserum was purified by affinity chromatography on a column containing coupled MeCP2 480–496 peptide and the affinity-purified antibody to MeCP2 was eluted.

The polyclonal antibody to phosphor–S438-MeCP2 (MeCP2-pS438) was generated by injecting New Zealand White rabbits with the KLH-conjugated peptide NH2-CMPRGGpSLES-CONH2. The antiserum was purified by affinity chromatography on a column containing coupled nonphosphorylated MeCP2-S438 peptide. The flow through was then passed over a second column containing coupled phosphorylated MeCP2-S438 peptide and the affinity-purified antibody to MeCP2-pS438 was eluted.

Characterization of the MeCP2 antibodies was performed by transfection of pRK7-FLAG MeCP2 and pRK7-FLAG MeCP2 (S438A) (0.1 µg each), singly or together, with the different CaMKII expression vectors (0.5 µg each) into LLC-PK1cells. Mock transfections were performed using an equal amount of filling plasmid. Whole-cell extracts (20 µg) were fractionated on 8% SDS-PAGE gels, immunoblotted and tested with either antibody to MeCP2 (1:1,000), the UP-MeCP2 (Upstate, 07013, 1:1,000), antibody to MeCP2-pS438 (1:1,000) or antibody to Flag (1:1,000). In a parallel set of experiments, the same cellular lysates were treated for 1 h at 37C° with calf intestine phosphatase (10 units) or assay buffer alone followed by PAGE gel electrophoresis.

Antibody to MeCP2-pS438 and antibody to MeCP2-pS421 (kindly provided by Z. Zhou and M.E. Greenberg[30], Harvard Medical School) produced similar results when tested on PVN sections (**Supplementary Fig. 8**).

Statistical analysis. Numerical data were analyzed by *t* tests or ANOVA, followed by Newman-Keuls *post hoc* test. In all cases, the nominal level of significance was $P \leq 0.05$. Correlations between AVP expression and CpG methylation were tested by Pearson's correlation coefficient.

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2.2 Chapter 2: Depletion of the Neural Precursor Cell Pool by Glucocorticoids

Yu S, **Patchev AV** et al., *Annals of Neurology*, 2010

2.2.1 Rationale

Glucocorticoids are well known to affect neuronal cell death and neurogenesis (Yu et al., 2008; Sousa et al., 2008). Previous work has shown that neuronal plasticity depends strongly (although not solely) on neuronal proliferation and differentiation from the neural precursor cell pool (Pittenger and Duman, 2008). The dentate gyrus within the hippocampal formation, is a brain area with a high degree of neuronal proliferation from multipotent precursors, especially during early ontogeny, but also in later life (Cameron and McKay, 1999). Albeit disputed, adult neurogenesis from the neuronal precursor cell pool has been ascribed a role in mediating the effects of (at least) some antidepressants (Hanson et al., 2011b; Pittenger and Duman, 2008). With regard to the use of glucocorticoids in obstetrics and perinatology, we examined the targeting of neuronal precursors for apoptosis by glucocorticoids and individual aspects of this process *in vitro*. We scrutinized whether neonatal application of corticosteroids to rat pups would affect the amount of neuronal precursor cells, and thus, hippocampal volume and function in later life.

2.2.2 Major findings

- *in vitro*
    - neural precursors, as well as immature neurons, express functional GR
    - GC induce apoptosis of neuronal cells in a dose-dependent manner
    - GC target proliferating, but also resting precursors for apoptosis
    - GC-induced apoptosis of neuronal precursors is mediated via caspase 3 and caspase 9, and mitochondrial reactive oxygen species (ROS) generation

- *in vivo*

    - neonatal GC administration leads to increased apoptosis in the subgranular zone (SGZ), where most NPCs are located
    - both quiescent and amplifying neuronal precursors in the SGZ express GR
    - neonatal GC treatment reduces the neurogenic capacity in later life by reducing the number of cells available for mitosis and proliferation
    - these changes are mirrored by corresponding growth retardation of the SGZ, as well as the granule cell layer, where more mature neurons are located

2.2.3 Conclusions

GC exposure during early ontogeny can affect brain plasticity in later life. Here we show that these effects also comprise apoptosis of the neural precursor and progenitor pool, thus reducing the cells available for neurogenesis in later life. These findings suggest the possibility that animals exposed to GC in early life might respond differently to stress and/or antidepressants in adulthood. The medical relevance of this work is epitomized by the fact that both, stress and antidepressants have been shown to affect hippocampal neurogenesis, while GC appear to be a key permissive component in this latter process.

2.2.4 Outlook / ongoing studies

To prove the hypothesis, that neonatal GC exposure selectively reduces the NPC pool and, thus, could lead to differential responses to stress and/or antidepressant medication, we carried out additional studies where animals treated neonatally with GC were followed up until 5-6 months of age and then subjected to either chronic unpredictable stress (known to reduce neurogenesis and increase apoptosis in the hippocampus (Sousa et al., 2008; Yu et al., 2008)) and/or daily Fluoxetine treatment (known to increase neurogenesis (Hanson et al., 2011b)). First results indicate that neonatally GC treated animals display differential HPA axis responses under basal circadian, as well as acute stress conditions. Currently, stereological evaluation of hippocampal cell numbers (including apoptotic and recently proliferated cells) and volumes is in progress. In addition to alteration of cell numbers, the observed volumetric changes indicate that neonatal

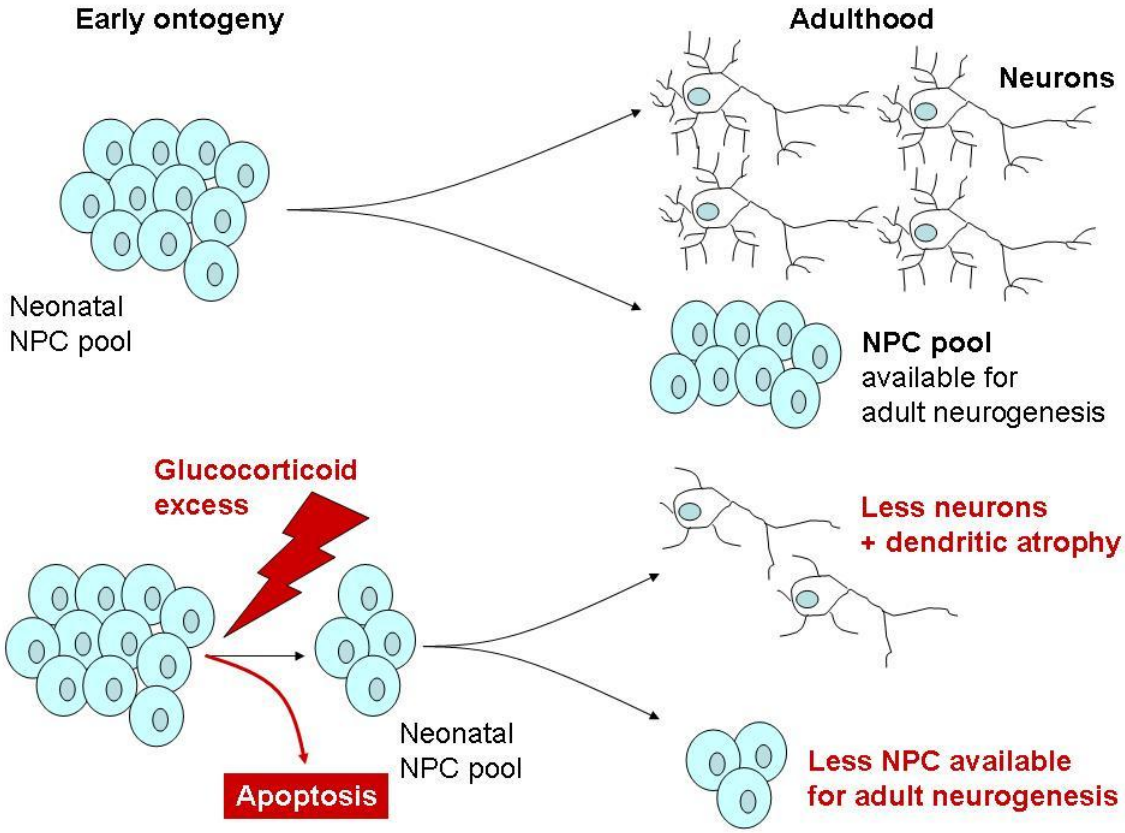
GC treatment also has long term (programming) effects on dendritic arborization and synaptic plasticity (Sousa et al., 2008). Further intriguing questions are exemplified by ***i)*** the neurochemical characteristics of the surviving proliferating cells; ***ii)*** their capacity to become integrated in functional networks derived from NPC pools that have not experienced neonatal GC impact, and ***iii)*** their resistance to noxious stimuli.

2.2.5 Contributions

- planning and performing of in vivo studies, including neonatal treatment, behavioral and endocrine analysis
- involvement in histological analysis and preparation of the manuscript.

Figure 2 Model of long-term effects of glucocorticoid-induced apoptosis of NPC during early ontogeny

Supraphysiological doses of GC during early ontogeny (here neonatal period) induce apoptosis of NPC, thus reducing the number of NPC in the pool available for adult neurogenesis in the hippocampus. In addition, recent observations indicate that not only hippocampal neuronal numbers, but also dendritic arborization are reduced in animals with a history of neonatal GC exposure.



Depletion of the Neural Precursor Cell Pool by Glucocorticoids

Shuang Yu, MD, PhD,[1] Alexandre V. Patchev, MD,[1] Yan Wu, MD, PhD,[1] Jie Lu, MD, PhD,[1] Florian Holsboer, MD, PhD,[1] Jing-Zhong Zhang, MD, PhD,[2] Nuno Sousa, MD, PhD,[3] and Osborne F. X. Almeida, PhD[1]

Objective: Glucocorticoids (GCs) are indicated for a number of conditions in obstetrics and perinatal medicine; however, the neurodevelopmental and long-term neurological consequences of early-life GC exposure are still largely unknown. Preclinical studies have demonstrated that GCs have a major influence on hippocampal cell turnover by inhibiting neurogenesis and stimulating apoptosis of mature neurons. Here we examined the fate of the limited pool of neural progenitor cells (NPCs) after GC administration during neonatal development; the impact of this treatment on hippocampal structure was also studied.
Methods: Phenotype-specific genetic and antigenic markers were used to identify cultured NPCs at various developmental stages; the survival of these cells was monitored after exposure to the synthetic glucocorticoid dexamethasone (DEX). In addition, the effects of neonatal DEX treatment on the neurogenic potential of the rat hippocampus were examined by monitoring the incorporation of bromodeoxyuridine and expression of Ki67 antigen at various postnatal ages.
Results: Multipotent nestin-expressing NPCs and Tα1-tubulin–expressing immature neurons succumb to GC-induced apoptosis in primary hippocampal cultures. Neonatal GC treatment results in marked apoptosis among the proliferating population of cells in the dentate gyrus, depletes the NPC pool, and leads to significant and sustained reductions in the volume of the dentate gyrus.
Interpretation: Both NPCs and immature neurons in the hippocampus are sensitive to the proapoptotic actions of GCs. Depletion of the limited NPC pool during early life retards hippocampal growth, thus allowing predictions about the potential neurological and psychiatric consequences of neonatal GC exposure.

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Acquisition and loss of hippocampal neurons are implicated in the regulation of cognition, mood, and neuroendocrine function.[1–4] Most likely, the availability of hippocampal neurons determines neuroplastic changes in the intrahippocampal circuitry as well as connectivity between the hippocampus and other cortical and subcortical areas.[5] The subgranular zone (SGZ) of the hippocampal dentate gyrus is endowed with a pool of neural precursor cells (NPCs) that can divide and differentiate into either neurons or glial cells.[2,6] Newly generated neurons integrate into existing hippocampal circuits[6] and facilitate learning and memory.[2–4] Neurogenesis tapers off over a lifetime and is regulated by intrinsic (eg, age[7–9]) and extrinsic signals (eg, stress[10,11]), whose actions are mainly mediated by glucocorticoids (GCs). Because the size of the NPC pool is a potentially important determinant of lifelong hippocampal function, there is considerable interest in the link between lifetime neurogenesis and cognitive deficits that result from exposure to high GC levels.[12,13]

The synthetic glucocorticoid receptor (GR) agonist dexamethasone (DEX) is commonly used in obstetrics

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Address correspondence to: Dr Shuang Yu and Dr Osborne F. X. Almeida, Max Planck Institute of Psychiatry, Kraepelinstrasse 2, 80804 Munich, Germany. E-mail:shuang@mpipsykl.mpg.de; osa@mpipsykl.mpg.de

Current address for Dr Wu is Department of Anatomy, Capital Medical University, Beijing, China.

Current address for Dr Lu is Department of Neurology, Harvard Medical School, Boston, MA.

From the [1]Max Planck Institute of Psychiatry, Munich, Germany; [2]Beijing Institute for Neuroscience, Capital Medical University, Beijing, China; and [3]Life and Health Sciences Research Institute, University of Minho, Braga, Portugal.

and neonatal medicine. Previously, we demonstrated that DEX induces cell cycle arrest[14]and apoptosis[15–17] in mature neurons of the dentate gyrus. In this study, we addressed the question of whether DEX can directly influence the survival of NPCs. In addition, we tested the hypothesis that neonatal DEX administration permanently depletes the neurogenic pool. Our results show that GCs target NPCs for apoptosis and that neonatal GCs markedly reduce the number of NPCs available for the generation of new neurons.

Materials and Methods

Drugs and Plasmid

DEX (Fortecortin, Merck, Darmstadt, Germany), was added to cultures for 48 hours (24 hours after transfection). The GR antagonist RU38486 (10μM; NHPP, Torrance, CA) was added 1 hour before DEX application. Mitotic cells were labeled (24 hours) with 5-bromo-2′-deoxyuridine (BrdU) (20μM; Sigma, St. Louis, MO). The specific caspase 3 and 9 inhibitors Ac-DEVD-cmk (1μM) and Ac-LEHD-cmk (30μM) were obtained from Calbiochem (Schwalbach, Germany) and applied 30 minutes before addition of DEX.

NPCs, neural progenitors, and astrocytes were labeled with pBSIISK-E/nestin–enhanced green fluorescent protein (EGFP),[18] pBSII SK-Tα1–green fluorescent protein (GFP),[19] and phosphorylated glial fibrillary acidic protein (pGFAP)-GFP[20] (courtesy of Drs. Hideyuki Okano, Freda Miller, and Helmut Kettenmann, respectively).

Primary Hippocampal Cultures and Transfection

Hippocampal cultures were prepared from Wistar rats (Charles River, Sulzfeld, Germany) on postnatal day (PND) 4, and transfected (~10% efficiency) 5 days after plating.[16]

Animals and Tissues

European Union and National Institutes of Health guidelines on animal care and experimentation were observed. Forty-eight male Wistar rats were housed under standard laboratory conditions. Rats received subcutaneous injections of either vehicle (saline) or DEX on PND 1–7 (DEX 200μg/kg/d on PND 1–3; 100μg/kg/d on PND 4–7). All animals received a single intraperitoneal injection of BrdU (50mg/kg) 24 hours before killing on PND 10, 18, or 28. Serial coronal cryosections (20μm), extending over the entire length of the hippocampal formation, were cut and mounted before sequential double-staining of every 8th section with antibodies against BrdU (1:200; DAKO, Hamburg, Germany) and Ki67 (1:500, Biotrend, Cologne, Germany); cell nuclei were counterstained with Hoechst 33342 (1μg/ml, 10 minutes).

Immunocyto- and Histochemistry

Cells or sections were fixed (4% paraformaldehyde), permeabilized (0.3% Triton-X100/phosphate-buffered saline), blocked, and incubated (4°C) with anti-BrdU (after treatment with 2 N HCl), nestin (1:1,000; Millipore, Goettingen, Germany), anti-TuJ1 (1:500; Babco, Richmond, CA), anti-MAP2 (1:500; Sigma), anti-doublecortin (DCX) (1:500; Santa Cruz, Heidelberg, Germany), anti-GFAP (1:1,500; DAKO or 1:4,000, Sigma), anti-NeuN (1:500; Millipore), O4 antibody (1:500; Millipore), anti-GR (1:300; M20, Santa Cruz), anti-Sox2 (1:300, Santa Cruz), cleaved caspase 3 (1:200; Cell Signaling/NEB, Frankfurt, Germany), and p47-phox (1:200, Millipore). Immunoreactivity was visualized using appropriate Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen). Cells were analyzed (ImagePro software, Media Cybernetics, Bethesda, MD) on an Olympus BX-60 microscope. Cell counts were performed on 10 individual microscopic fields ($0.072mm^2$), randomly chosen across 2 diameters of each coverslip (×400 magnification). An average of 1,000 cells or 100 transfected cells was sampled on each coverslip; results shown represent values from 6–9 coverslips/treatment.

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry (with fluorescein isothiocyanate- or Texas Red-conjugated avidin; Vector, Burlingame, CA), immunostaining for cleaved (active) caspase 3, or Hoechst 33342 staining. Those cells showing morphological signs of DNA fragmentation[17,21] were considered to be apoptotic.

Stereology

StereoInvestigator (MicroBrightField, Williston, VT) was used to estimate the volumes of different subdivisions of the dentate gyrus and cell densities (N_V) in the SGZ of the dentate gyrus. The total number of BrdU+ or Ki67+ NPCs in the SGZ was derived from the product of N_v and total SGZ volume. To identify BrdU and Ki67 double-stained cells, sections were examined (XY, YZ, and XZ views) by confocal microscopy (Olympus IX81 LSM, Hamburg, Germany).

Statistics

Numerical data (shown as mean ± standard error of the mean) were subjected to 2-tailed Student *t* tests or analysis of variance and appropriate post hoc analysis (SPSS Inc, Chicago, IL). The level of significance was preset at $p < 0.05$.

Results

Phenotypic Identity and GR Expression in Hippocampal Cultures

After 6 days in vitro (DIV), hippocampal cultures expressed markers specific to NPCs (~40% nestin+) and immature neurons (~35% TuJ1+and DCX+); approximately 10% of the cells were young neurons (NeuN+), and 15% were astrocytes (GFAP+) or oligodendrocytes (O4+) (Fig 1A). Immunoreactive GR was detectable in NPCs and immature/young neurons (Fig 1B and C). GR expression was observed in ~45% of nestin-GFP–trans-

fected NPC and ~65% of Tα-tubulin-GFP–labeled immature neurons (Fig 1B); stimulation with DEX resulted in translocation of immunoreactive GR to the nucleus, suggestive of its transcriptional potential (Fig 1B, inset). Treatment of cultures with DEX resulted in a dose-dependent induction of apoptosis that was preventable by pretreatment with the GR antagonist RU38486 (Fig 1D); because consistently robust effects were observed at a dose of 10^{-5}M, this dose was chosen for all subsequent in vitro experiments.

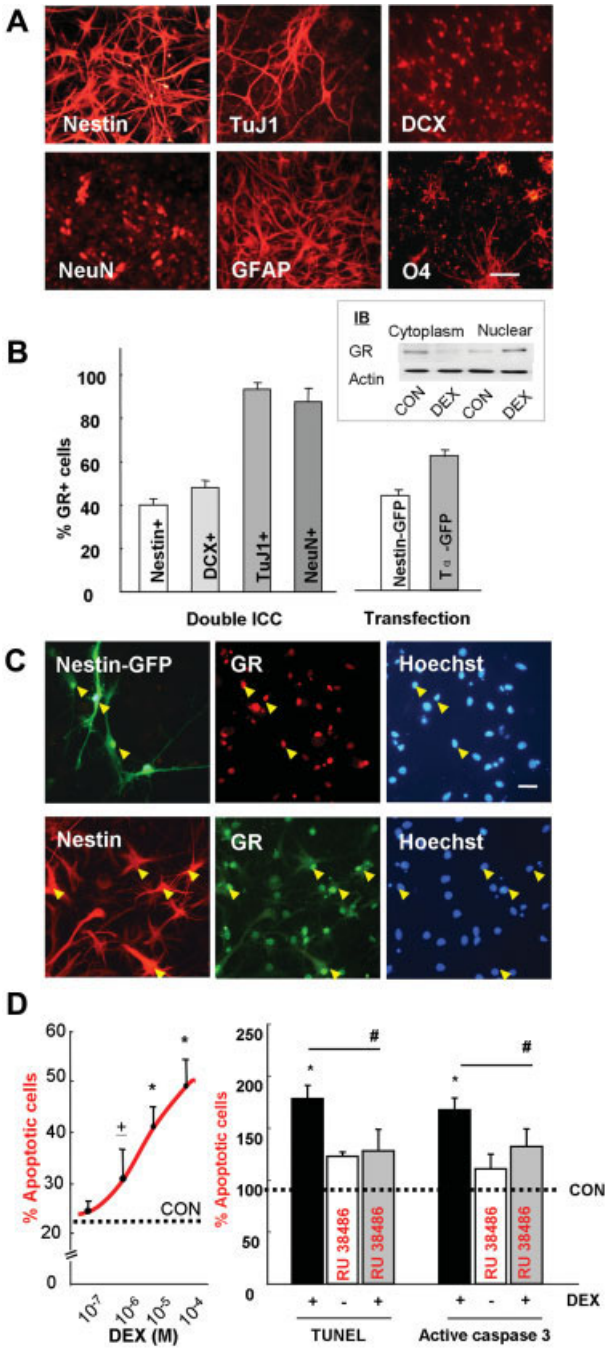

FIGURE 1: Immature hippocampal cells are sensitive to glucocorticoids. (A) After 7 days in vitro (DIV), primary hippocampal cultures, derived from postnatal rats aged 4 days, were comprised of ~40% neural precursor cells (NPCs, labeled with antinestin) and ~35% immature neurons (stained with anti-TuJ1 or anti-doublecortin [DCX]); <10% of the cells stained with anti-NeuN, a marker of young neurons (NeuN), and <20% of the cells were astrocytes and oligodendrocytes (stained with antibody O4). (B) Immunoreactive glucocorticoid receptor (GR) was localized in both NPCs and neuronal progenitors; shown are the percentage of GR-expressing cells in the different cell populations, including NPCs (nestin-positive cells identified by immunocytochemistry or cells transfected with nestin-green fluorescent protein [GFP]) and immature neurons (stained with anti-DCX or anti-TuJ1, or transfected with Tα-tubulin-GFP), as well as neurons (stained with anti-NeuN). The inset is an example of an immunoblot (IB) of cytoplasmic and nuclear fractions probed with GR antibody; note the increased GR signal in the nucleus (vs cytoplasm) in lysates from cells that had been treated with glucocorticoid (dexamethasone [DEX], 10^{-5}M). (C) Representative images of nestin-GFP–transfected and anti-nestin–stained cells coexpressing GR are shown; also shown are images from the same sets of cells after staining of the cell nuclei with Hoechst dye 33342; arrowheads point to identical cells in each row. (D) The dose-dependent induction of apoptosis by DEX is shown in the left-hand panel; these results are based on terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of apoptotic cells. Consistently robust responses are obtained at a dose of 10^{-5}M (used in all subsequent experiments). The right-hand panel shows that similar results are obtained when apoptosis is evaluated by either TUNEL histochemistry or immunocytochemistry for the active (cleaved) form of the executioner caspase, capsase 3. Note that the apoptotic actions of DEX can be significantly attenuated by pretreatment (30 minutes) of cells with the GR antagonist RU38486 (10^{-5}M), indicating mediation by GR. All numerical data are depicted as mean ± standard deviation. *$p < 0.05$ vs control (CON), #$p < 0.05$ vs DEX. Scale bar = 50μm in (A), 20μm in (C). GFAP = glial fibrillary acidic protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Regulation of NPC and Postmitotic Hippocampal Cell Fate by GCs

Neuroplasticity depends on the availability of NPC.[6] Whereas neurogenesis is implicated in recovery from stroke,[22] reduced proliferative capacity of hippocampal cells is associated with epilepsy,[23] impaired cognition,[2] and depression.[24,25] We show here that DEX reduces the number of immunocytochemically identified NPC (by ~39%), neuroblasts (~39%), and immature neurons (~54%) ($p < 0.05$, in all cases; Fig 2A).

We previously demonstrated that GCs induce apoptosis in hippocampal cells in culture[16,17] and that GC-induced apoptosis in situ is prominent in the SGZ, where NPC reside and proliferate.[21,26] To examine the hypothesis that apoptosis leads to a reduction in NPC and immature neuron numbers, we next treated hippocampal

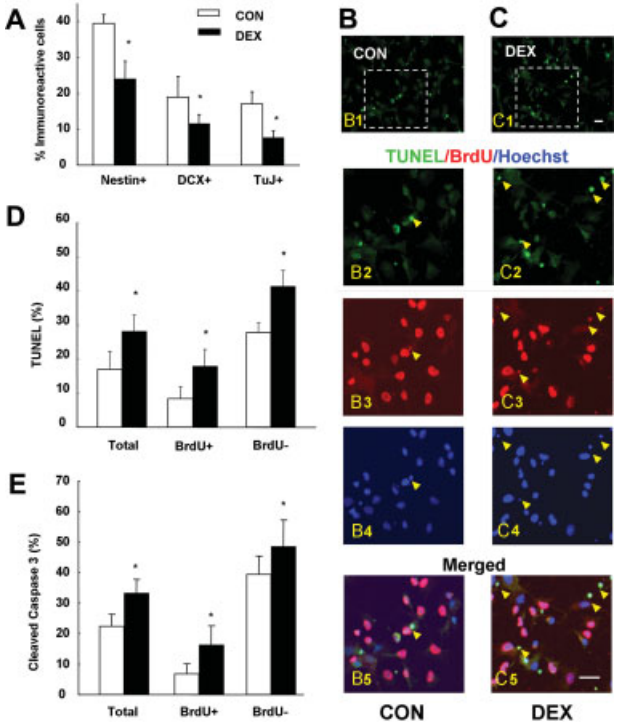

FIGURE 2: Proliferating and resting cells are targets of glucocorticoid-induced apoptosis. (A) Exposure of DIV 7 hippocampal cultures to dexamethasone (DEX) (10^{-5}M) leads to a significant loss of neural precursor cells (nestin-positive) and immature neurons (doublecortin [DCX]/TuJ1-positive). (B–C) Representative images of cells that were treated simultaneously with 5-bromo-2′-deoxyuridine (BrdU) (20μM) and DEX (10^{-5}M) before staining 24 hours later for BrdU (to mark cells born in the preceding 24 hours) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). TUNEL are shown in control (CON) (B1) and DEX-treated cells (C1), respectively; panels B2–B5 and C2–C5 are higher magnifications of the areas marked by the dotted boxes in B1 and C1, respectively; B2 and C2 show apoptotic cells, B3 and C3 show BrdU-incorporating cells, and B4 and C4 show nuclear staining with Hoechst dye, in CON and DEX-treated cells, respectively; panels B5 and C5 show merged images of B2–B4 and C2–C4, respectively. Arrowheads point to identical cells in each column and exemplify apoptosis (TUNEL-stained) in recently-proliferated cells (BrdU-stained). (D) Quantitative analysis of TUNEL staining in proliferative (BrdU+) and resting (BrdU−) cells. (E) Comparable data to those shown in (D) were obtained when cells were double-labeled for BrdU and cleaved (active) caspase 3. All numerical data are shown as mean ± standard deviation. Asterisks indicate significant differences vs CON (untreated) cells ($p < 0.05$). Scale bars: 20μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cultures with the cytosine analog BrdU (to reveal recently proliferated cells[27]) and DEX for 24 hours before quantifying apoptosis (identified by TUNEL or activated caspase 3 immunoreactivity) in recently proliferated (BrdU-labeled[27]) cells. As compared with untreated cells (Fig 2B1–B5), DEX-treated cells showed greater colocalization of BrdU and TUNEL signals (Fig 2C1–C5). Evaluation of individual merged images revealed significantly increased apoptosis among both mitotic (BrdU+, control [CON], 8.4 ± 3.4%; DEX: 17.8 ± 5.0%; $p < 0.05$) and resting (BrdU−, CON: 27.6 ± 3.0%; DEX: 41.0 ± 4.1%; $p < 0.05$) cell populations after GC treatment (Fig 2D). The results obtained with TUNEL histochemistry were corroborated by cleaved (activated) caspase 3 immunocytochemistry (Fig 2E).

Phenotype-Specificity of the Apoptotic Actions of GCs

NPC proliferate and differentiate along either neuronal or glial lineages.[6] Given the intrinsic characteristics of NPC and the heterogeneous nature of primary hippocampal cultures (Fig 1), we here analyzed the cell phenotypes targeted for GC-induced apoptosis in mixed hippocampal cultures transfected with specific plasmids that would facilitate distinction between NPC (nestin-EGFP) and neuronal progenitors (Tα1-GFP). Exposure of cells to DEX produced a significant increase in TUNEL-labeled apoptotic cells among the NPC (Fig 3A–C, G–I, and U; $p < 0.05$) and neuronal progenitor (Fig 3D–F, J–L, and U; $p < 0.05$) cell populations; the TUNEL results were confirmed by staining for cleaved (activated) caspase 3 immunoreactivity (Fig 3M–P, Q–T, and V; $p < 0.05$). In all cases, the apoptotic actions of DEX were attenuated when cells were pretreated with the GR antagonist RU 38486, indicating their mediation by GR (Fig 3U, V). Interestingly, astrocytes marked with GFAP-GFP did not succumb to the apoptotic effects of DEX (Fig 3U).

Mitochondrial Mechanisms Mediate GC-Induced Apoptosis in NPC

The data showing that DEX treatment leads to an activation of caspase 3 (Fig 2E, Fig 3M–T, and V, and Fig 5B–D) in NPC hinted at involvement of the mitochondrial or "intrinsic" pathway of apoptosis.[15] These findings were confirmed in hippocampal cultures using pharmacological inhibitors of caspase 3 and its upstream caspase, caspase 9 (Fig 4A). Examining events upstream of the caspases, we observed that DEX treatment dose-dependently increases the ratio of proapoptotic *bax* to antiapoptotic *bcl-2* mRNA expression, without influencing the *bax*:bcl_{XL} mRNA expression ratio (Fig 4B); the latter findings are consistent with the fact that the predominant antiapoptotic protein in developing neurons is Bcl-2 rather than Bcl_{XL}.[26]

The mitochondrial proteins Bax and Bcl-2 act in a rheostatic manner to regulate the integrity of the mitochondrial permeability transition which is particularly sensitive to perturbation by reactive oxygen species (ROS). Measurement of ethidium intercalation into DNA

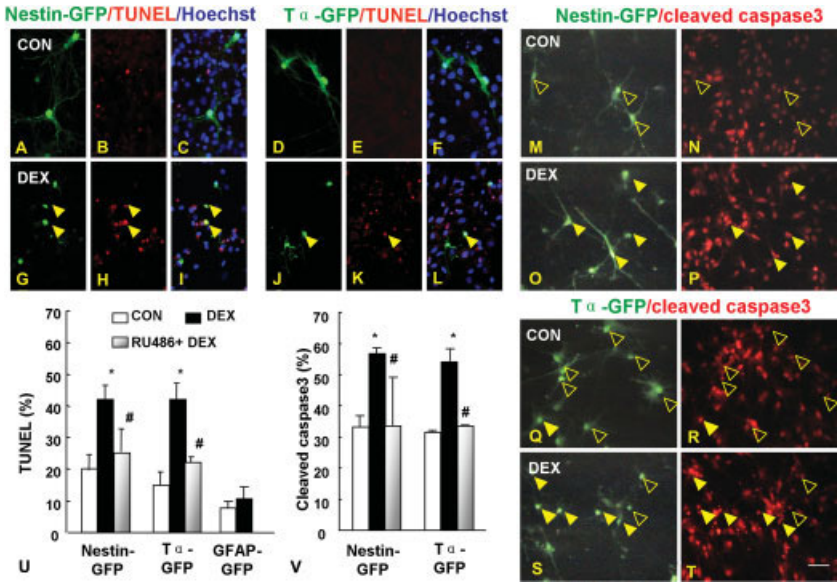

FIGURE 3: Neural precursors and neuronal progenitors are driven into apoptosis by dexamethasone (DEX). Cultures were transfected with either nestin-green fluorescent protein (GFP) or Tα-tubulin-GFP plasmids to label neural precursors or neuronal progenitors, respectively. The percentage of cells staining positively for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) or activated caspase 3 among the nestin- or Tα-tubulin–labeled populations were counted to examine how each phenotype was influenced by DEX. Representative images are shown from TUNEL-stained cells that had been previously transfected with nestin-GFP (A–C and G–I) or Tα-tubulin-GFP (E–F and J–L); control (CON) cells are shown in A–C and D–F, and DEX-treated cells are shown in G–I and J–L, where solid arrowheads indicate apoptotic GFP+ cells, detected by TUNEL and Hoechst staining. Examples of activated caspase 3 staining in specifically tagged neural precursor cells (nestin-GFP) and neuronal progenitor (Tα-tubulin-GFP) subpopulations are shown in M–T; open arrowheads indicate activated caspase 3−/GFP+ cells, and solid arrowheads point to activated caspase 3+/GFP+ cells. Numerical analysis of these data is shown in U and V. Note that astrocytes labeled with glial fibrillary acidic protein (GFAP)-GFP do not undergo DEX-induced apoptosis (U). All numerical data are given as mean ± standard deviation. *$p < 0.05$ vs CON, #$p < 0.05$ vs DEX. Scale bars: 20μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

showed that ROS generation represents a mechanism through which DEX induces apoptosis (Fig 4C). Additionally, DEX stimulates ROS production in NPC (Fig 4D and E); this effect is accompanied by a translocation of membrane-associated $p47^{phox}$ (an essential component of the nicotinamide adenine dinucleotide phosphate oxidase complex required for the production of superoxide anions) (Fig 4F) and reductions in the activities of Cu^{++}/Zn^{++} superoxide dismutase and glutathione, 2 key antioxidant enzymes (Fig 4G).

Depletion of the NPC Pool by GC Treatment During Peak Neurogenesis In Vivo

Hippocampal neurogenesis occurs at a high frequency during early postnatal life.[28] However, NPC have limited self-renewal capacity,[29] and the NPC pool from which new neurons are generated diminishes exponentially with age[7,9]; GCs are thought to at least partially contribute to the latter phenomenon.[28] On the other hand, proliferating hippocampal cells were previously reported to express GR only sparsely.[30] As shown in Figure 5A, numerous cells in the neonatal dentate gyrus express GR along a gradient that increases from the SGZ to the inner layers of the granule cell layer (GCL), where more mature granule neurons are localized. Importantly, DEX treatment provoked a 60% increase in apoptosis (increase in active caspase 3 immunoreactivity) in the SGZ (Fig 5B–D; $p < 0.05$). Two types of NPC are found in the SGZ: quiescent neural precursors (QNP; GFAP-positive, proliferate relatively slowly) and amplifying neural precursors (ANP; GFAP-negative, display high proliferative activity).[31] Accordingly, it was considered important to investigate if QNP and ANP might be differentially sensitive to glucocorticoids. Both QNP and ANP express nestin and Sox2, but whereas nestin levels in ANP diminish over time, Sox2 expression is maintained at relatively steady levels in both NPC subtypes and serves as a more reliable marker of NPC (QNP: GFAP+/Sox2+; ANP: GFAP−/Sox2+). Exploiting these characteristics, colocalization studies showed that a similar proportion of QNP and ANP express GR (Supplementary Fig S1A–C), suggesting their similar vulnerability to DEX.

We subsequently assessed the impact of neonatal GC administration on the proliferative capacity of the dentate gyrus in later life by performing stereological

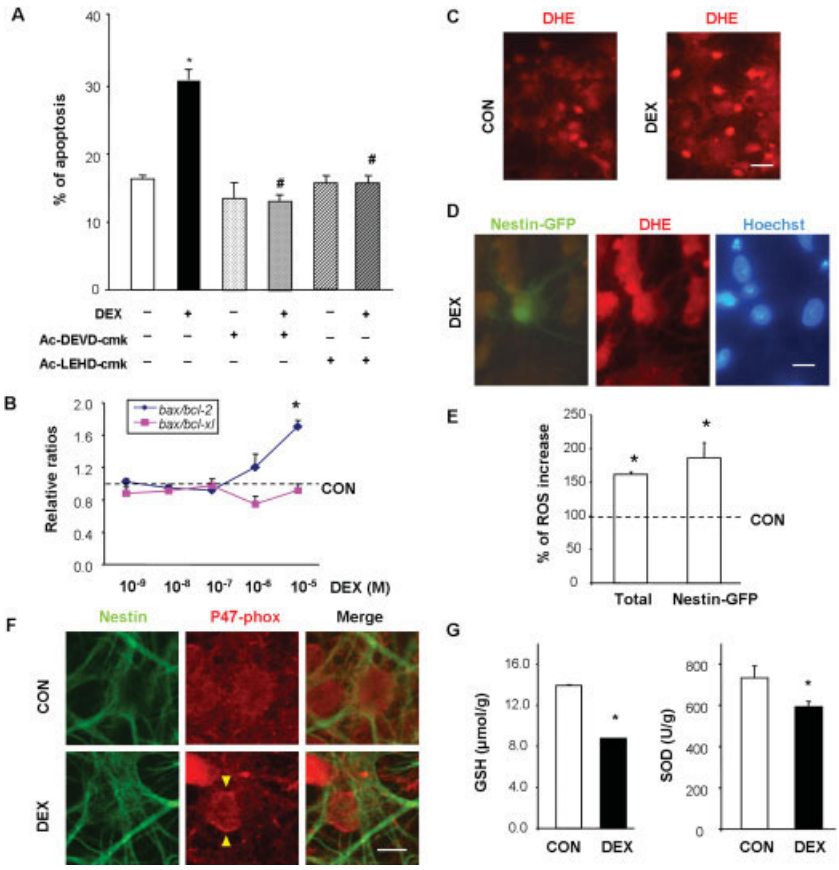

FIGURE 4: Dexamethasone (DEX) induces neural precursor cell (NPC) apoptosis by increasing reactive oxygen species (ROS) production, perturbation of the mitochondrial membrane potential, and subsequently activation of the intrinsic apoptotic pathway. (A) Pretreatment with either Ac-DEVD-cmk (caspase 3 inhibitor) or Ac-LEHD-cmk (caspase 9 inhibitor) rescues NPC from DEX-induced apoptosis. (B) Expression levels of Bcl-2 family members were measured by quantitative polymerase chain reaction after exposure of cultures to various doses of DEX; dose response curves, in terms of the ratio of proapoptotic *bax* to anti-apoptotic *bcl-2* or *bcl-xl*, reveal that DEX first produces a significant increase in the *bax*:*bcl-2* ratio at a dose of 10^{-5}M, and that the ratio of *bax*:*bcl-xl* is not influenced by DEX treatment. (C) Treatment of NPC cultures with DEX (10^{-5}M) stimulates ROS production, indicated by the intercalation of ethidium into DNA (red fluorescence). (D) Confirmation of DEX-stimulated ROS production in identified NPC that were labeled with nestin-GFP. (E) Treatment of primary cultures with DEX increases ROS production, as measured by dihydroethidium (DHE) staining; note NPCs, marked with nestin-green fluorescent protein, also show significantly increased levels of ROS in response to DEX. (F) Immunostaining for the p47-phox, a subunit of nicotinamide adenine dinucleotide phosphate oxidase, showing localization of the signal from the cytoplasm to the plasma membrane (arrowheads) in NPCs (identified by nestin immunostaining) after DEX treatment. (G) Treatment of cultures with DEX leads to significant reductions in 2 key antioxidant enzymes, glutathione (GSH) and superoxide dismutases (SOD); enzyme activities were normalized to protein concentrations of the cell extracts. All numerical data are depicted as mean ± standard deviation. *$p < 0.05$ vs control (CON), #$p < 0.05$ vs DEX. Scale bar = 20μm in C, 10μm in D and F. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

counts of the number of BrdU- and Ki67-stained cells in the SGZ at PND 10, 18, and 28 (Fig 6A–C). The results of this analysis revealed that neonatal treatment with DEX results in a significant reduction in the number of cells available for mitosis at any given time ($p < 0.05$; Fig 6D), suggesting a depletion of the NPC pool by neonatal DEX. Interestingly, the absolute differences between the number of proliferating cells in the SGZ of both control and DEX-treated rats diminished over time (but remained significantly different), probably reflecting age-related decreases in proliferative activity (Fig 6D) and the fact that a subpopulation of NPC that do not express GR (see Supplementary Fig S1) may escape the apoptotic actions of neonatal GC treatment.

Because expansion of the GCL occurs primarily during early postnatal life, we next carried out a stereological assessment of the volumes of the SGZ and GCL. This analysis revealed that SGZ volumes of DEX-treated animals were significantly smaller ($p < 0.05$), despite similar volumetric increments over time (14–20%) (Fig 6E); the latter suggests proliferation by residual NPC that were spared from the apoptotic effects of neonatal DEX. Although both controls and DEX-treated animals showed significant increases in the volumes of their GCL between

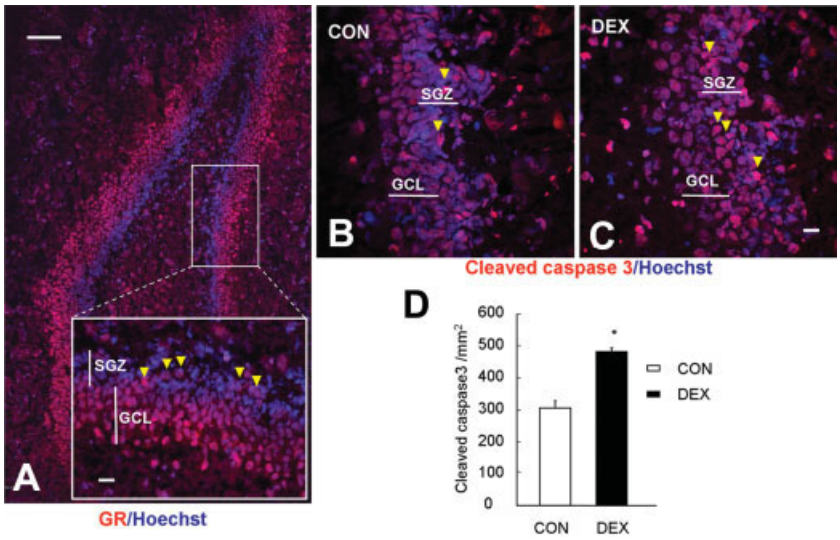

FIGURE 5: Neonatal glucocorticoid treatment induces apoptosis in the subgranular zone (SGZ) in situ. (A) Immunohistochemistry for glucocorticoid receptor (GR) in the dentate gyrus of PND10 rats revealed a gradient of staining intensity, from the germinative SGZ (high) to the granule cell layer (GCL) (low); the area enclosed by the white box is enlarged in the inset, where the arrowheads mark examples of GR-positive neural precursor cells at the hilus-SGZ border. (B–C) Confocal images of activated caspase 3 immunostaining in a section from the dorsal portion of the dentate gyrus of a control (CON) (B) and a dexamethasone (DEX)-treated (C) rat (PND10); the arrowheads point to examples of cells showing immunoreactivity in the SGZ. (D) Numerical analysis of sections from CON (n = 7) and DEX-treated rats (n = 8) stained for activated caspase 3 activity in SGZ; data shown are mean ± standard error of the mean. *p < 0.05 vs CON. Scale bar: 1mm in (A) and 20μm in the inset of (A), and (B) and (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the ages of 10 and 28 days (Fig 6E, $p < 0.05$), GCL volumes in the DEX-treated animals were significantly smaller than in controls (Fig 6E, $p < 0.05$). In sum, these results suggest that reduced neurogenesis and subsequently, reduced cell acquisition in the GCL, result in a marked retardation of GCL development in animals exposed to neonatal DEX (Fig 6E).

Discussion

Several neurological and psychiatric disorders are hallmarked by hippocampal dysfunction. The last decade has witnessed compelling evidence for a link between hippocampal function and cell turnover in the postnatal hippocampus.[6,13,24,25,32] Neuronal turnover in the hippocampus is a dynamic process involving neurogenesis and apoptosis in the germinative layer (SGZ) of the dentate gyrus[3]; stress and elevated GC levels inhibit neurogenesis and stimulate apoptosis in the hippocampus.[11,12,21,26] Although GCs are known to interfere with the neural cell cycle,[14] it is not known whether GCs target NPC for apoptosis. Accordingly, we here examined the incidence of apoptosis in hippocampal cultures that were genetically marked with developmental phase-specific markers to identify proliferating multipotent NPC and NPC destined to become neurons. In addition, we studied the consequences of neonatal treatment with DEX, a synthetic GC (when neurogenesis and apoptosis occur at high frequency[28]) on dentate gyrus development in situ.

The presented results demonstrate that both NPCs and neuronal progenitors are subject to DEX-induced apoptosis. The actions of DEX were shown to be mediated by GRs, which are expressed by NPCs, by neuronal progenitors and mature neurons (in culture), and by QNP and ANP cells residing in the SGZ; notably, the SGZ displays a prominent apoptotic response to DEX. It is important to note, however, that because GR expression by NPCs is not ubiquitous, a subpopulation of NPCs may be (at least transiently) spared from the actions of DEX. However, given the finite self-renewing properties of NPCs,[29] disruption of the lifelong cycle of neuronal birth and therefore, sustained deleterious effects on hippocampal growth and function, is a plausible scenario being investigated in a long-term study. Meanwhile, analysis of the mechanisms through which DEX induces NPC apoptosis revealed a role for the mitochondrial pathway. Consistent with previous findings,[33] our results indicate that the proapoptotic actions of DEX are initiated by an increase in ROS levels and concomitant decreases in the cellular defenses against oxidative stress. Through their disruption of the mitochondrial membrane potential, these events subsequently lead to activation of caspase 9 and caspase 3. Notably, we show that DEX treatment results in increased activation of the "executor caspase,"

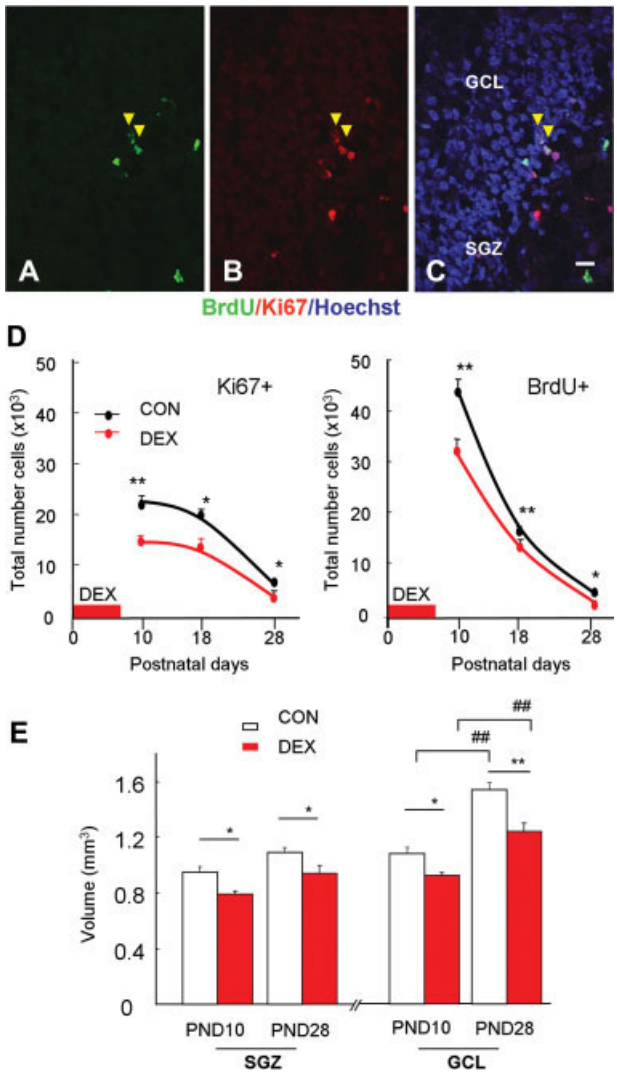

FIGURE 6: Glucocorticoid treatment in neonatal life results in a sustained reduction of neurogenic capacity. (A–C) Confocal images showing double staining with anti–5-bromo-2′-deoxyuridine (BrdU) (A) and anti-Ki67 (B). Arrowheads indicate cells colabeled with the BrdU and Ki67 antibodies. Hoechst 33342 staining (C) was used to identify cell nuclei and to help delineate the subgranular zone (SGZ) and granule cell layer (GCL). (D) Dexamethasone (DEX) treatment (200μg/kg/d on postnatal day [PND] 1–3, tapering to 100μg/kg/d) on PND 1–7 results in a significant reduction in the number of proliferating cells, as judged by immunostaining of BrdU-incorporating cells and Ki67-immunoreactive cells on PND 10, 18, and 28; note the progressive decline in proliferating cells in controls over the time period examined. (E) The volume of the SGZ and GCL of rats exposed to DEX on PND 1–7 is significantly smaller when estimated on PND 10 and 28. Note that the increase in GCL volume between PND 10 and PND 28 was greater in controls (CON) than in neonatally DEX-treated rats. All numerical values are mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$, as compared with age-matched controls; ## indicates significant difference between PND 10 and PND 28 ($p < 0.01$). PND 10: CON, n = 7; DEX, n = 8. PND 18: CON, n = 8,; DEX, n = 8. PND 28: CON, n = 8; DEX, n = 9. Scale bar: 20μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

caspase 3, in NPCs in culture and in the SGZ of the intact hippocampus.

The capacity of the hippocampus to produce new neurons declines markedly with age.[7–9,34] Whereas previous work reported an 80% decrease in the neurogenic capacity of the hippocampus between 1 and 22 months of age,[7–9,34] our results show an even steeper decline (~92%) between PND10 and 28. Thus, the hippocampus undergoes its most dynamic structural organization during the early postnatal period, with a precipitous depletion of the NPC pool[9] that probably reflects changes in the milieu that normally encourages NPC proliferation.[35] Given that NPCs are vulnerable to the apoptotic actions of DEX (this study), and have a limited capacity for self-renewal,[29] as well as the fact that the dentate gyrus increases in neuronal number and volume for at least 1 year,[36] it was considered important to examine whether DEX influences the in vivo NPC pool in a transient or sustained fashion. We observed that neonatal DEX treatment induces a sustained reduction in the number of mitotic cells and, importantly, retards the volumetric growth of the SGZ and GCL. This finding is consistent with previous reports in rats and rhesus monkeys.[37–39] On the other hand, postnatal neurogenesis and granule cell volumes appear to be unaltered by prenatal exposure to DEX,[40] and neurogenesis is only transiently inhibited when DEX is administered to adults.[41] These observations suggest that early postnatal life may represent a window during which NPCs are particularly sensitive to DEX, and that exposure to DEX during this period results in a protracted retardation of dentate gyrus development.

The paradigm of chronic DEX administration during perinatal life is clinically relevant; there is convincing evidence that glucocorticoids during early childhood lead to impairments of neuromotor functions and cognition, as well as head and somatic growth.[42,43] This study shows that the hippocampus endures increased levels of neuronal apoptosis, retarded growth, and sustained reductions in the rate of neurogenesis when DEX is administered during neonatal life; moreover, an earlier study associated such treatment with reduced forebrain expression of synaptic proteins and disruption of the ontogeny of neurotransmitter systems.[37] Since lifetime cognitive performance relies on plasticity (including neurogenesis) in the hippocampus,[2,4,6,34] the sustained depletion of the NPC pool by neonatal DEX is likely to have a major impact on lifetime learning and memory. Lastly, early life experiences that stimulate endogenous glucocorticoid secretion and interfere with neuroplasticity are established etio-

pathogenic factors in a number of psychiatric conditions, best exemplified by major depression.[1,13]

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2.3 Chapter 3: Probing the role of estrogen receptor isoforms in neonatal programming of neuroendocrine and behavioral functions

Patchev AV et al., *Endocrinology Studies*, 2011

2.3.1 Rationale

The HPA axis in rodents displays strong sexual dichotomy, with females showing higher basal and stress induced corticosteroid secretions, but also a more effective GC negative feedback. Previous work from our lab has shown that the neonatal sex-steroid milieu in the rat is crucially involved in the sex-specific organization of the HPA axis (Patchev et al., 1995). The discovery of the estrogen receptor β (ERβ) (Kuiper and Gustafsson, 1997), as well as the capacity of both ER isoforms to induce differential transcriptional programs depending on the cell phenotype and mutually curb their transcriptional effects when co-expressed (Matthews and Gustafsson, 2003) prompted us to ask, whether isoform-selective ER activation might have differential consequences for sex-specific HPA axis organization and function. In earlier studies we have shown that selective neonatal ERα or ERβ activation in female rats leads to distinct neuroendocrine and behavioral effects relevant to reproduction (Patchev et al., 2004). Neonatal ERα activation lead to defeminization of several aspects of gonadal function, sexual behavior and morphological features of sensitive brain areas, while ERβ effects were confined to abrogation of the ovarian cycle and changes in neuronal populations which control gonadotropin secretion patterns. In this context, and in view of the finding that ERβ is predominantly expressed in brain areas involved in the neuroendocrine stress response (Laflamme et al., 1998), we hypothesized that selective neonatal activation of ER isoforms might differentially affect sex-specific HPA axis organization and function in later life.

2.3.2 Major findings

- the validity of the experimental model was confirmed by demonstration of sex dichotomy in several aspects of HPA axis function and by their abrogation by neonatal administration of the non-selective ER agonist estradiol in female rats

- neonatal isolated activation of any of the ER isoforms could not emulate the effects of estradiol

- selective ERα-activation was associated with impaired GC negative feedback and signs of increased behavioral anxiety in adulthood

- selective ERβ-activation led to increased basal GC secretion and blunted circadian oscillations, as well as a hypoanxious phenotype

- selective neonatal ERα activation lead to stronger gonadal axis impairments, while ERβ activation was associated with a preservation of residual ovarian sex hormone secretions.

- Homologous down-regulation of the corresponding ER isoform (and, thus, subsequent male-like insensitivity) by neonatal ligand exposure was manifest only in animals treated with ERα agonist and the non-selective agonist estradiol

2.3.3 Conclusions

Defeminization of HPA axis function is a result of an orchestrated interplay between both ER isoforms. In contrast to the sex-specific organization of reproductive behaviors and neuroendocrine functions, the sex dichotomy of HPA axis function appears to depend also on superimposed effects of gonadal secretions in adulthood. Our data strongly indicate that the degree of preservation of female gonadal secretions and, corollary, activational effects of estrogens, in animals with a history of neonatal ER activation accounts for the manifestation or abrogation of sex differences in HPA axis function.

2.3.4 Outlook / ongoing studies

In future studies we plan to explore the hypothesis that sex dimorphisms in emotional and cognitive behaviors, like those in HPA axis function, are secondary to activational effects of sex-steroids, rather than products of their organizational action. To this end, females will be neonatally exposed to estradiol or isoform-selective ER agonists; in adulthood the animals will be ovariectomized and supplemented with estradiol. We expect that only animals with a history of neonatal ERβ activation will display female-like HPA axis function. Furthermore, the importance

of androgen receptor signaling as mediator of behavioral and neuroendocrine masculinization should be evaluated in greater detail.

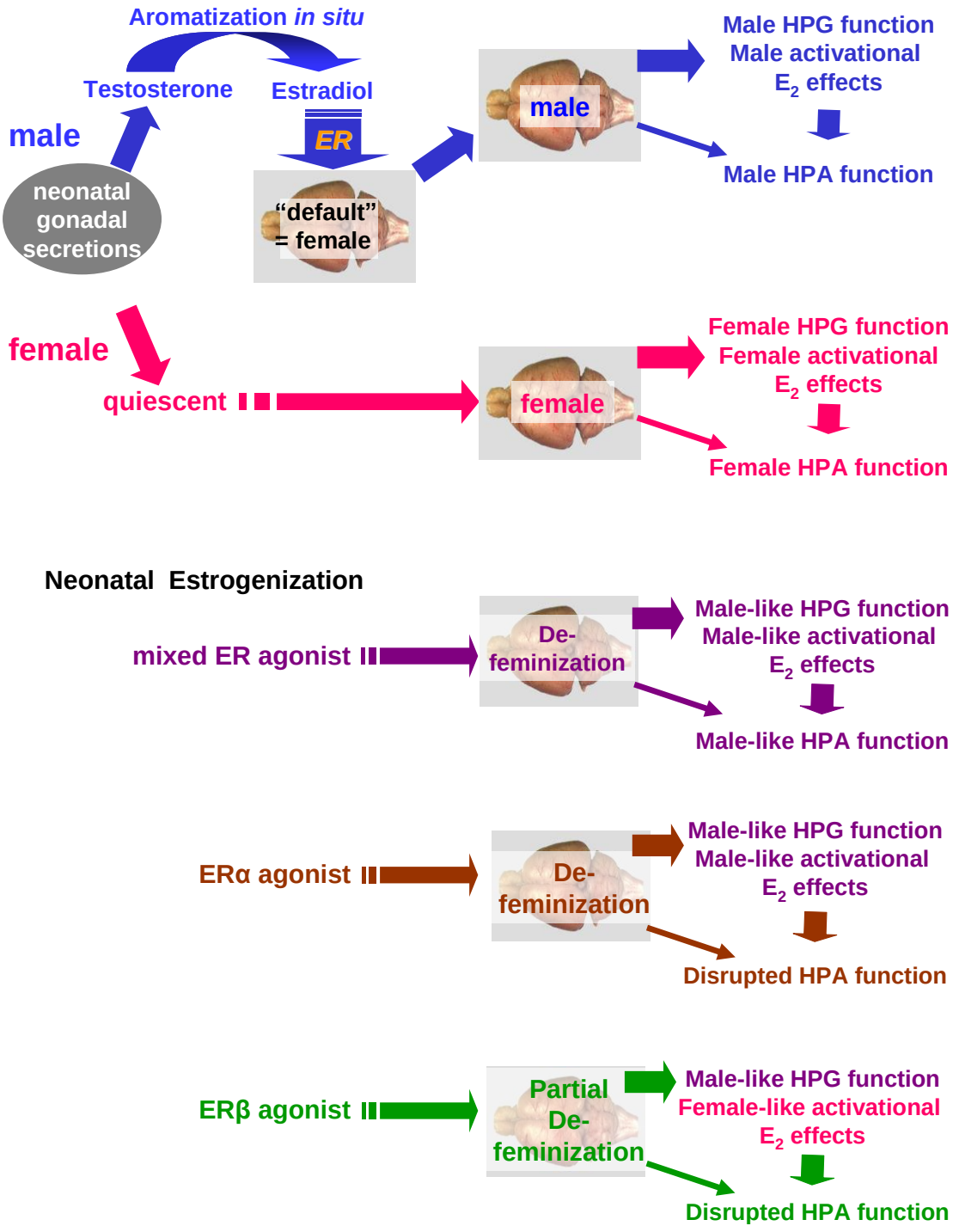
Remark: Unfortunately, due to changes in institute infrastructure in 2009 precluding the use of rats as experimental models, we could not complete these experiments. The alternative use of mice may become problematic, as sex-specific dimorphisms, which serve as validation criteria for the organizing efficacy of isoform-selective ER agonists are not reliably expressed in the murine brain (Bonthuis et al., 2010).

2.3.5 Contributions

- Performing of all in vivo studies (including day to day treatment, blood sampling and behavioral analysis)
- Supervision of a master student (A. Wolff-Muscate) on a daily basis.
- Molecular and histological analysis of brain specimens
- Performing endocrine measurements
- Writing the manuscript

Figure 3 **Selective ER isoform activation leads to distinct disruption, rather than sex-specific organization, of HPA axis function**

Sex-specific HPA axis function seems to be strongly affected by activational sex-steroid effects, and thus depends on the organization and function of the gonadal (HPG) axis, although direct organizational effects of sex-steroids on discrete pathways within the HPA axis can not be excluded. Neonatal estrogenization of female rats with estradiol (a mixed ER agonist) leads to male-like gonadal function and activational effects (e.g. ER expression in the brain), thus leading to male-like HPA axis function. None of these effects could be reproduced by selective ER isoform activation, which resulted in distinct disruption profiles of HPA axis function.



Probing the role of estrogen receptor isoforms in neonatal programming of neuroendocrine and behavioral functions

Alexandre V. Patchev,[1] Annemarie Wolff-Muscate,[1,2] Dieter Fischer,[1] Osborne F.X. Almeida[1]

[1]Max Planck Institute of Psychiatry; [2]Institute of Developmental Genetics, Helmholtz Center, Munich, Germany

Abstract

Sex differences in the activity of the hypothalamus-pituitary-adrenal (HPA) axis in rats are programmed by neonatal estrogens; exposure of female neonates to estradiol (E_2) leads to overt defeminization of endocrine and behavioral functions in adulthood. E_2 activates both estrogen receptor isoforms (ERα and ERβ); these are widely expressed in the brain, and differentially regulate HPA axis activity in adulthood. However, the contributions of each ER isoform to the sex-specific organization of the neural mechanisms governing HPA axis function remain unknown. ERα, ERβ agonists (PPT and DPN, respectively) or E_2 were administered to female rats on days 1-10 of life. Animals subsequently underwent endocrine (HPA axis and reproductive) and behavioral profiling (anxiety-related and reproductive) in adulthood, and patterns of expression of relevant genes were monitored in limbic structures post mortem. Exposure of neonatal females to PPT or DPN led to distinctly different HPA secretory profiles, neither of which completely recapitulated the effects of E_2. Thus, whereas impaired glucocorticoid negative feedback was the most prominent effect of PPT treatment, increased basal corticosterone secretion was the most obvious characteristic of DPN-treated animals. Behavioral analysis revealed higher anxiety levels in PPT-treated animals, similar to those observed in E_2-treated female neonates and control males; in contrast, DPN treatment was associated with reduced anxiety-like behavior. Parallel treatment-specific alterations in the expression of the genes encoding mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hippocampus and amygdala and altered expression of ERβ mRNA in discrete brain regions, as well as disturbed ovarian activity, were also found; together, they suggest potential mechanisms that could account for the different endocrine and behavioral phenotypes observed.

Defeminization of HPA axis activity and associated anxiety-related behavior depends on balanced activation of ERα and ERβ during early postnatal life, rather than on the activation of a specific ER isoform. Long-term E_2-, PPT- and DPN-induced alterations in the expression levels of GR and MR in the hippocampus and amygdala, as well as disrupted ovarian activity appear to be largely responsible for eliciting and maintaining the aberrant endocrine and behavioral phenotypes induced by estrogenization of neonatal females.

Introduction

Understanding the neurobiological basis of sex differences in the activity of the hypothalamus-pituitary-adrenal (HPA) axis is of medical relevance given the association between excessive glucocorticoid (GC) secretion and mood and anxiety disorders, conditions that occur more frequently in women.[1] Women and female rodents secrete higher GC levels under both basal and stressful conditions; however, in contrast to males, healthy females show more efficient GC negative feedback regulation of adrenocorticotropin (ACTH) and glucocorticoid receptors (GR) in the pituitary, corticotrophin-releasing hormone (CRH), arginine vasopressin (AVP) and GR in the hypothalamus, and GR in the hippocampus.[2-4] In adults of both sexes, these molecules are subject to dynamic regulation by gonadal steroids such as estradiol (E_2). In females, cyclical fluctuations in the secretion of sex steroids contribute to the regulation of glucocorticoid secretion[5-6] and a variety of behaviors in rodents[7] and primates, including humans; the influence of estradiol (E_2) on these functions is well known.[8] On the other hand, neonatal exposure of female rats to E_2 results in the expression of a male-like HPA axis function.[3] Since neonatal rats are considered to have a default female status, it is thought that the male phenotype results from the so-called *organizing* actions of neonatal estrogen,[9] a view supported by the observation that neonatal castration of males prevents manifestation of masculine endocrine and behavioral features.[10] Adult female gonadal secretions have been shown to affect HPA axis function through so called activational effects, whose magnitude and quality strongly depend on the *organizing* effects during early ontogeny.[2]

Estrogen actions are mediated by estrogen receptors (ER), of which there are two major isoforms (ERα and ERβ). While ERα are predominantly expressed in brain nuclei implicated in the control of reproductive hormone secretion and behavior, ERβ are found in regions that are responsible for the regulation of non-reproductive functions, including HPA axis activity.[11-12] Since E_2 activates both ERα and ERβ in a relatively non-selective manner (EC50 values: 50 pM and 200 pM for ERα and ERβ respectively),[13] questions regarding the individual contributions of each ER isoform to the organization of sex differences in HPA axis function remain open. This study addressed this issue by selectively activating ERα and ERβ with 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT; EC50 of 200 pM at ERα 410-fold higher affinity for ERα than ERβ)[14] and 2,3-bis(4-Hydroxyphenyl)-propionitrile (diarylpropionitrile; DPN; EC_{50} of 0.85 nM at ERβ; 170-fold higher affinity for ERβ than ERα),[15] respectively. In addition to monitoring GC secretion under differing conditions, we also monitored a number of pathways involved in the regulation of the HPA axis and of anxiety-related behavior; the latter is influenced by adrenal and gonadal steroids.[16-17] Our results highlight the importance of co-activation of both ER isoforms during sex-specific organization of the brain since activation of just one isoform results in erroneous programming of both neuroendocrine and behavioral

Correspondence: Dr. Alexandre Patchev, Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany.
Tel: +49.89 30622.397; Fax: +49.89.30622.461.
E-mail: patchev@mpipsykl.mpg.de

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functions. These findings are interesting from an environmental health perspective, as many environmental pollutants and endocrine disrupting compounds show differential affinities for the two ER isoforms.[18-19] Further, our experiments draw attention to the fact that the disruptive effects of neonatal estrogenization paradigms on ovarian secretions and their receptive targets must be considered when interpreting the results from such experiments. Specifically, our results hint that sex differences of HPA axis function arise from impairment of activational estrogenic effects due to impairment of sex-steroid secretion and ER expression patterns in the brain, which are the consequence of the neonatal sex-steroid milieu.

Materials and Methods

Animals and treatment paradigms

All experiments were conducted in compliance with the Code of Ethics of The Endocrine Society and European Union Directive on Animal Experiments (Directive 2010/63/EU); specific procedures were approved by the ethics committee of the Government of Upper Bavaria, Germany (*Permit 2531-22-07*). Timed pregnant Wistar rats were purchased from Charles River Laboratories (Sulzfeld, Germany) on gestation day 15 and were housed individually under standard conditions (lights on: 18.00, lights off: 6.00). On the day of birth, litters were culled (8-10 pups), with equal distribution of males and females across litters. On postnatal days 1-14 (PND 1-14), litters were assigned to one of four treatment groups: vehicle (peanut oil), estradiol benzoate (EB, 7.5 µg; n = 13), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, ERα agonist, 50 µg; n = 10), or 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN, ERβ agonist, 50 µg; n=14). Estradiol benzoate (Sigma Aldrich, Deisenhofen, Germany), PPT and DPN (both from Tocris, Bristol, UK) were initially dissolved in absolute ethanol and peanut oil (final ethanol: 0.001%) and injected subcutaneously in a volume of 0.1 mL on every second day. Choice of doses was based on the relative binding affinities of PPT and DPN to ERα and ERβ respectively[14-15] and the relative transcriptional efficacies of these compounds compared to previously used compounds.[20] Upon weaning on PND 21, animals were ear-marked and housed in groups of 4 under an inversed light rhythm. Ovarian cyclicity was monitored (vaginal smear cytology) from post natal days 80 to 121 and female sexual behavior was assessed between days 114 to 121. Animals were tested for locomotor and anxiety-related behaviors in the open field[21] and elevated plus maze[22] starting at ca. 130 days of age, with an interval of at least 1 week between each test. All methods of behavioral analysis are described below. Blood samples (tail vein) were collected for evaluation of HPA axis activity and glucocorticoid negative feedback (PND 130); serum was stored at -20°C until assayed for hormones. Animals were killed on PND 150.

Assessment of female sexual behavior

Female sexual behavior was evaluated according to established protocols.[20] In brief, vasectomized, sexually experienced male Wistar rats were placed in the testing cage and allowed to habituate for 5 min before being presented with estrous females. The number of mounts, lordosis responses and ejaculations were used to compute the lordosis quotient over an observation period of 5 min.

Assessment of anxiety-related behavior

Thigmotaxis was evaluated (5 min) in an open field arena (LxBxH: 70×70×50 cm; non-reflecting white PVC) according to established protocols.[21] Randomly-cycling non-treated females were used as controls (to ensure a normal distribution of the phases of the oestrous cycle throughout the experiment) and both tests were performed under 100 lux illumination. Central and peripheral line crossings as well as time spent in the central area of the arena were scored. Anxiety-related behavior was evaluated in the elevated plus maze test[22] (LxBxH: 50×10×40 cm, with open-arm edges 0.5 cm high; placed 70 cm above the floor). The number of entries into, and the time spent in the open compartments of the maze were evaluated over a period of 5 min. Events in the open field apparatus and elevated plus maze were video-recorded and subsequently scored by an investigator blind to the treatments.

Characterization of HPA axis activity

Basal and stress-induced corticosterone secretion was monitored in serial blood samples as reported elsewher.[23] Serial blood samples (ca. 20 µL) were obtained while animals were in their home cages over approximately 20 s.

Samples for estimation of diurnal fluctuations in corticosterone were collected at the circadian zenith (06:00) and nadir (18:00). Immediately thereafter, animals were exposed to an emotional stressor for 2 min; to this end, animals were placed in an empty cage and exposed to an air puff delivered with a hair dryer. Blood samples were obtained 30 and 180 min later, to determine maximal corticosterone responses and shut-off of the endocrine response to stress. After a resting period of 3 days, animals were subjected to a dexamethasone suppression test (DST). For this, animals were given a bolus intraperitoneal (i.p.) injection of dexamethasone (Fortecortin®, Merck, Darmstadt, Germany; 10 µg/kg BW in a volume of 0.2 mL) at 24:00. Animals were blood sampled at 06:00 (the expected time of the circadian peak of corticosterone secretion). Serum samples were stored at -20°C until hormone assay.

Tissue processing

Animals were sacrificed at the circadian zenith of HPA axis activity (06:00) by rapid decapitation. Brains were rapidly removed from the skull, snap-frozen in pre-chilled isopentane and kept at -80°C until further processing. Six serial coronal (10 µm) cryosections were prepared from the PVN (bregma -1.53 to -1.78) amygdala (bregma -1.78 to -2.0) and dorsal hippocampus (bregma -2.45 to -4.60) and micropunches from the remaining parts of these areas of interest were obtained as previously described.[24] Sections and micropunches were stored at -80°C until further processing.

RNA isolation and qPCR

RNA was isolated from micro-dissected brain areas using RNAeasy® kits (Qiagen, Hilden, Germany), and 100 ng RNA were used for cDNA synthesis (RevertAid® kit; Fermentas, St. Leon-Rot, Germany). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green I Master mix on a LightCycler 480 (Roche Applied Science, Mannheim, Germany). Expression levels of mRNAs of interest were normalized against levels of Mas mRNA since preliminary studies showed that Mas *per se* is not regulated by sex or hormonal status (*data not shown*). Primer sequences are listed in Table 1 (5'-3').

GR and MR mRNA expression in hippocampus and amygdala

Labeled ribonucleotide probes for the detection of GR and MR protein-encoding transcripts were produced from linearized plasmids using *in vitro* transcription kits with T7, T3 and Sp6 RNA polymerases (Promega, Madison, WI) and [^{35}S]-dUTP (Perkin Elmer, Rodgau, Germany). The GR and MR expression plasmids were a generous gift from Dr. J. L. Arriza.[25-26] Cryosections were permeabilized and hybridized according to published protocols.[27] Autoradiograms (BioMax MR; Kodak, Rochester, NY) were analyzed by densitometry on two sections per animal, using the NIH software Scion Image Beta 4.2.0. Individual averaged transmittance levels were converted to specific radioactivity by third-order polynomial equations generated from co-exposed 14C

standards (ARC, St. Louis, MO, USA).

Hormone measurements

Serum corticosterone levels in serial blood samples, and estradiol, progesterone and luteinizing hormone (LH) concentrations in probes derived from trunk blood, were determined using commercially available radioimmunoassay (corticosterone: DRG Instruments, Marburg, Germany) or enzyme immunoassay (estradiol and progesterone: Beckman Coulter, Krefeld, Germany; LH: Millipore, Schwalbach, Germany) kits.

Statistics

Data are presented as either group means ± SEM or scatter plots with medians. Group means were compared by either parametric or non-parametric 1-way ANOVA and appropriate post-hoc tests (Tukey-Kramer or Kruskal-Wallis, respectively). The threshold of significance was defined as $P<0.05$.

Results

Programming versus disorganization of HPA axis activity

Several aspects of HPA axis function differ markedly in the two sexes. For example, previous studies showed that females secrete higher levels of corticosterone under both baseline and stressful conditions.[2-3] Further, those studies demonstrated that these endocrine profiles are subject to defeminization by neonatal exposure of female rats to estradiol benzoate (EB).[3,4] As shown in Figure 1, those earlier findings with EB were reproduced in the present work, in which neonatal estrogenization resulted in attenuated corticosterone secretory responses to stress (Figure 1A, $P<0.0001$, $F=26.4$) as well as reduced night time (zenith) baseline levels of corticosterone (Figure 1B, $P<0.0001$, $F=45.5$). Together, these results attest to the ability of neonatal EB to program the neuroendocrine system to elicit phenotypically male HPA axis responses in rats with a female genotype.

Since EB non-selectively activates both ERα and ERβ, HPA axis activity in adulthood was next assessed in females that had been exposed to selective agonists of either ERα (PPT) or ERβ (DPN) during neonatal life. As compared to normal female rats, PPT-treated animals had significantly lower daytime baseline corticosterone levels ($P < 0.01$, $F = 62.2$) although night time levels did not differ between the two groups (Figure 1B). Notably, neither PPT nor DPN treatments reproduced the effects of EB (daytime corticosterone: DPN > EB > PPT; night-time corticosterone: DPN > PPT > EB; Figure 1B). Interestingly, vehicle-, PPT- and DPN-treated rats all responded to an acute stressor with significant increases in corticosterone secretion. However, the between-group magnitudes of response differed remarkably (approximately 5-fold, 8-fold and 2-fold in the vehicle-, PPT- and DPN-treated groups, respectively; Figure 1A). In terms of relative magnitude of response to stress, the DPN-treated animals showed closest resemblance to the EB-treated group; however, it should be noted that the DPN group displayed high basal corticosterone levels, and that the attenuated corticosterone response to stress in DPN-treated rats is unlikely to be the result of reduced steroidogenic capacity since the levels of the mRNAs encoding for critical regulators of adrenocortical steroidogenesis (StAR protein, P450scc, 3β-hydroxysteroid dehydrogenase, and the ACTH receptor) were unchanged (data not shown). Considered together with the results obtained with EB, it is concluded that whereas dual occupation of ER during neonatal life programs the female HPA axis to express a male-like phenotype, selective activation of either ERα or ERβ results in malprogramming of the central mechanisms that regulate HPA axis function.

Table 1. Primer sequences (5'-3') used for quantitative polymerase chain reaction.

Gene	Forward primer sequence	Reverse primer sequence
Mas	AGTACCGTCGTGGCTGCTGAGAA	GGCGTTGTCCATGGCGGTCAT
SCC	TGCAGCTGCCTGGGATGTGATT	ATGGAGGTCGTGTCCACCCCTCC
StAR	GGCGTCGGAGCTCTCTACTTGGTTC	ACCTTGCCCACACCTGGCAC
3β-HSD	TTGTGGTGAGTCAGAAGACCAGGG	TGCTCCTGTCACCAGGCAGC
ACTHR	TTGTGACCATGCGACGCACC	CATGGTGATGCCGCTCCCTGT
ERα	GGCTGCGCAAGTGTTACGAA	CATTTCGGCCTTCCAAGTCAT
ERα	TTCCCGGCAGCACCAGTAACC	TCCCTCTTTGCGTTTGGACTA
GR	ACCTCGATGACCAAATGACC	AGCAAAGCAGAGCAGGTTTC
MR	CGTACAAACATACGAACAGCCA	TAGAACCTCTGCCAACTCTGTC

Mas, NM_053825; SCC, steroid side-chain cleavage; StAR, Steroid acute regulatory protein; 3 -HSD, 3β-hydroxysteroid dehydrogenase; ACTHR, Adrenocorticotropin (ACTH) receptor; ERα, Estrogen receptorα; ERβ, Estrogen receptorβ ; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

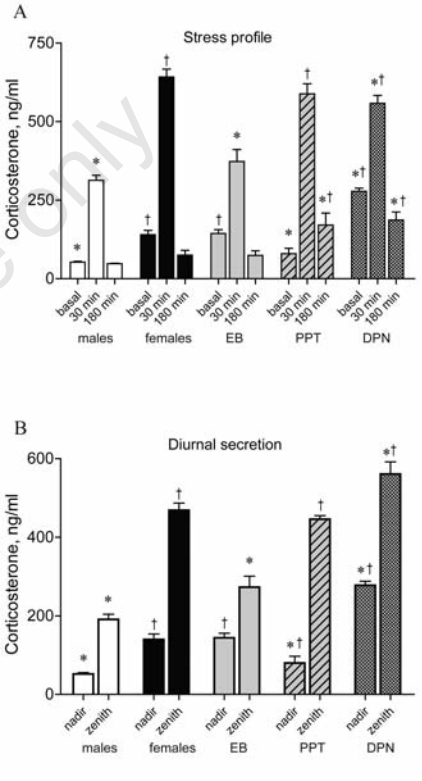

Figure 1. Corticosterone secretory profiles. Neonatal female rats were exposed to estradiol benzoate (EB), PPT (selective ER agonist) or DPN (selective ER agonist) and their basal and stress-induced corticosterone levels assessed during adulthood (PND 100). Comparisons were made with vehicle-treated female and male rats: A) depicts corticosterone levels at the circadian nadir, and 30 and 180 min after exposure to a brief emotional stressor; B) diurnal trough and peak levels of corticosterone secretion under quiescent conditions. One-Way-ANOVA were performed for each sampling time point: basal (nadir): F=62.2; 30 min: F=26.4; 180 min (Kruskal-Wallis ANOVA) H=26.3; zenith: F=45.5. Bars represent mean ± SEM of 9-14 animals/group. Asterisks indicate significant ($P<0.05$) differences as compared to control vehicle-treated females, crosses indicate significant ($P<0.05$) differences vs. control males.

Disruption of central mechanisms regulating HPA axis function

Homeostatic control of corticosterone secretion is maintained through a series of regulatory loops that are sensitive to the negative feedback actions of corticosterone. The dexamethasone suppression test (DST) serves as a powerful tool to assess the efficacy of corticosteroid negative feedback at both brain and pituitary levels.[28] Here, administration of the DST revealed that, as compared to vehicle- EB- and DPN-treated females, PPT-treated females display impaired corticosteroid-mediated negative feedback (Figure 2A, $P<0.0001$, $F=10.1$). Since males are known to be less sensitive to glucocorticoid negative feedback in the DST,[28] it is interesting to note that neonatal PPT treatment of females resulted in even greater insensitivity to dexamethasone ($P<0.001$).

While this impaired response to the negative feedback actions would be expected to result in increased basal corticosterone levels, PPT-treated females displayed significantly reduced levels of this hormone under basal conditions, as compared to control females (Figure 1B). In marked contrast to the PPT group, DPN-treated females showed high basal levels of corticosterone (Figure 1B) and were unimpaired in the DST (Figure 2A). These differences in feedback efficacy imply differential roles of ERα and ERβ in the programming and, possibly, regulation of corticosteroid feedback mechanisms.

The hippocampus is implicated as a major site of corticosteroid negative feedback.[29] It is richly endowed with the two types of corticosteroid receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptors. MR and GR differ in their affinities for corticosterone and together contribute to the maintenance of homeostasis in the HPA axis under basal and stressful conditions.[30] Analysis of GR and MR mRNA transcripts in the hippocampi of PPT- and DPN-treated animals indicated differential regulation of the two receptors by the ERα- and ERβ-selective ligands: as compared to vehicle-treated females, PPT- and DPN-treated females showed reduced levels of GR and MR expression, respectively (Figure 2B and 2C, P<0.0001, F=16.6 and P<0.0001, F=11.3 respectively). Together with the above-reported differences in baseline corticosterone secretion and sensitivity to corticosteroid feedback in the PPT- and DPN-treated groups, these observations are consistent with the suggestion that MR are responsible for maintaining HPA axis activity under resting conditions whereas GR are responsible for mediating corticosteroid negative feedback when corticosterone levels exceed a certain threshold.[30]

Opposing behavioral effects of isoform-selective neonatal ER activation

The amygdala is another important site of corticosteroid actions. This brain region is not only implicated in the control of emotional behaviors such as anxiety but also of HPA axis activity. However, in contrast to the hippocampus, the amygdala exerts a positive drive on the HPA axis.[31] Chronically increased levels of corticosterone are closely linked to the expression of anxiety-related behaviors[32] and, given the results described in the previous section, it was predicted that rats neonatally exposed to DPN would show the highest levels of anxiety. In this study, neonatal administration of PPT and DPN did not significantly influence amygdaloid GR mRNA transcript levels (as compared to vehicle-treated females; Figure 2E). In contrast, amygdaloid MR expression was significantly down- and upregulated after neonatal exposure to PPT and DPN, respectively (Figure 2D, P<0.01 and P<0.0001 respectively, F=22.2). In the present study, anxiety-related behavior was evaluated in the open field (OF) arena and elevated plus-maze (EPM) by monitoring the following standard parameters, validated to reflect anxiety-related behavior in rodents:[21,22] time spent by animal exploring the center area of the unfamiliar OF and in the open arms of the EPM as well as frequency of these events. When compared to untreated males and PPT-treated females, random-cycling control females showed less anxiousness in terms of the number of entries into (P<0.01, H=33.4) and time spent in the open arms (P<0.01, F=29.9) of the EPM (Figure 3A, 3B and 3C). However, DPN-treated animals displayed significantly less anxiousness than

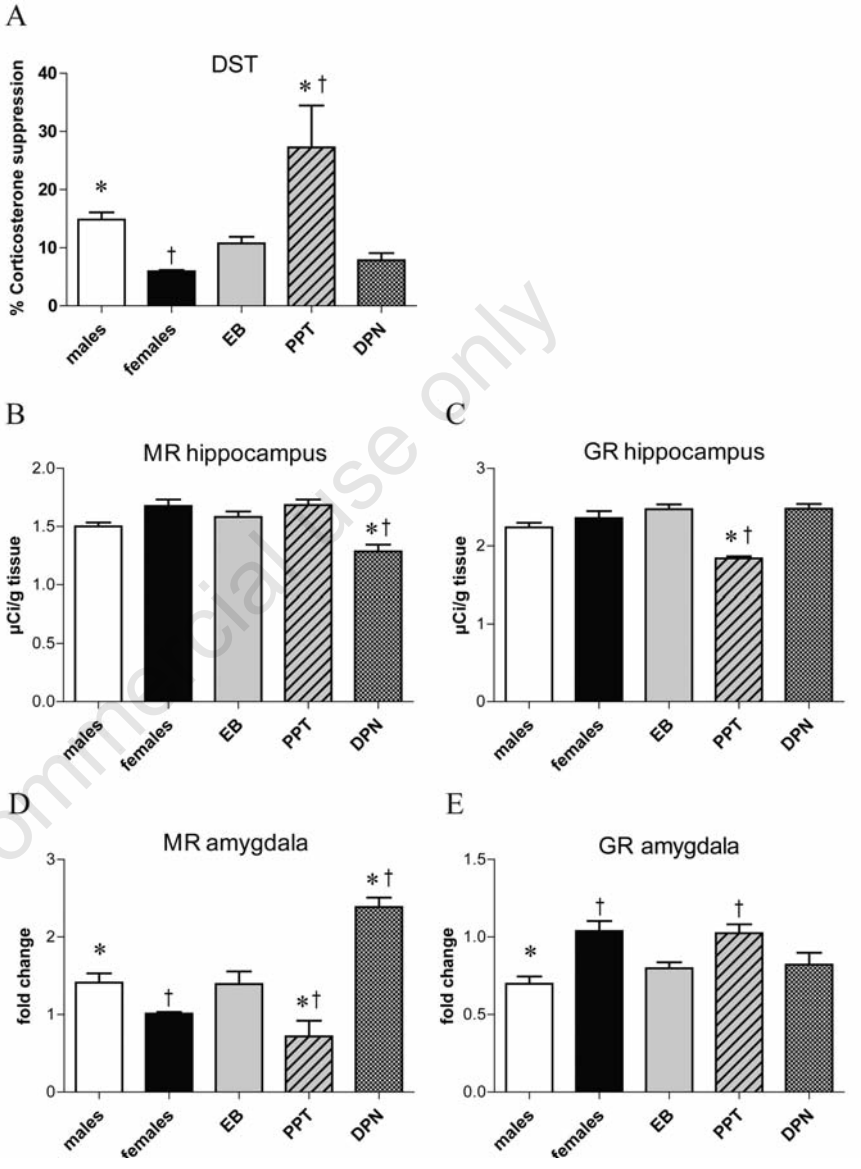

Figure 2. Glucocorticoid negative feedback and its molecular correlates. A) Efficacy of glucocorticoid negative feedback was evaluated by the dexamethasone suppression test (DST). Animals were given 10μg/kg BW dexamethasone 6 h before blood samples were collected at the time of the daily peak in corticosterone secretion. Data are presented as a percentage of each individual's peak level of corticosterone secretion on the previous (dexamethasone-free) day. One-Way-ANOVA: F = 10.1; asterisks indicate P<0.05 vs. control females, crosses indicate significant difference vs. control males (n=9-14 animals/group); mRNA expression levels of mineralocorticoid (B, D) and glucocorticoid (C, E) in the hippocampus and amygdala, respectively. mRNA transcripts in the hippocampus were assessed using semi-quantitative *in situ* hybridization histochemistry. Tissue punches from the amygdala were used to quantify mRNA levels by qPCR; values were normalized against those obtained in control females to yield fold-differences. One-Way-ANOVA: B) F = 11.3; C) F = 16.6; D) F = 22.2; E) F=6.5. All data are shown as mean ± SEM of 5-6 animals/group; asterisks indicate significant differences from control females (P<0.05), crosses indicate significant (P<0.05) difference vs. control males.

the vehicle-treated controls on these measures (Figure 3A, 3B and 3C, P<0.001 respectively). These results show that activation of ERβ in neonatal females results in a hypo-anxious phenotype despite overt hypercorticism. The analysis of the data from the OF test yielded a similar picture: DPN- and vehicle-treated females spent more time exploring the central area of the arena (a sign of reduced anxiety-related behavior) than males, EB- and PPT-treated animals (Figure 3D, P<0.001, F=6.22).

Modulatory influence of ovarian steroids

Neonatal estrogenization is known to abolish ovarian cyclicity by inducing the so-called *interrupted persistent estrus syndrome*[33] and we previously showed that activation of either ER isoform results in persistent estrus in adulthood.[20] The above-described mismatches between behavior and endocrine phenotype in the PPT- *vs* DPN-treated animals led us to consider the potential importance of differential alterations in gonadal status, resulting from isoform-selective neonatal estrogenization, in the observed behavioral phenotypes. The isoform-selectivity of the different neonatal treatments was verified by assessing female sexual behavior. We found that, whereas neonatal treatment with either EB or PPT results in a loss of female sexual behavior, neonatal exposure to DPN does not influence this parameter (*data not shown*); these results are consistent with our previous findings using other ERα and ERβ agonists.[20] As shown in Figure 4A, estradiol levels in adult females that had been exposed to PPT or DPN during neonatal life were not markedly different from those found in random cycling control females; on the other hand, neonatal EB treatment resulted in significantly reduced levels of estradiol secretion (P<0.001, H=13.13). Interestingly, serum progesterone levels were significantly reduced only in the EB and PPT-treated animals (P<0.001 and P<0.0001 respectively, H=22.8), but not in the DPN-treated group; these findings support the view that neonatal exposure to the ERβ-selective agonist does not abolish the steroid secretory activity of the adult gonad. While ovarian cyclicity (as judged by vaginal epithelial cornification) was abolished by all of the neonatal estrogenization paradigms (*data not shown*), the degree of ovarian dysfunction, as judged by gonadotropin (LH) secretion (Figure 4C, P<0.001, F = 10.4), ovarian histology (*not shown*) and ovarian and uterine weights (Figure 4D, P<0.0001, F=48.6 and P<0.0001, F=30.2 respectively) was graded: EB > PPT > DPN. It is interesting to note that, although elevated HPA axis activity is frequently associated with impaired reproductive function, the DPN-treated animals showed the least degree of ovarian disruption despite their high levels of corticosterone secretion (Figure 1B). Sex differences in basal and stress-induced anxiety are well-known and estrogens have been implicated in the regulation of anxiety in humans and rodents.[34] Experiments using either pharmacological or genetic approaches have suggested that ERβ mediate the anxiolytic effects of estrogens.[17,35-37] The latter, together with the above-reported hypo-anxious state of DPN-treated animals prompted us to examine ERβ expression in the amygdala. As shown in Figure 4E, amygdaloid levels of ERβ mRNA are sexually differentiated, with females displaying higher ERβ expression as compared to males (P<0.01, F=17.3). Generally, exposure of neonatal females to EB, PPT or DPN resulted in a significant reduction of ERβ mRNA levels in the amygdala (Figure 4E, P<0.0001, P<0.001 and P<0.001 respectively), but the degree of down-regulation was significantly less in the DPN-treated animals as compared to the EB- and PPT-treated groups (P<0.01). The latter suggests that neonatally DPN-treated animals are more responsive to estrogens, thus providing an explanation for their lower levels of anxiety. Further, since ERβ are implicated in mediating the ability of estrogens to drive the HPA axis at the level of the hypothalamic PVN,[17] it is interesting to note that ERβ mRNA levels in the PVN were least downregulated by DPN *vs* EB and PPT (Figure 4F, P<0.01, F=9.28). Together, the ERβ expression data in the amygdala and PVN offer a plausible mechanistic basis for the mismatch between the behavioral and endocrine phenotypes of the DPN-treated animals.

Discussion

Sexual differentiation of the mammalian brain results from activation of estrogen receptors (ER) by estradiol (E_2) during perinatal life.[9] Estradiol binds to both ER isoforms and is crucial for their transcriptional activity.[13] ERα and ERβ are expressed in a tissue-specific manner in peripheral tissues; in the brain, ERα and ERβ show discrete patterns of distri-

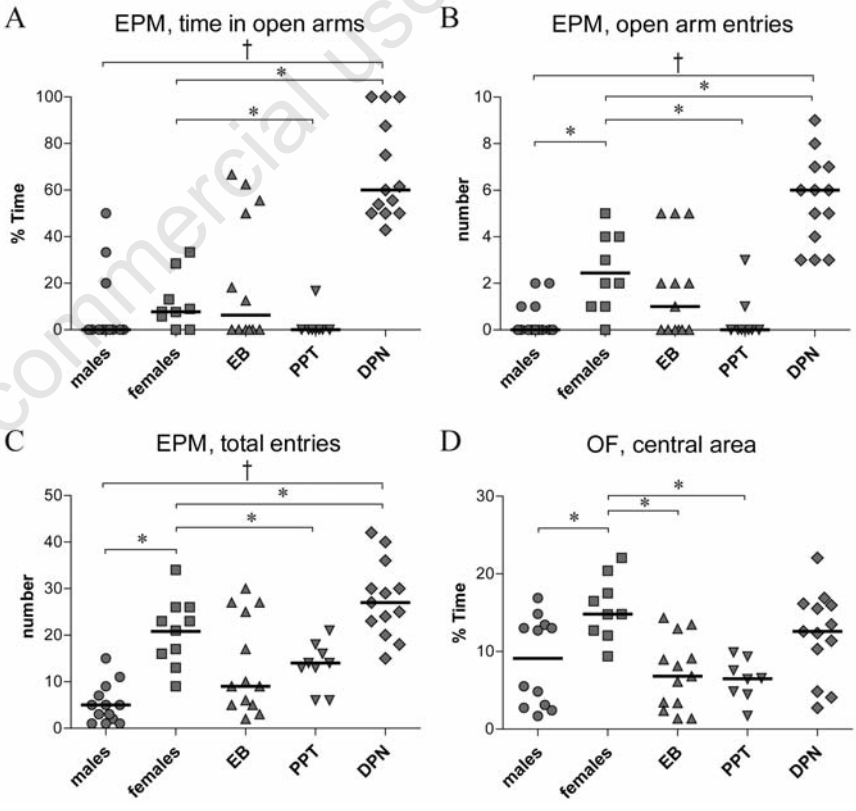

Figure 3. Differential effects on anxiety-related behavior. Measures of anxiety-related behavior were obtained in an open field arena and the elevated plus maze (EPM); animals were monitored in each test over a total of 300 s and behaviors were scored by an observer who was blind to the treatments. A) Percentage of time spent in the open arms of the EPM (Kruskal-Wallis-ANOVA H = 29.9); B) Number of entries into the open compartments of the EPM (Kruskal-Wallis-ANOVA H=33.4); C) Total number of entries into either open or closed arms of the EPM, serving as an index of locomotor activity (Kruskal-Wallis-ANOVA H = 31.9); D) Percentage of time spent in the center of an unfamiliar OF (One-Way-ANOVA F = 6.22). The results of each individual are plotted (horizontal lines show group median values), asterisks indicate P<0.05 vs. control females, crosses indicate significant (P<0.05) difference vs. control males.

bution.[11,12] While ERα are predominantly involved in the regulation of reproductive behavior and hormone secretion as well as growth and maintenance of peripheral reproductive tissues, ERβ are implicated in the control of a variety of non-reproductive functions, including the regulation of emotion and cognition.[17]

Estradiol can activate both ERα and ERβ[13] and current evidence suggests that estrogen actions are determined by cooperative as well as antagonistic actions of the two receptor types.[38] Previous studies in animals with targeted deletions of ERβ indicated that this ER isofrom is a crucial mediator of the anxiolytic effects of estrogens.[17,35,39] In addition, genetic and pharmacological approaches have demonstrated a role for ERβ in the regulation of corticosterone secretion. On the other hand, mice with ERα or ERβ null mutations do not display clear sexually differentiated HPA axis phenotypes. Accordingly, the goal of this study was to attempt to understand the relative contributions of each ER isoform to the sexual differentiation of the neural substrates responsible for regulation of HPA axis activity and anxiety. Based on the well-established paradigm of neonatal estrogenization of the female rat with E_2 – which results in the expression of clear male-like behavioral and neuroendocrine profiles[2,9] – we here treated neonatal female rats with selective ERα (PPT) or ERβ (DPN) agonists and analyzed their behavioral and endocrine phenotypes during adulthood. The specific features examined included activity of the HPA axis and expression of anxiety-related behavior. Sex differences have been described in both of these functions[2,40,41] and, in addition, elevated HPA axis activity is positively correlated with increased emotionality and susceptibility to depression and anxiety in humans and animals.[1,42]

As compared to females, males secrete lower amounts of corticosterone under basal conditions and in response to stressful stimuli.[2] Further, glucocorticoid negative feedback is less efficient in males than in females and thus, shut-off of the HPA axis response to stress is more sluggish in males.[2,4,28] We previously showed that neonatal administration of E_2 defeminizes these measures of HPA axis function in female rats,[4] a result reproduced in the present work. Our results also show that neonatal activation of either ERα or ERβ does not defeminize, but clearly disrupts the mechanisms governing HPA axis activity. Interestingly, the two agonists resulted in opposing endocrine phenotypes: whereas animals exposed to neonatal PPT showed female-like corticosterone secretory response to stress, those exposed to DPN presented with hypersecretion of corticosterone under resting conditions and a relatively blunted endocrine response to stress. Despite these anomalies, the DPN-treated group did not show alterations in their ability to respond to the negative feedback actions of glucocorticoids, as judged by their normal post-stress shut-off of corticosterone secretion and their responses in the DST. In contrast, the animals that had been exposed to neonatal PPT showed marked impairment in terms of glucocorticoid negative feedback.

The actions of corticosterone are mediated by MR and GR; these nuclear receptors are negatively regulated by corticosterone and play a key role in maintaining homeostasis in the HPA axis. Both MR and GR are strongly expressed in limbic regions such as the hippocampus and amygdala where they act to regulate emotional and cognitive behaviors.[30] In addition, MR and GR are expressed in the

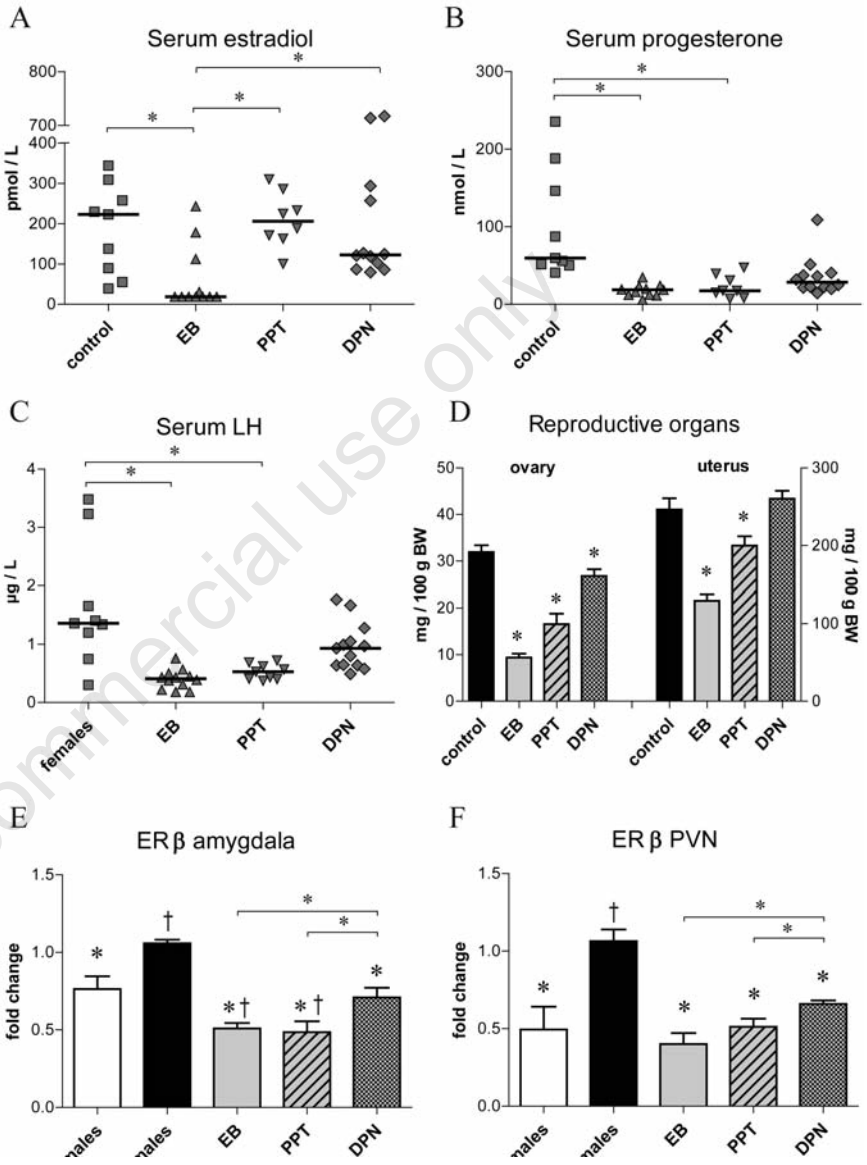

Figure 4. Descriptors of reproductive function and ERβ expression in the amygdala and hypothalamus. Reproductive parameters evaluated included A) serum estradiol, B) progesterone, C) luteinizing hormone (LH), and D) ovarian and uterine weights. The latter were normalized to body weight (BW) at the time of sacrifice. Individual hormone values and group medians are depicted in A-C). Kruskal-Wallis-ANOVA: A) H = 13.1; B) H = 22,8; C) (One-Way-ANOVA): F=111.4 (asterisks indicate P<0.05 vs. control). Data in D) are means ± SEM (n = 9-14); significant differences from control females are denoted by asterisks (P<0.05; One-Way-ANOVA ovaries: F=48.6; uteri: F=30.2). Expression levels of ERβ mRNA in tissue punches from the amygdala (E) and paraventricular nucleus of the hypothalamus (PVN) (F) were obtained by qPCR and values (fold change) are shown normalized against those obtained in control females. Bars represent group means ± SEM of 5-6 animals per group. One-Way-ANOVA E) F = 17.3; F) F = 9.3. Asterisks indicate significant differences vs. control females, crosses indicate significant differences vs. control males (P<0.05).

hypothalamus; within the hypothalamic paraventricular nucleus (PVN), MR and GR are important for inhibiting the central neuropeptidergic (CRH, AVP) drive on the pituitary-adrenal unit.[43] While MR are suggested to be responsible for maintaining corticosterone levels under basal conditions, GR are implicated in restoring physiological levels of corticosterone secretion following stress.[30] Given the above-mentioned disruption of HPA axis regulation, it was considered important to gain some insight into the contributory mechanisms by analyzing *MR* and *GR* expression in the hippocampus. Our finding that hippocampal MR mRNA expression is reduced in animals given DPN during neonatal development provides an explanation for the elevated basal levels of corticosterone secretion in these animals. Neonatal exposure to PPT resulted in a downregulation of *GR* expression in the hippocampus, providing a potential mechanistic explanation for the impaired negative feedback efficacy of corticosteroids in the PPT-treated animals.

As already alluded to, chronically elevated levels of corticosterone are frequently associated with a hyperanxious state.[32] Most studies consider impaired glucocorticoid negative feedback as a factor that contributes to this correlation.[44] Intriguingly, our assessment of anxiety in animals that had undergone selective neonatal activation of ERα or ERβ does not support the view that anxiety is a direct correlate of HPA axis activity. On the one hand, we found that neonatal activation of ERα with PPT leads to increased anxiety, an effect that could be explained by the fact that PPT-treated animals are poor responders in the DST and show exaggerated endocrine response to stress; the latter is believed to be a precipitating factor in anxiety disorders.[45] On the other, we observed reduced anxiety in the DPN-treated rats; these animals showed chronically elevated baseline corticosterone secretion but normal endocrine responses to stress and the DST.

Several groups have suggested a role for GR in the regulation of anxiety. For example, conditional overexpression of *GR* in the dentate gyrus of the mouse hippocampus reportedly increases anxiety-related behavior as measured in the elevated plus maze,[46] while *GR* knockout mice display reduced anxiety-related behaviors.[47,48] In contrast, forebrain- or amygdala-targeted overexpression of *MR* is reported to reduce anxiety in rodents.[49] In this respect, it is notable that animals in which ERα were activated by PPT during neonatal life display significantly reduced levels of amygdaloid MR mRNA as compared to vehicle-treated controls; in contrast, neonatal PPT treatment did not elicit any changes in *GR* expression in this brain area. Interestingly, the hypo-anxious state observed in DPN-treated rats was associated with a >2-fold upregulation of MR expression in the amygdala and we propose that increased MR levels in the amygdala, resulting from neonatal activation of ERβ, serve to reduce anxiety. Amygdaloid MR may also act to buffer against the high levels of corticosterone experienced by animals exposed to the ERβ agonist during neonatal development by reducing the availability of corticosterone at GR (cf. the MR-GR balance hypothesis proposed by de Kloet and colleagues[30]) or the efficacy of GR activity.[50,51] In addition the increased MR expression in the amygdala of neonatally DPN-treated animals might account for the sluggish acute adrenocritical stress response in these animals (Figure 1A); previous work described a dampening of stress-induced corticosterone secretion in rats overexpressing amygdaloid MR.[49]

Although plausible explanations can be found for the apparently dissociated endocrine and behavioral profiles observed in adult rats whose ERα or ERβ had been activated during neonatal life, it is important to consider other factors that could have contributed to the development of the specific phenotypes. The neonatal treatments in the present study were used to study the so-called *organizational* actions of early estrogens on HPA axis function and expression of anxiety. However, results from our previous studies showing that ERα or ERβ also differentially organize reproductive development cannot be ignored.[20] Here, we found that, whereas neonatal E_2 treatment results in hypogonadotropic hypogonadism, anovulatory ovaries and persistent estrus as expected,[20,52,53] neonatal exposure to PPT and DPN, while also causing persistent cornification of vaginal epithelia, only partially disrupts ovarian activity; in particular, the DPN-treated animals did not differ markedly from vehicle-controlled rats in this respect and continued to secrete amounts of estrogen that would be sufficient to exert so-called *activational* actions. Numerous studies have shown that estrogens sex-dependently stimulate the HPA axis in adulthood and there is evidence that low estrogen levels are associated with increased anxiousness in humans and animals.[7,34,54] In light of previous studies that described the anxiolytic actions of ERβ agonists,[36] the present finding that ERβ are significantly less downregulated in the amygdala of DPN- treated animals indicates that preserved ERβ signaling in the amygdala contributes to the anxiolytic phenotype in the DPN-treated animals. This assumption is further supported by the finding that gonadal secretory activity was not completely abolished in this group, despite the cytological observation of persistent estrus. While levels of estradiol secretion were similar in PPT-treated and control animals, it should be noted that the PPT-treated group showed the highest degree of ERβ downregulation and high levels of anxiety-related behavior. Accordingly, the distinct behavioral and endocrine phenotypes expressed in adult females that had experienced selective neonatal stimulation of either ERα or ERβ likely result from the *activational* effects of residual estrogen secretion.

Taken together, the present results show that isoform-selective ER activation during neonatal life does not *per se* contribute to sex-specific organization of the HPA axis, but rather leads to dysregulation of the central mechanisms governing corticosterone secretion under basal and stressful conditions, and dissociate the usual relationship between corticosterone levels and anxiety. In contrast to individual activation of ERα and ERβ, dual activation of both ER isoforms with E_2 in neonatal females produces a male-like phenotype in which low levels of corticosterone are associated with reduced anxiety-related behavior. Further, our results identify hippocampal and amygdaloid MR and GR expression patterns as correlates of the disrupted endocrine and behavioral profiles. Nevertheless, the molecular and cellular pathways and mechanisms through which neonatal estrogenization exerts its sustained effects on the expression of MR and GR remain to be elucidated. While epigenetic marking of ER-responsive gene loci may account for the sustained effects of neonatal estrogen exposure,[55] the present work indicates that both HPA axis activity and anxiety in neonatally PPT- and DPN-treated animals remain subject to regulation by residual ovarian secretions acting at central ERβ. Since many environmental endocrine disruptors activate ERα and ERβ[18,19] these findings may be of wider relevance, beyond the present interest in sexual differentiation of the brain and regulation of the endocrine response to stress and stress-related behavior.

Lastly, the present findings have implications for human health since dysregulation of the HPA axis is associated with the pathogenesis of mood and anxiety disorders, both of which show a higher prevalence in women.[1]

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3. Discussion

The present work illustrates three different aspects of the ontogenetic programming of the neuroendocrine stress response and adaptive behavior. We demonstrate that neonatal exposure to either stress or GC has profound and long-lasting effects on the structural and neurochemical organization of brain mechanisms which are essential for homeostasis. The experimental challenges (stress, glucocorticoid excess or sex-steroid application) we applied during the same period of early ontogeny (neonatal period) differentially alter the activity thresholds of homeostatic sensors and elicit adaptive responses of inappropriate magnitude throughout later life. As many of our observations suggest the existence of sex differences in the quality and degree of manifestation of symptoms of mal-adaptation, the examination of the contribution of sex hormones to neonatal organization and adult modulation of endocrine and behavioral responsiveness elucidates certain novel aspects of the interaction between neuroendocrine circuits in this context. Although the link between the three studies described above might appear elusive at first glance, they address three major facets of brain programming during early ontogeny: substrate morphogenesis, neurochemical properties, and editing of the sensitivity to physiological hormone secretions. The focus on the mal-programming of the HPA axis is justified by its paramount involvement in the pathophysiology of several disorders of ill-adaptation, especially such affecting mood and cognition, which currently account for the greatest disability rates in industrialized countries (Kessler, 2012; Larson, 2010).

3.1 Physiological programming of brain functions

As already stated in the introduction, programming of physiological functions determines the bandwidth of homeostatic processes in later life. Especially during early, sensitive (and, thus, vulnerable) time windows of development endogenous and exogenous factors affect virtually every organ system and program its later functionality. Programming on the one hand represents the organization of biological functions by endogenous signals according to the genetic blueprint (e.g. sexual differentiation of the brain (Dörner, 1983; Arnold and Breedlove, 1985), on the other hand it is a result of proper or impaired capacity to re-set a system following its response to an external stimulus (e.g. metabolic changes in response to intrauterine nutritional status (Hanson et al., 2011a; Gluckman et al., 2009).

Programming through exogenous stimuli pre-sets physiological sensory gauges and thresholds for elicitation of responses to external stimuli, thus warranting the ability to discriminate and evaluate the significance of sensory input and perform adequate responses. For instance intrauterine mal-nutrition in humans and animals will cause specific effects on feeding and metabolic functions to preserve energy homeostasis in a certain nutritional environment (Lillycrop et al., 2010; Gluckman et al., 2011; Kyle and Pichard, 2006).

If assuming that programming through environmental factors represents focusing of physiological functions to ensure survival in a certain environmental setting, it is conceivable that the more demanding the programming environment is, the more restrictive the programmed phenotype will become at the cost of confined homeostatic flexibility and adaptability. The paradigm of programming through neonatal stress used in our studies would thus translate into endocrine and behavioral alterations which reflect re-definition of homeostatic set points, but are unnecessary and, even, pathogenic in a stress-free environment (Kaufman et al., 2000). Intriguingly, in animals with a history of neonatal stress, exposure to chronic stress in adulthood results in apparent improvements in certain behavioral domains (anxiety, cognitive functions), as well as endocrine response to stress, indicating that these animals might be able to cope with certain stressors in adulthood better than those without stressful experience as neonates (*Patchev, Sousa, Almeida, unpublished observations;* Karatsoreos and McEwen, 2011). While from an anthropomorphic point of view this seems plausible (a larger stress-coping repertoire in those who grew up on the "wrong side of the tracks"), from a biological and medical perspective the elucidation of the mechanisms which account for the apparent resilience towards stress represents a challenging issue. Indeed, the insensitivity and, often questionable, validity of behavioral readouts in rodents (Sousa et al., 2006; Anisman and Matheson, 2005; Matthews et al., 2005) do not allow a comprehensive examination of all facets of the stress-coping ability and, to even lesser extent, the conduction of comparisons between individuals with different neonatal history.

Another open question (provided this phenomenon proves valid) is whether enhanced stress-coping ability following neonatal stress becomes equally manifest in all stress modalities. In this context the possibility should be considered that individuals with a history of adverse experience in early life might be able to cope better with stressors of given modalities and/or high

intensities, while displaying deficits in situations or stressors where these conditions are not met.

It should be pointed out that programming of neuroendocrine and behavioral responses is not limited to the intrauterine or neonatal period (Andersen, 2003). Many studies are showing that adolescence and puberty in rodents and primates are permissive windows of time for both endogenous and exogenous programming stimuli. Over the last decades we have learned, that sexual differentiation and organization in mammals is not "finalized" until the organism goes through puberty where certain behavioral and endocrine functions are subject to ultimate organization by physiological sex steroid secretions (Schulz et al., 2009; Vigil et al., 2011). Adolescence and puberty also represent critical periods of increased vulnerability to the programming effects of stress (Beardslee et al., 2012; Lupien et al., 2009; Koenig et al., 2011) and mal-nutrition (Pervanidou and Chrousos, 2012; Must and Strauss, 1999). We and others have shown that adolescent rodents respond differently to stress than adults and that chronic stress during puberty has long-term effects on emotional and cognitive behaviors as well as neuroendocrine functions in later life (*Patchev, Sousa, Almeida unpublished observations;* Romeo, 2010).

However, with increasing age of the organism, a higher intensity or duration of a stressor is required for the induction of sustained after-effects in adaptive capacity. For instance, a stressful challenge which produces life-long alterations in learning and coping style when applied during the peripubertal time window, elicits similar, but reversible effects when applied in adults, with spontaneous recovery after 6 weeks thereafter (*Patchev, Sousa, Almeida unpublished observations;* Sousa and Almeida, 2012). It cannot be ruled out, however, that during adulthood and senescence the brain may remain liable to the programming effects of stress, depending on its duration and intensity.

Taken together, physiological programming of brain functions requires endogenous and exogenous stimuli of appropriate intensity and duration, acting at the proper target substrate within a defined period of time. This programming pre-sets the quality and magnitude of future endocrine, metabolic and behavioral responses to environmental challenges, thereby determining our homeostatic flexibility and adaptability. If the orchestrated sequence of programming processes is disturbed, the adaptability of the homeostatic rheostat might become insufficient and result in overt pathology (i.e. mal-adaptation as a result of mal-programming).

3.2 Consequences of mal-programming and their implication in mental disorders

Mal-programming of the HPA axis and, consequently, the responsiveness to stress have been increasingly linked to disorders of the brain (Aguilera, 2011; Mesquita et al., 2009; Saveanu and Nemeroff, 2012). Many studies have demonstrated associations between stress and hypercorticism, and the prevalence, severity and susceptibility to therapeutic interventions of depression and other mood disorders (de Kloet et al., 2005). Importantly, correction of HPA axis dysfunction shows (albeit with major inter-individual variability and in still poorly defined clinical sub-forms of the disease) beneficial additive therapeutic effects to classical antidepressants (Schatzberg and Lindley, 2008).

Stress during childhood and adolescence have been identified as risk factors for depression, and in these cases signs of hypercorticism are more common than in the general population of patients with a history of depressive episodes (Kaufman et al., 2000; Joyce et al., 2007; Penza et al., 2003). In view of the confirmed role of stress as precipitating factor in the pathogenesis of affective disorders, the identification of this cluster of patients points at a potential causality connection between, or a facilitating role of mal-programming of the HPA axis function by early life stress experience and the emergence of this mental condition.

Stress and glucocorticoids also strongly affect cognitive functions and growing evidence suggests that both are risk factors for cognitive disorders such as Alzheimer's disease (Sotiropoulos et al., 2008b; Yu et al., 2008). Work from our labs and others has identified direct links between glucocorticoids and amyloidogenic pathways, resulting in hyperphosphorylation of Tau and the increased production and deposition of pathogenic amyloid β fragments, the hallmark of Alzheimer's disease (Sotiropoulos et al., 2011; Catania et al., 2009). These findings suggest that a mal-programmed, hyperactive HPA axis could tilt neuronal homeostasis towards amyloid overproduction and thus increased risk of developing or faster progression of imminent AD (Sotiropoulos et al., 2008b). Importantly, both AD and depression show higher prevalence in women, thus implying the existence of sex-specific risk factors in these pathological states (Angst et al., 2002; Vest and Pike, 2012). Sex differences in HPA axis function appear plausible candidates for the role of such gender-specific predisposing factors, however scrutiny of human data fails to show association between sex-specific

dichotomies in cortisol secretion and the expression of depressive behavior (Hinkelmann et al., 2012).

Although female rodents display a behavioral repertoire that seems to reflect lower incidence of anxiety- and depression-related behaviors (Dalla et al., 2011), the large fluctuation of test endpoints in the course of the ovarian cycle make experimental work in female rodents more bias-prone (e.g. task acquisition which requires several days of training and the fulfillment of the learning criteria shows large variations in female mice, with the outcome strongly depending on the gonadal endocrine milieu at the time of retrieval testing) (*Patchev, Almeida, Sousa unpublished*). Research in non-human primates has also failed to reproduce the observation of higher vulnerability to stress-related disorders in human females (Willard and Shively, 2012). Furthermore, with the incidence of both depression and Alzheimer's disease increasing with age, the contribution of menopausal sex hormone deficiency has been vigorously debated in the past (Llaneza et al., 2012). The involvement of gonadal hormones in the regulation of mood, cognition and stress-related endocrine functions is well known (Dalla et al., 2011; Luine, 2008; Patchev and Almeida, 1998), and it is conceivable that life stages with abrupt changes in sex hormone secretions might be associated with increased incidence of depression in both sexes (Bebbington et al., 2003).

This allows for the speculation that the interactions between gonadal hormones and stress-related behavioral and endocrine functions throughout the lifespan might represent a vulnerable homeostatic set-point at which stress might exert mal-programming effects. Our studies on the programming effects of estrogens on the HPA axis function and anxiety suggest that, rather than perinatal organization, endogenous gonadal secretions might exert a stronger leverage on these outcomes. Still, speculations on the revenant issue of the interplay between the adrenal and gonadal axes in humans are, nonetheless, incongruous, also in view of the scarcely elucidated importance of progestins as endogenous buffers of glucocorticoid effects in the brain (Patchev and Almeida, 1996).

Evidence in support of the organizing capacity of ER isoform-selective ligands on neural circuits controlling gonadal and, secondarily, adrenal functions prompts the question of mental health consequences of exposure to endocrine disruptors. Endocrine disruptors (e.g. compounds with xenoestrogenic activity) are ubiquitous in form of environmental pollutants but also items of every-day use and affect human and wild-life health (Flint et al., 2012; Yang et al., 2006).

Epidemiological evidence hints at a possibility that teratogenic effects of xenoestrogen exposure during early life might represent risk factors for affective disorders in adulthood (Crews and McLachlan, 2006; Weiss, 2011). The well-known potential of these compounds to disrupt adult gonadal function extends the question of xenoestrogen significance in the pathogenesis and neuroendocrine epiphenomena of mental disorders to life phases beyond sexual maturity.

3.3 Mechanisms of programming and mal-programming

Programming of the stress-sensing threshold, response capacity and endurance to noxious challenges can occur at any level of the HPA axis.

Here we show that stressful experience during early ontogeny leads to epigenetic marking (hypomethylation at specific CpG islands of the enhancer region) of the AVP gene, a key trigger of the neuroendocrine stress response (Volpi et al., 2004). We also gathered preliminary evidence (*manuscripts in preparation*) that such epigenetic alterations with functional relevance possibly affect other genes, whose products comprise almost the entire effector cascade (e.g. CRH and POMC), as well as the gauge which accounts for the efficient reset of the system (e.g. GR).

Epigenetic mechanisms comprise, amongst others, posttranslational histone protein modifications, as well as DNA methylation (Jaenisch and Bird, 2003). Initially it was suggested, that while histone modifications are transient, DNA-methylation changes are persistent (Razin, 1998; Razin and Riggs, 1980). Recent evidence, however, reveals that DNA-methylation is a rather dynamic process (Métivier et al., 2008; Unternaehrer et al., 2012), with DNA and histone modifications being highly interdependent in terms of regulation of gene expression (Razin, 1998; Bird, 2001; Jaenisch and Bird, 2003). While mechanisms of active DNA de-methylation at CpG islands have not been shown in mammals yet, recent findings indicate that DNA methylation has to be actively maintained, e.g. by a restrictive histone confirmation that also leads to the site-specific recruitment of DNA methyl trasnferases (Chen and Riggs, 2011). Taken together these findings evince that programming through epigenetic mechanisms is unlikely to act in an “on-off” fashion, but rather comprises orchestrated signaling processes that maintain methylation marks depending on the cell type and its paracrine and endocrine milieu.

One intriguing observation in our study was that neonatal stress-induced upregulation of AVP mRNA became manifest on postnatal day 10 (last day of stress exposure), while no measurable alteration in CpG methylation could be documented at that time point. Since, however, inactivation of MeCP2 was observed at this time in AVP neurons, we presume that the increased AVP expression might represent (at least in part) a process of Ca^{2+} - CamKII- driven phosphorylation. At 6 weeks of age hypomethylation of the AVP enhancer region was clearly present; this finding indicates that the intracellular mechanisms that had been set off by the stressful experience required several weeks to "establish" the epigenetic footprint on that particular gene locus. Similar (and even longer) time courses were observed also with regard to other HPA axis-related genes (CRH, POMC, GR). At this stage we can only speculate about the control of the temporal pattern of the AVP enhancer CpG hypomethylation. One possibility is that the chromatin structure has been transiently altered in a mode that would allow passive de-methylation of critical gene loci through signaling mechanisms that also contribute to MeCP2 phosphorylation at Serine 438 (Zhou et al., 2006); similar mechanisms have been described in cancer and pluripotent stem cells (Shoemaker et al., 2011).

An alternative assumption is that neonatal stress exposure results not only in transient changes of intracellular signaling, but also in altered neuronal "hard wiring". Pertaining to the parvocellular AVP neurons, this would mean that the afferent input into these neurons has been changed by the neonatal stress procedure, leading to persistent activation of AVP gene expression and also allowing for passive demethylation of the AVP gene. Several studies have demonstrated that neonatal stress leads to lasting alterations in the monoaminergic input to the hypothalamus (Liu et al., 2000), which in turn could prompt altered activity and, ultimately, epigenetic changes in parvocellular CRH and AVP neurons (Pacak et al., 1995). This assumption would suggest that the epigenetic marking seen in neuroendocrine cells are secondary to stress-induced alterations in distant neural sensors of stress (Liu et al., 2000; Kaufman et al., 2000).

The question of the substrates affected by mal-programming of the HPA axis by neonatal stress is not resolved by the identification of the hypomethylated AVP gene enhancer: even with the proof of functional significance, it might represent merely one of several candidates. With regard to rodent models, however, it is prudent to remark that it is highly unlikely that these effects of neonatal stress are a direct consequence of altered glucocorticoid secretions during the

neonatal period. Mechanisms of endocrine regulation in rodents undergo substantial maturation *ex utero*, with adrenal glands becoming functional only around postnatal day 14 (Sapolsky and Meaney, 1986). Basal and stress-induced GC secretions in newborn rodents are barely detectable, a phenomenon which coined the term "stress hypo-responsive period" (Vázquez, 1998; Sapolsky and Meaney, 1986). It is pertinent to mention that this term is to a certain extent misleading, as neonatal mice and rats respond to stress in several modalities (e.g. ultrasonic vocalizations (Hofer, 1996) and catecholamine release (Sullivan, 2003) in response to maternal separation) except for the inability of their immature adrenal cortex to mount a full-scale secretory response. Still, the importance of GC in the process of mal-programming of the HPA axis responsiveness merits further attention. Exaggerated secretory responses to stressful challenges are a hallmark of disturbed HPA function. While impairment of the neonatal organization might not depend on GC secretions, the resultant sustained adrenal hypersecretion in adulthood is associated with structural, behavioral and endocrine aberrations which are ascribed to the phenotype induced by early life stress, but may reflect the consequences of sustained adrenocortical activation (Murgatroyd et al., 2009; Sousa and Almeida, 2012). The distinction between such “bystander” effects from those laid down during neonatal mal-programming remains a challenging issue.

Neonatal stress and exposure to non-physiological glucocorticoid doses seem to produce divergent, even partly opposite, effects. We here show that neonatal treatment with the GR agonist dexamethasone leads to hippocampal atrophy and growth retardation in later life, due to reduction of the neural precursor cell pool. In contrast, neonatal stress has been shown to increase hippocampal neurogenesis in adulthood (Hays et al., 2012). While the consequences of the pharmacological impact are readily explicable, the mechanisms which account for increased neurogenesis remains elusive. It can not be ruled out that they are due to compensatory mechanisms which are set off by the neonatal adverse experience. Still the possibility remains that the mal-programmed phenotype might reflect impaired efficacy of such compensatory mechanisms. It remains intriguing that neonatal stress is associated with impaired hippocampal function on the one hand (*Patchev, Sousa, Almeida unpublished observations*; Marco et al., 2012; Mesquita et al., 2009), and increased neurogenesis on the other (Hays et al., 2012). This prompts questions of the functional features of the post-stress newborn neurons, of their capacity to integrate into functioning hippocampal circuits or whether hippocampal deficits in neonatally stressed animals would be augmented if their adult

neurogenesis is blocked. These open questions also indicate that, while morphogenic aspects (e.g. cell numbers, proliferation, migration, cell death, cell plasticity) are subject to neonatal programming (Kaufman and Charney, 2001; Kaufman et al., 2000), the structural alterations might have long-term consequences (and, even, exert programming themselves) in distant and not immediately affected brain regions.

Dealing with programming of complex neuroendocrine systems and neuronal circuits one might be mislead in terms of non-justified emphasis on singular processes (i.e. “hens and egg” issue). This is exemplified by our studies on sex-specific and estrogen-dependent differentiation of reproductive (Patchev et al., 2004) and HPA axis functions (Patchev et al., 2011). We and others have shown that estrogens with ERα activity have the ability to produce defeminization of sexually dimorphic brain areas by either inducing apoptosis (Kato et al., 2012; Tsukahara, 2009; Lephart et al., 2003) or increasing proliferation and migration (Jacobson and Gorski, 1981), whereas compounds with ERβ selectivity affect only certain morphological sex-specific features (Patchev et al., 2004). These findings offer a plausible explanation of how neonatal exposure to estradiol (a non-selective ER ligand) or ERα-specific agonists leads to a defeminization of structures accounting for both, cyclic operation mode of the GnRH pulse generator (AVPV) and proceptive sexual behavior (SDN-POA), whereas exposure to ERβ agonists is capable to abolish only cyclic gonadotropin secretion, but not female sexual behavior. The fact that animals neonatally treated with ERβ agonists displayed residual ovarian secretions and intact behavioral responsiveness to estrogens was interpreted merely as corroboration of the differential organizing efficacy of isoform-selective estrogens (Patchev et al., 2004). However our recent study on the contributions of ER isoforms to the sex-specific programming of the HPA axis (Patchev et al., 2011) shows that the consequences of neonatal estrogenization on HPA axis activity in adulthood are secondary to the degree of impairment of gonadal function, rather than to specific organizational effects of estrogens on brain areas of relevance in HPA axis regulation.

The differential (and, in some aspects, opposite) effects of selective ER agonists on HPA axis function and emotional behaviors cannot be ascribed to "once-and-forever" consequences of structural alterations, especially in view of the different adult sex steroid milieu in rats treated neonatally with ERα- and ERβ-selective ligands. Residual (albeit not genuinely cyclic) ovarian estrogen and progestin production and, most important, preserved sensitivity to estrogens in

several brain areas of behavioral relevance in rats neonatally exposed to ERβ agonists support the view that sex-specific features of HPA and behavioral responses to stressful challenges are decisively shaped by the adult gonadal secretions, thus pointing at the importance of the so-called activational effects of sex hormones. Certainly, direct teratogenic effects of neonatal estrogenization at some levels of HPA axis cannot be ruled out and deserve further attention. Notwithstanding this, sex-dimorphic endocrine and behavioral responsiveness to stress is apparently based on programming (organizing) effects which target primarily the gonadal neuroendocrine axis and its responsiveness to estrogens, thus preparing the ground for differential modulation of HPA axis activity by adult (activational) actions of physiological sex hormone secretions (Arnold and Breedlove, 1985). It is pertinent to underline that in earlier studies sex-specific programming of the HPA axis (Patchev et al., 1999; Patchev and Almeida, 1998) might have erroneously exaggerated the importance of neonatal organization, while neglecting the superimposed effects of adult sex hormone secretions. These conclusions, however, were all based on experiments using the non-selective ER agonist estradiol, which acts in an all-or-none fashion in this model and precludes the preservation of residual ovarian function in adulthood; the insight presented in our last study was made possible by the discovery of novel pharmacological tools and thus have added to our understanding of the mechanisms of establishing a sex-specific HPA axis phenotype.

In summary, we demonstrate that programming of the HPA axis occurs at many morphological, biochemical and systemic function levels, e.g.

- at the epigenetic level, where stable or dynamic epigenetic alterations (DNA methylation and histone modifications) lead to sustained alteration of gene expression. The mechanisms leading to the establishment and maintenance of these changes, as well as the possibilities of their reversibility (e.g. pharmacological or behavioral) still need to be explored in greater detail.

- at the structural level, where neonatal glucocorticoid exposure can affect neuronal survival and neurogenesis by altering the availability of neural precursors. Still, the possibility that neuronal cell death or birth might result from sequential activation (or failure) of pre-existing compensation mechanisms and associated cell programs deserves future exploration.

- at the systemic functional level, where the structural and epigenetic changes might lead to differential responsiveness of neuronal populations to external or endogenous signals, which in turn might lead or contribute to the expression of the mal-programmed phenotype.

3.4 Ongoing and future studies

The present work has assessed different mechanistic components of programming of the brain during the neonatal sensitive period. The focus of the work, due to personal interest, medical relevance and institutional bias was laid on the programming and mal-programming of HPA axis function and its emotional behavioral correlates. This work has raised several interesting questions, which should be subject of future studies.

3.4.1 Methylation marks and HPA axis function – cause or consequence?

As discussed above, the question remains whether the "epigenetic footprint" of neonatal stress is a cause or a consequence of the observed HPA axis phenotype. In our observations, the establishment of DNA-methylation marks on genes expressed in the PVN and involved in the HPA axis regulation required several weeks to become manifest. This suggests the existence of a time lag between the "funneling" of neonatal stress effects into the neuroendocrine neurons of the PVN and the occurrence of epigenetic changes. Another possible explanation could be that the neonatal stress-induced hyperactivation of the HPA axis leads to a sustained hypersecretion of glucocorticoids which, in turn, affect plasticity, hard-wiring and ultimately DNA-methylation in relevant brain areas following discrete spatio-termporal patterns.

One way to dissect these interdependent effects would be to adrenalectomize neonatally stressed animals at the youngest possible age (e.g. around 3-4 weeks) and to supplement the animals with low physiological doses of corticosterone. This would allow to differentiate behavioral and molecular (i.e. neurochemical and epigenetic) changes that occur as direct responses to neonatal stress and not to the hypercorticism that results from it.

While such an experiment would add more to our knowledge of the mechanisms triggered by neonatal stress, it is also burdened by certain bias. For one, adrenalectomy at such young age

is not only a procedural challenge in mice, but might also lead to disruption of maturational processes. Recent studies suggest that adrenarche also occurs in rodents and, like in primates, precedes physiological pubertal transition (Pignatelli et al., 2006).

Furthermore, the lack of circa- and ultradian oscillations of corticosterone secretion over long periods might *per se* affect neuronal morphology and neurochemistry and, ultimately, behavior.

An alternative approach, albeit bearing similar difficulties, would be the targeted deletion of GR in specific brain areas, e.g. by means of stereotactic virus-delivered shRNA. Such a GR knockdown in the PVN would allow the distinction between direct effects of hypercorticism and the "paracrine" effects of other brain areas projecting to the PVN.

Currently we presume that the neonatal stress-induced alterations in the quality of these projections are responsible for the initiation of the epigenetic changes in the PVN, with GR signaling in response to a hyperactive HPA axis supporting the establishment or maintenance of the DNA-methylation in the AVP and CRH genes.

Similarly, as we suspect that neonatal stress-induced methylation-marks need to be actively maintained, one could hypothesize that if they were to be artificially induced in an otherwise healthy organism, they would disappear with time. For instance, using a combination of conventional and conditional transgenic approaches, it could be possible to insert a hypomethylated CpG island in the AVP enhancer region (similarly to our in vitro studies (Murgatroyd et al., 2009)) in the parvocellular division of the PVN of an otherwise completely healthy control adult animal (not exposed to neonatal stress). It would then be interesting to study the time course during which the artificially inserted methylation mark will be changed back to the physiological level, assuming that the maintenance of neonatal stress-induced methylation marks results from other effects of neonatal stress on brain structure and function.

3.4.2 What does not kill you makes you stronger?

Similar mechanistic studies would also appear important in the context of the programming of adult neurogenic capacity of those cells of the NPC pool that survive neonatal GC treatment. Although we show that the majority of NPC express GR *in vivo* and *in vitro*, not all NPC are forced into apoptosis by dexamethasone treatment. This indicates that there are subpopulations of NPC that are not sensitive to the pro-apoptotic effects of GC.

Also neurons derived from these NPC surviving neonatal GC treatment might be less sensitive to deleterious effects of GC exposure in adulthood.

To answer these questions neonatal animals could be injected with detectable synthetic nucleoside analogues (e.g. Bromodeoxyuridine (BrDU) and Iododeoxyuridine (IrDU)) at different time points of the dexamethasone treatment (e.g. at the beginning and the end) in order to label cells that have been born under and after GC exposure. Tissue sampling at different ages and subsequent analysis of the cellular phenotype of all cells that have incorporated BrDU and/or IrDU will allow for identification and characterization of these cells and their life span (e.g. whether neurons born from NPCs that have survived neonatal GC treatment die earlier or later and how do they react to subsequent challenges like stress or GC exposure).

The later question seems important also in terms of epigenetic marking. For instance, it is possible that in NPCs that survive neonatal GC treatment, methylation marks which determine the epigenetic status of GC responsiveness are established on critical fragments of the GR gene or GR responsive genes. If this is the case, it would be important to scrutinize the emergence, maintenance and stability of these epigenetic marks in the NPCs, as well as in their postmitotic daughter cells.

Both, the responses to neonatal stress and GC exposure might be differentially expressed depending on the sex of the individual. The sex hormone milieus in neonatal males and females are very likely to differentially affect the consequences of stress and GC during this period of ontogeny. We have recently documented sex-specific methylation patterns of the CRH promoter in parvocellular PVN neurons (*Menger, Patchev, Spengler, Almeida; unpublished observations*). Although our work on the role of isoform-selective ER activation suggests that activational sex steroid effects during adulthood largely account for the sex-specific differences observed in HPA axis and emotional behaviors, we can not rule out the contributions of direct organizational effects during early life.

It appears important to scrutinize the roles of different sex steroids (e.g. estrogens, androgens and progestins) in the establishment of ELS-induced sex-specific methylation marks. Using pharmacological and surgical tools, as well as conventional and conditional transgenesis, it could be assessed whether the sex-specific effects of ELS on DNA-methylation can be reversed or, even, blocked. Similarly, the effect of sex and sex steroids should be evaluated in the context

of neurogenesis, e.g. are neonatal females more or less sensitive to the pro-apoptotic effects of GC on NPC?

The cross-talk between steroid receptor signaling cascades is well known, and there are many possible points of interaction between ER, progesterone receptor (PR, which is strongly induced by ERα-activation) and GR signaling (Uht et al., 1997). Mechanistic dissection and understanding of these interactions and their relevance for structural, neurochemical, epigenetic and, ultimately, endocrine and behavioral outcomes might help to unravel the basis of sex-differences in the prevalence of psychiatric and neurological disorders.

3.4.3 Stress during different stages of early life – programming vulnerability or resilience?

Another related question which has attracted our attention is the analysis of the programming effects of stress during later life stages, e.g. puberty, adulthood and senescence, and the cumulative effects of several stress episodes throughout critical stages of life.

Our hypothesis is that stress during different critical windows of time will differentially (age-specifically) program or damage endocrine and behavioral outcomes.

We have shown that neonatal stress does not seem to affect anxiety-related behaviors in adulthood, whereas stress exposure during puberty leads to increased anxiety. Other behavioral traits, e.g. mood (depression-like behavior) and cognition (spatial learning) are similarly affected by neonatal and peripubertal stress.

A history of stress exposure during neonatal life produces clear symptoms of HPA axis dysregulation: increased basal GC secretion, hyperreactivity to acute stress and delayed shut-off of the endocrine stress response, due to impaired GC negative feedback. In ongoing studies we are trying to identify the most vulnerable control point within the HPA axis, at which the impairment of GR signaling translates into reduced negative GC feedback efficacy in both models.

Interestingly, animals exposed to neonatal stress do not show a down-regulation of GR mRNA expression at any supra-pituitary level of HPA axis regulation. In the PVN these animals actually show increased GR mRNA. Functional tests of GR sensitivity demonstrated a dose-dependent

increase in the expression of SGK-1 and FKBP5 (GR responsive genes) in the PVN of animals with a history of neonatal stress, indicating that GR signaling in the PVN of these animals is, in reality, increased (*Bockmühl, Kuczynska, Patchev, et al.; in preparation*). Ongoing studies aim to understand the reasons for the discrepancy between impaired GC negative feedback efficacy and enhanced GR expression and signaling in the PVN of these animals. One of the present assumptions that increased GR signaling in the PVN is a sign of an effort to compensate for reduced GR efficacy at a higher neural level of HPA axis regulation.

It is conceivable that the (mal-)adaptive endocrine and behavioral responses induced by neonatal or peripubertal stress exposure might render the organism more vulnerable to the detrimental consequences of stress in adulthood. This assumption was originally supported by our finding of increased endocrine and behavioral responsiveness to acute stress in animals with a history of neonatal or peripubertal stress.

Surprisingly, however, animals with a history of neonatal or peripubertal stress failed to show symptoms of aggravated emotional or cognitive deficit when exposed to chronic unpredictable stress in adulthood. Unexpectedly, when exposed to CUS in adulthood, animals with an early-life stress history displayed improved spatial learning, indistinguishable from that of controls with no history of ELS. In contrast, in animals with peripubertal, but not neonatal, stress experience exposure to CUS in adulthood resulted in depression-like behavior in the forced swim test.

The hyper-reactive HPA axis phenotype resulting from early-life stress exposure was not further altered by subsequent adult CUS challenge.

Taken together these findings indicate that the animals with a history of ELS show a differential behavioral response to chronic stress in adulthood, depending on the age at which ELS was applied. The apparent behavioral improvement following adult CUS exposure, however, cannot be associated with amelioration of the ELS-induced HPA axis disturbance.

Future studies should address the mechanisms which account for the differential programming of behavioral phenotype by neonatal and peripubertal stress. Another challenging task would be to understand why adult chronic stress differentially affects mood and cognitive functions depending on the individual ELS-history of the animal (e.g. neonatal *vs.* peripubertal stress).

Figure 4 Summary of behavioral and endocrine phenotypes of combinatorial stress exposure

The figure shows a summary of behavioral and endocrine outcome in adult animals which have undergone neonatal, peripubertal or adult chronic stress and combinations thereof. Adult chronic stress in animals with a history of neonatal stress exposure does not alter depression-like behavior, however improves spatial learning back to levels displayed by control animals. In contrast the same adult CUS procedure in animals with a history of chronic stress during puberty leads to apparent improvement in spatial learning, but also reduces depression-like behavior to levels below those of control animals. The question remains, if those apparent improvements are reflecting a better coping of the animals with stressful situations, or are epiphenomena of increased vulnerability, which are erroneously interpreted as improvements.
In the rows, where only one stressor is applied (either neonatal, peripubertal or adult) arrows indicate change vs. controls. In the two lower rows (combinatorial stress procedures) arrows indicate change vs. the phenotype induced by ELS. Question marks indicate either inconclusive findings or parameters that have not been assessed yet.

STRESS			BEHAVIOR			HPA axis function		
neonatal	peripubertal	adult	anxiety	depression-like	cognition	basal	acute stress response	GC feedback efficacy
✔			↔	↑	↓	↑	↑	↓
	✔		↑	↑	↓	↑	↑	↓
		✔	↑	↑	↓	↑	↑	↓
✔		✔	?	↔	↑	↔	↓	?
	✔	✔	?	↓	↑	↔	↔	?

At this stage, we cannot denote the apparent improvement in certain behavioral outcomes that results from adult CUS exposure as “resilience”; however, we also cannot exclude that animals with a history of ELS cope better (or at least differently) with adult chronic stress. For instance, the strong increase in active behavior in the FST (an apparent reduction of depression-like behavior) after CUS exposure of peripubertally stressed mice, might actually reflect increased arousal, locomotion drive or, merely, general hyperactivity in these animals.

We also cannot generalize the apparent beneficial effects of CUS in animals with ELS history to all other stress modalities. Perhaps other forms of superimposed stress (e.g. social defeat or isolation, chronic pain, infections etc.) may cause stronger deleterious effects in animals with an ELS background.

As both, early life traumas, as well as repeated stress exposure over the lifetime have been identified as major risk factors for depression, and depression itself is a recognized risk factor for cognitive disorders, the dissection of the above-described, complex phenotypes resulting from our combinatorial stress models represents an issue of major medical interest.

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Annex

Riedeman, Patchev et al., Corticosteroids: way upstream. *Molecular brain* **3**:2. DOI:10.1186/1756-6606-3-2

Molecular Brain

REVIEW Open Access

Corticosteroids: way upstream

Therese Riedemann[1,2], Alexandre V Patchev[1], Kwangwook Cho[2], Osborne FX Almeida[1*]

Abstract

Studies into the mechanisms of corticosteroid action continue to be a rich bed of research, spanning the fields of neuroscience and endocrinology through to immunology and metabolism. However, the vast literature generated, in particular with respect to corticosteroid actions in the brain, tends to be contentious, with some aspects suffering from loose definitions, poorly-defined models, and appropriate dissection kits. Here, rather than presenting a comprehensive review of the subject, we aim to present a critique of key concepts that have emerged over the years so as to stimulate new thoughts in the field by identifying apparent shortcomings. This article will draw on experience and knowledge derived from studies of the neural actions of other steroid hormones, in particular estrogens, not only because there are many parallels but also because 'learning from differences' can be a fruitful approach. The core purpose of this review is to consider the mechanisms through which corticosteroids might act rapidly to alter neural signaling.

The protagonists and their roles

Corticosteroids are the main humoral mediators of stress and their increased secretion in response to adverse stimuli normally results in a cascade of physiological and behavioral homeostatic mechanisms that allow survival and the activation of defense mechanisms against future insults. They facilitate arousal and the appropriate channeling of physiological resources; primarily, corticosteroids act to conserve essential salts, stimulate gluconeogenesis and lipid metabolism, cardiovascular and pulmonary function and erythropoeisis and bone turnover, while inhibiting, among others, reproductive and ingestive behaviors as well as immune responses [1]. Thus, corticosteroids are well suited to serve the fight-or-flight response (first described by Walter B. Cannon in 1915).

Corticosteroids (CS) are primarily produced by the adrenal glands although recent studies suggest that they may also be synthesized in the brain [2,3]. The term 'corticosteroids' embraces two prototypic steroids with distinct biological functions: glucocorticoids (cortisol in most large mammals, corticosterone in rodents and other taxa), named because of their gluconeogenic properties, and mineralocorticoids (primarily aldosterone), named for their role in the regulation of the salt-water balance. Like other steroid hormones, corticosteroids are small, lipophilic molecules (ca. 300 Da) that are derived from cholesterol. Their physical properties facilitate their passage across the blood brain barrier where they act to maintain brain structure (they are implicated in the regulation of neuronal cell birth, differentiation and apoptosis, as well as dendritic arborization and synaptic function), and integrate a variety of behavioral and physiological processes, including their own secretion. In this respect, they serve as messengers between the periphery and brain, but also between the external and internal environments and the brain.

The hypothalamo-pituitary-adrenal axis embraces the feedforward and feedback neuroendocrine mechanisms that regulate CS production and synthesis (Figure 1). Neural inputs trigger the release of adrenocorticotrophic hormone (ACTH) from the pituitary which, in turn, stimulates adrenocortical synthesis and secretion of CS. Although CS are not stored in a readily-releasable pool, it is estimated that adequate amounts of CS can be released into the bloodstream within minutes of appropriate neural stimuli. Noxious (stressful) stimuli are the primary triggers of neural firing that result in increased CS release. On the other hand, CS are secreted according to strictly-regulated circadian rhythms that are dictated by the central nervous system. More recently, CS have been found to have ultradian rhythmic patterns of release. Such patterns are most likely maintained through dynamic cross-talk between the peripherally-produced CS and centrally-driven regulatory

* Correspondence: osa@mpipsykl.mpg.de
[1]Max-Planck-Institute of Psychiatry, Kraepelin Str. 2-10, 80804 Munich, Germany

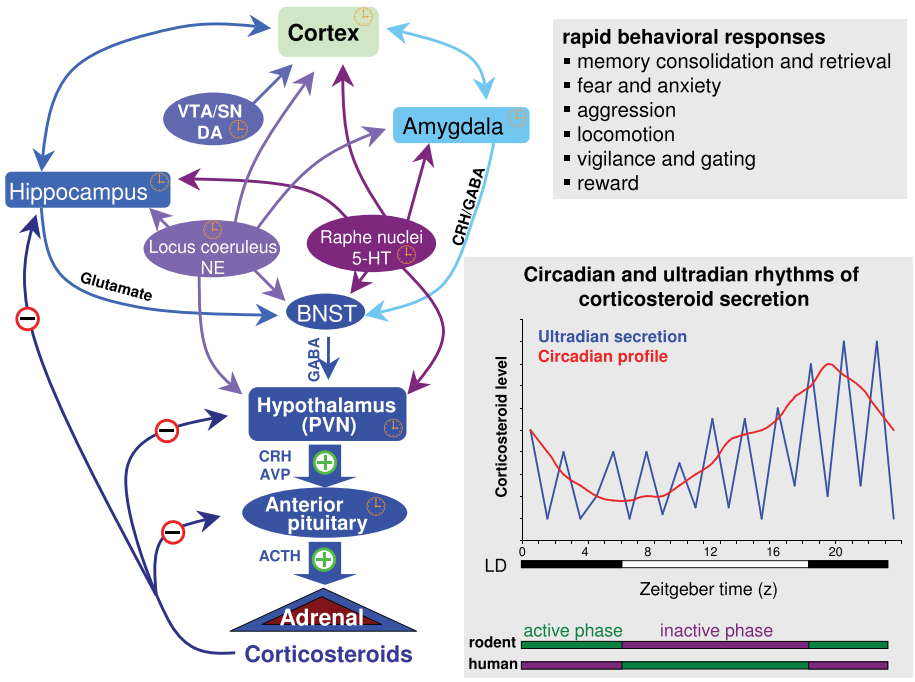

Figure 1 Schematic representation of the hypothalamo-pituitary-adrenal (HPA) axis and its neuronal inputs. Corticotropin-releasing hormone (CRH)- and arginine vasopressin (AVP)-expressing parvocellular neurons in the paraventricular nucleus (PVN) project to pituitary (via the median eminence) where they stimulate adrenocorticotrophic hormone (ACTH) synthesis and secretion, subsequently triggering corticosteroid synthesis and release from the adrenal cortex. Besides acting in the brain to regulate various behaviours, corticosteroids fine-tune the subsequent pattern (amplitude and duration) of corticosteroid secretion; they activate their cognate receptors in the pituitary, hypothalamus and hippocampus and bed nucleus of the stria terminalis (BNST, a relay between the hippocampus/amygdala and the PVN) to restrain, and in the amygdala to enhance, adrenocortical secretion. Monoaminergic transmitters, namely, norepinephrine, serotonin and dopamine released from midbrain nuclei (the locus coeruleus [LC], raphé and ventral tegmental area [VTA] and substantia nigra [SN], respectively) exert modulatory effects on all brain regions involved in the control of the HPA axis. 'Plus' signs *(green)* indicate positive drive on the HPA axis; 'minus' signs *(red)* represent sites of corticosteroid negative feedback; 'clock' signs denote neuronal populations known to respond rapidly to corticosteroids. Corticosteroids are secreted rhythmically, displaying ultradian and circadian patterns. The circadian peak coincides with the onset of the daily activity cycle (dark phase in rodents, light phase in humans). While the physiological and behavioural significance of the ultradian rhythms of corticosteroid secretion is still unclear, it is plausible that they serve to dynamically fine-tune the regulation of the HPA axis and thus, to facilitate adaptive processes. *LD*, light-dark cycle.

mechanisms; they are also likely important integrators of normo-physiological functions [4].

Since corticosteroids come on stage within 3-7 minutes of first perception of a stressor [5], they may be considered to be secondary or auxiliary players in comparison to monoamines (in particular, epinephrine and norepinephrine) whose actions are initiated within milliseconds to seconds [6] i.e. corticosteroids are secreted during the first stage of the 'general adaptation syndrome', a concept introduced by Hans Selye in 1946. However, since corticosteroids act against the background of increased monoamine secretion, it is thought that they act to fine-tune the organism's response to stress [7] and to facilitate signal-to-noise discrimination. Moreover, unlike the transient monoamine response, corticosteroids exert sustained actions on cellular activity and behavior, and therefore are essential for ensuring the orchestration of a coordinated adaptive response as well as 'preparedness' of the organism to cope with future challenges.

Although corticosteroids are often thought of in negative terms because of their causative role in diseases such as diabetes, hypertension, osteoporosis and immune suppression, they are essential for adaptation to stress and for maintaining physiological processes. With respect to brain structure and function, corticosteroids play an important role in maintaining hippocampal cell numbers under basal conditions; this is illustrated by robust observations that removal of corticosteroids by extirpation of the adrenal glands results in massive apoptosis, with parallel increases in neurogenesis, within the granule cell population of the hippocampus [8]. On the other hand, stress and elevated levels of glucocorticoids inhibit the generation of new granule neurons [9]. Another aspect that suggests an important role of corticosteroids in normo-physiology is the well-pronounced

circadian pattern of corticosteroid secretion. These rhythms are robust and bi-directionally tightly coupled to the individual's sleep-activity and feeding cycles, while being entrained and maintained by the daily light-dark cycle.

The magnitude and duration of the humoral response to stress is tightly coupled to the nature (quality, intensity and duration) of the stressor, as well as the context in which it occurs. Depending on context (e.g. the prevailing physiological or psychological state, as well as history of the individual), stressors may trigger excessive corticosteroid secretion over an extended duration; in such cases, the response switches from being an adaptive one into a maladaptive one, marked by transient or chronic pathology. Major depression and cognitive impairment are two conditions that represent the so-called stress-induced disorders of the brain. The first of these seems to reflect a sub-optimal stress-coping strategy and may largely originate from impairments of the mechanisms contributing to the homeostatic negative feedback processes that act to protect the organism against excessive exposure to corticosteroids; frequently, depressed mood is accompanied by impaired cognition and hyperemotionality, indicating that stress impacts on multiple, inter-related neural circuits. A number of human and animal studies have demonstrated the disruptive effects of excessive corticosteroid secretion on cognition [10-12]. There is now strong evidence that the latter involve structural changes, including severe reductions in the dendritic arborization of hippocampal and prefronto-cortical neurons [13-15]. and synaptic loss [16-18]. In addition, recent studies indicate that stress may initiate neurodegenerative processes that increase the risk for severe cognitive deficits such as those seen in dementia of the Alzheimer type [19]. Lastly, chronically elevated levels of corticosteroids interfere with central and pituitary integrators and regulators of the hypothalamo-pituitary-adrenal (HPA) axis, resulting in impaired corticosteroid negative feedback and sustained corticosteroid secretion [20].

The soliloquy we've come to know and love

Glucocorticoids and mineralocorticoids fulfill their characteristic biological functions through the mediation of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), respectively. Both of these receptors are present in the brain; while GR are expressed ubiquitously (most strongly in the hippocampus), MR are more discretely distributed (strongly expressed in certain hippocampal subfields and the septum, and moderately expressed in the amygdala and hypothalamic paraventricular nucleus) [21]. The MR has a 7-10-fold greater affinity for corticosterone as compared to the GR [22]. It is thus estimated that the MR is some 80% occupied under basal conditions, and that the GR only becomes activated when corticosterone levels rise during the daily circadian peak of corticosterone secretion or after stress. Although aldosterone may be synthesized in the brain [2,3], it should be noted that brain MR do not normally 'see' their prototypic endogenous ligand; aldosterone is produced in the periphery at concentrations that are too low to have a direct impact on the brain and in any case, the hormone does not easily cross the blood-brain barrier. On the other hand, it should be mentioned that ligand availability is subject to local regulation through activation/deactivation of cortisol/corticosterone through the actions of 11β-hydroxysteroid dehydrogenase [23].

The MR and GR belong to the phylogenetically ancient superfamily of nuclear receptors, all of which are transcriptional factors. For the sake of clarity, we will herein refer to nuclear MR and GR as nMR and nGR, respectively. Whereas the unliganded nMR is primarily localized in the nucleus, the unoccupied nGR resides in the cytoplasm and only translocates to the nucleus upon ligand activation. This process depends on the dissociation of a host of chaperone and co-chaperone molecules, including heat shock protein 90 (hsp90) as well as on the inclusion of a nuclear translocation signal in the receptor protein [24]. Like other nuclear receptors, nMR and nGR are organized according to canonical modules, including a ligand binding domain (LBD), a DNA binding domain (DBD), and two activation functions (AF-1 and AF-2) at their N- and C-terminals, respectively. The various domains share considerable homologies (homology between nMR and nGR: ~57% in LBD; ~94% in DBD). Interactions of the DBD with hormone response elements (HRE) in the promoters of specific genes result in the induction or repression of gene transcription and subsequently, changes in the expression of proteins that influence cellular functions. Homologies also exist within the HRE sequence of various nuclear receptors, and receptor recruitment and interactions with specific co-regulator proteins (co-activators/-repressors) may endow these structurally similar receptors with differing specificities and potencies.

Stage props

Transcriptional and translational effects of corticosteroid receptor activation have been demonstrated using drugs such as actinomycin D and cycloheximide, respectively. On the other hand, demonstration that nGR mediate corticosteroid effects have relied on the use of the antagonist mifepristone (RU 38486, also a potent antagonist of progesterone receptors), while spironolactone or oxoprenoate (RU28318) have been used to demonstrate mediation through nMR. Other potentially useful additions to the pharmacological toolbox for

studying events mediated by nGR and nMR include established chaperone inhibitors of hsp90 (e.g. cisplatin and geldanamycin; [25]) and of the FK506-binding proteins (e.g. GPI1046; [26]).

Drop scene[b]

The mode of action of corticosteroids summarized above, i.e. involving gene transcription and translation, may be generalized to all steroid hormone receptors, including those for estrogens. Since nuclear receptors become transcriptionally active upon ligand activation, their actions are, by definition, slow in onset and potentially long-lasting (hours to days, or even months); at best, gene transcription and translation require a minimum of 20-30 minutes (translation takes longer than transcription) [27]. However, steroids have been implicated in the elicitation of a number of 'rapid' or 'fast' physiological and behavioral responses to external stimuli; some examples of fast steroid-mediated responses and the mechanisms thought to underlie their actions are presented in Additional File 1. Historically, the idea that steroids can rapidly alter neuronal excitability and conduction stemmed from work on the actions of sex steroids by Kawakami and Sawyer in 1959 [28] and Woolley and Timiras in 1962 [29].

As a rule, fast responses are considered to be those that occur within the first 20 minutes of increased steroid secretion, i.e. in a much shorter timeframe than that required for effects on gene transcription and protein synthesis. Somewhat erroneously, these fast actions are referred to as 'non-genomic'; in fact, rapidly triggered signaling cascades may ultimately converge in the nucleus to regulate gene transcription and protein synthesis. Distinction between the 'fast' and 'slow' actions of steroid hormones is more of mechanistic than of behavioral or physiological importance, since the latter are the integrated manifestations of sequential events. Viewed from this perspective, the rapid actions of steroids may be considered as 'primers' of the substrates responsible for the manifestation of transcriptional events triggered by nuclear receptors; kinase cascades activated during early phases of steroid action and which lead to the phosphorylation of regulatory sites of nuclear receptors [30-32] are a good example of such priming functions.

Many of the changes in behavior and brain physiology that are listed in Additional File 1 reflect rapid responses of the hippocampus to steroid hormones. For example, corticosteroids have been consistently shown to influence cognition and their effects are thought to result from their ability to directly or indirectly alter the excitability of hippocampal neurons. The hippocampus has been extensively studied for a number of pragmatic reasons. The input-output connections of the different hippocampal subfields are well defined, making their electrophysiological study convenient. Of all brain areas, the hippocampus has been best studied in the context of long-term potentiation (LTP) and long-term depression (LTD), the electrophysiological correlates of learning and memory, functions in which the hippocampus is strongly implicated [[33-35]; see Figure 2 and Additional File 2]. The hippocampus also serves as an important homeostatic regulator of the HPA axis upon which it exerts a strong negative drive [36,37] through the mediation of nMR and nGR [38].

Although the attention paid to the hippocampus is justifiable because of its role in the regulation of many behavioral and physiological processes, it should be remembered that it constitutes only part of a complex neuronal network that underpins physiology and behavior in normal and pathological states. For example, although the hippocampus plays an important role in the regulation of the HPA axis, it should be noted that other brain areas such as the prefrontal cortex [39], amygdala and bed nucleus of the stria terminalis, under the modulatory influence of monoamines from the hindbrain [40], contribute to the control of corticosteroid secretion; all these areas have reciprocal connections with the hippocampus and express nGR.

Several studies have begun to define how corticosteroids and other steroids act on different brain structures to produce integrated and adaptive behavioral and physiological responses, e.g. the prefrontal and orbito-frontal cortices (executive functions, including attention, behavioral flexibility, declarative memory, decision making [41,13,14,42]), thalamus (processing and gating of sensory input [43], amygdala (evaluation of emotional load of sensory input and regulation of fear [44], ventral striatum (motivation and reward [45] and decision-making [42]), and the cerebellum (learning of motor tasks [46]. Of these, the amygdala, involved in the control of fear, aggression and cognition (see Additional File 1), has been the most intensively studied. Interesting work by Roozendaal and colleagues has demonstrated a cross-talk between rapid GC and noradrenergic signaling in contextual memory consolidation [44,47] and suggests that endocannabinoids are key mediators of this cross-talk [48].

Putative membrane receptors - pirates with legs to stand on?

The message that emerges from the previous section is that nuclear receptors, acting as transcriptional factors, are unlikely to mediate rapid actions of the sort listed in Additional File 1. Nevertheless, the identity of the molecular entity that allows rapid transduction of steroid signals remains elusive. Interestingly, some of the fast responses to corticosteroids are reportedly attenuated in

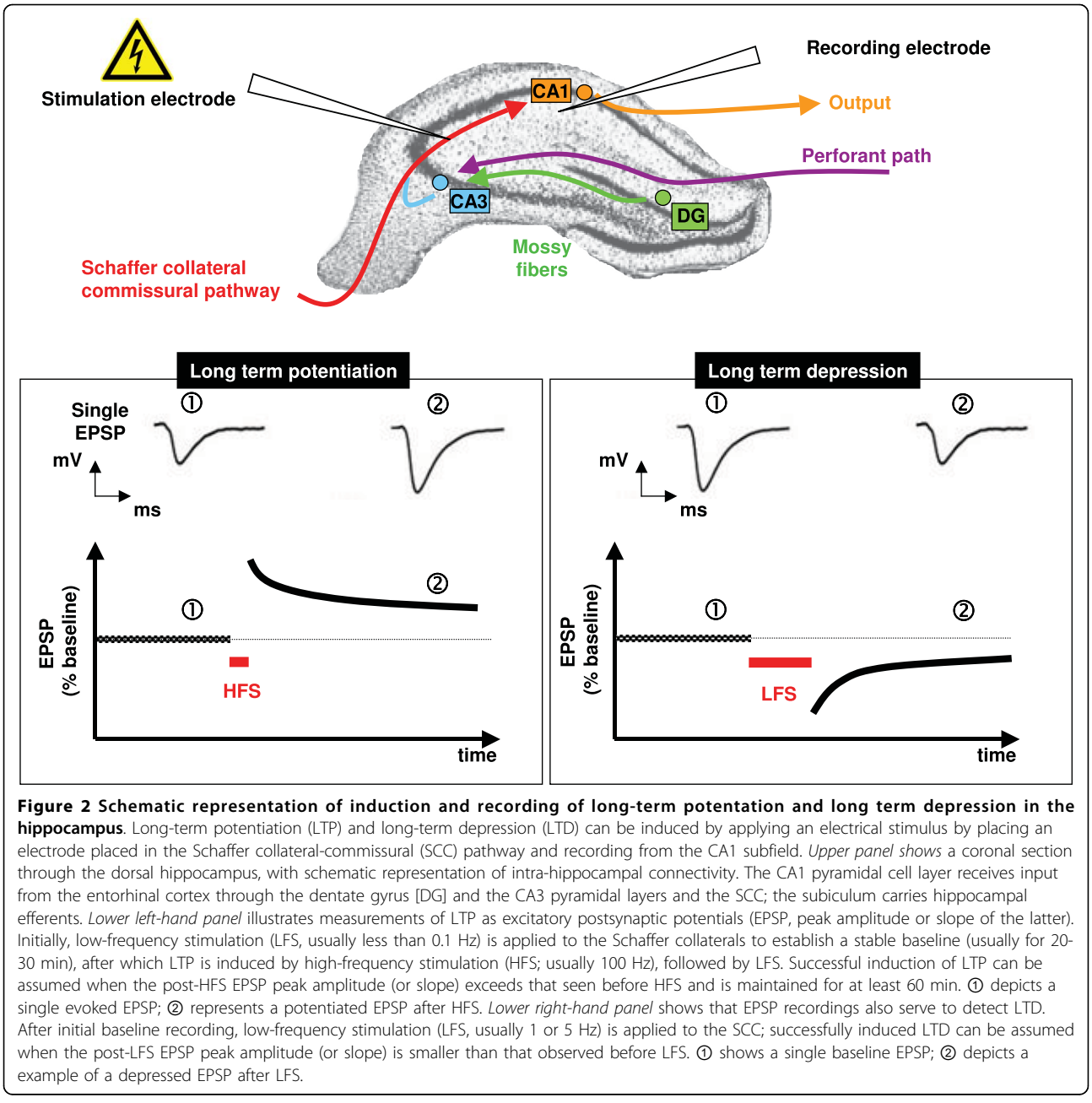

Figure 2 Schematic representation of induction and recording of long-term potentation and long term depression in the hippocampus. Long-term potentiation (LTP) and long-term depression (LTD) can be induced by applying an electrical stimulus by placing an electrode placed in the Schaffer collateral-commissural (SCC) pathway and recording from the CA1 subfield. *Upper panel shows* a coronal section through the dorsal hippocampus, with schematic representation of intra-hippocampal connectivity. The CA1 pyramidal cell layer receives input from the entorhinal cortex through the dentate gyrus [DG] and the CA3 pyramidal layers and the SCC; the subiculum carries hippocampal efferents. *Lower left-hand panel* illustrates measurements of LTP as excitatory postsynaptic potentials (EPSP, peak amplitude or slope of the latter). Initially, low-frequency stimulation (LFS, usually less than 0.1 Hz) is applied to the Schaffer collaterals to establish a stable baseline (usually for 20-30 min), after which LTP is induced by high-frequency stimulation (HFS; usually 100 Hz), followed by LFS. Successful induction of LTP can be assumed when the post-HFS EPSP peak amplitude (or slope) exceeds that seen before HFS and is maintained for at least 60 min. ① depicts a single evoked EPSP; ② represents a potentiated EPSP after HFS. *Lower right-hand panel* shows that EPSP recordings also serve to detect LTD. After initial baseline recording, low-frequency stimulation (LFS, usually 1 or 5 Hz) is applied to the SCC; successfully induced LTD can be assumed when the post-LFS EPSP peak amplitude (or slope) is smaller than that observed before LFS. ① shows a single baseline EPSP; ② depicts a example of a depressed EPSP after LFS.

the presence of pharmacological antagonists of nGR (RU 38486 [49] or nMR (spironolactone [50,51]). These findings suggest certain homologies between the classical nuclear receptors and the putative receptors mediating the rapid actions of these steroids. Nevertheless, the existence of another class of receptors, with distinct chemistries and cellular localizations, and that are not sensitive to the above-named antagonists, cannot be dismissed.

Several mechanisms that may account for membrane-mediated transduction of the rapid actions of estradiol have been proposed (see Figure 3). Substantial evidence supports the view that classical nuclear estrogen receptors (nER of which there are two isoforms, ERα and ERβ) are integrated into, or in close proximity of, the cell membrane. One hypothesis is that palmitoylation facilitates the interaction of these receptors with caveolins, a family of proteins that associate with cholesterol and sphingolipids to form caveolae within the plasma membrane and which are implicated in signal transduction. While some authors describe protein-protein interactions of such membrane-associated nER with other membrane proteins as a mechanism to explain rapid estrogen signaling [52-54], others propose mediation by a membrane-

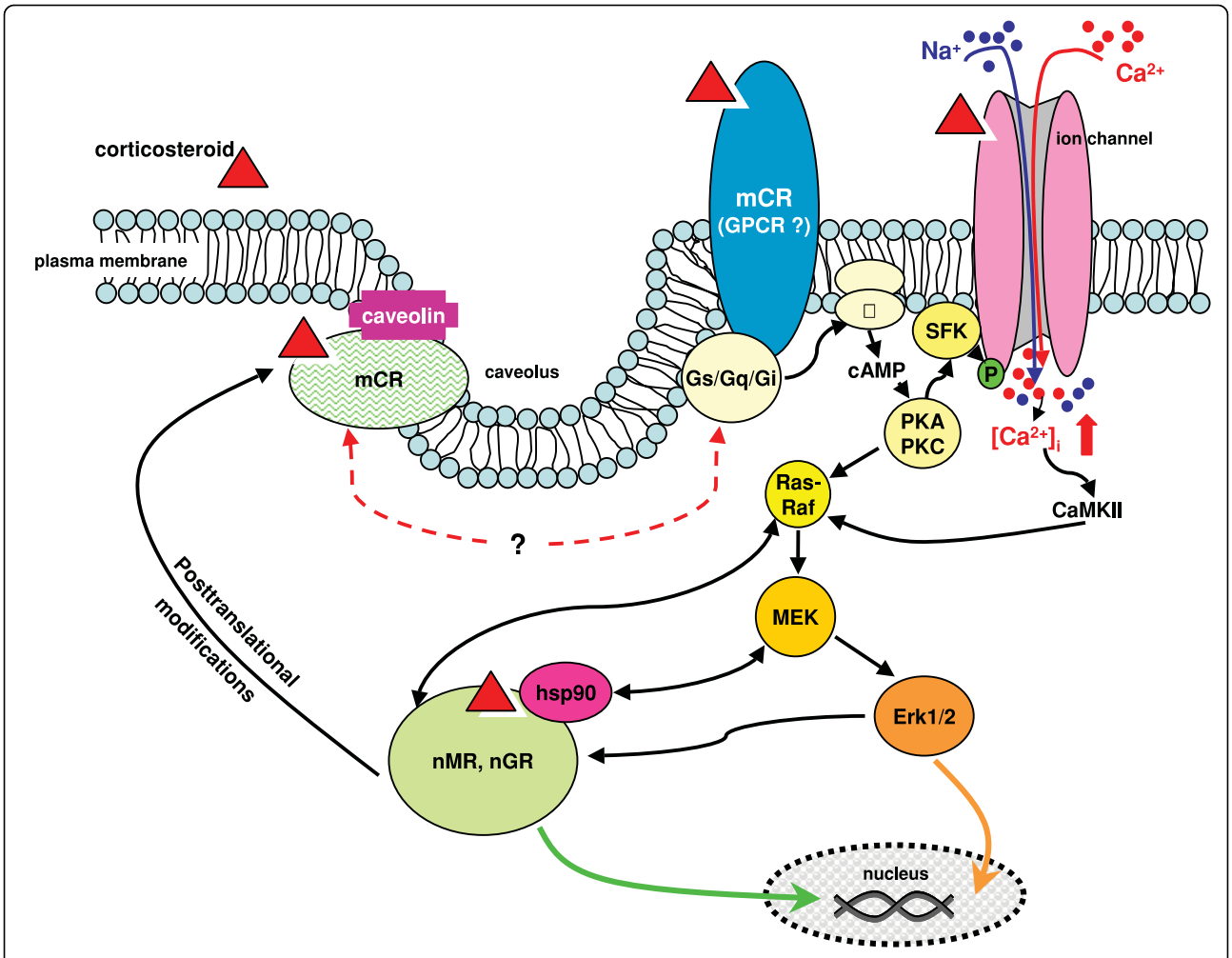

Figure 3 Schematic representation of corticosteroid-triggered multiple tentative rapidly influencing neuronal function. Corticosteroids are represented by red triangles. Nuclear GR (nGR) interact with caveolins [cf. [81]]; the interaction probably depends on posttranslational modifications of nGR to yield so-called membrane corticosteroid receptors (mCR). Alternatively, CS-initiated intracellular signaling cascades may result from corticosteroid binding to proteins embedded in the plasma membrane, e.g. G-protein-coupled receptors (GPCR) [75] which, upon activation, activate protein kinase A (PKA) and protein kinase C (PKC) in turn. Other evidence points to membrane-bound corticosteroid binding proteins that interact with members of the src family of kinases (SFK) to activate the mitogen-activated protein kinase (MAPK) pathway and/or modulate the activity of other membrane-associated proteins, e.g. NMDA receptors and other ion channels with potential steroid binding sites [76,85,86,222]. Under basal conditions, nGR are tethered in the cytoplasm in the form of a protein complex that includes the chaperone heat shock protein 90 (hsp90) which itself may directly interact with Src kinases and the MAPK kinase, MEK [cf. [83]]. Additionally, direct interactions between the nGR and Ras which may be functionally relevant have been described [84]. Finally, MAPK-mediated phsophorylation of nGR may influence the transcriptional activity of nGR [32]. Thus, corticosteroid actions at the plasma membrane can converge and prime or potentiate hormonal actions on gene transcription.

bound ER (mER) that is coupled to a $G\alpha_q$ protein. Evidence for the latter includes the observation that estradiol induces activation of the phospholipase C- protein kinase A (PLC-PKC-PKA) pathway in nER knockdown mice [55]. The same investigators demonstrated rapid electrophysiological effects of STX, a diphenylacrylamide-based selective estrogen receptor modulator, in nER knockout animals; STX, which does not bind to either isoform of the nER, proved to be more potent than estradiol in their *in vitro* and *in vivo* test systems [55,56].

While no mER has been cloned and characterized to date, GPR 30, an orphan G protein-coupled receptor (GPCR), has been identified as a potential transducer of estrogen signals that originate at the cell membrane [57,58]. GPR 30 was shown to display similar structural characteristics to other membrane receptors [57], but was nevertheless viewed with a certain amount of skepticism. For example, the nER antagonist ICI 182,780 exerts agonistic effects on this receptor [59] and neurons from GPR 30 knockout mice still display rapid responses to

estradiol [60], the latter finding suggesting that GPR 30 may co-exist alongside (an)other mER with unique pharmacological properties. Notably, in an extension of their earlier work, Revankar et al. [61] exploited chemical biology to explore the subcellular localization of GPR 30 and its signaling potential; on the basis of observations in 4 cancer cell lines, they discarded the notion that sufficient GPR 30 is localized at the plasma membrane and rather suggested that GPR 30 localized in the endoplasmic reticulum serves as an intracellular transmembrane receptor for estrogen.

Interestingly, Toran-Allerand and colleagues [62,63] described a high affinity (K_D for estradiol: 1.6 nM) caveolin-associated protein in the plasma membranes of neonatal (but not adult) neocortical and uterine tissues. This so-called ER-X seems to come closer to meeting the expectations of a distinct mER insofar that it cannot be blocked by ICI 182,780 [62]; moreover, these authors found that experimentally-induced ischemic stroke in adult animals is accompanied by an upregulation of ER-X in the brain, suggesting that the ER-X mediates the neuroprotective actions ascribed to estrogens.

It is tempting to hypothesize, that the mediators of rapid corticosteroid effects may share similar basic properties and mechanisms with the proposed membrane-associated estrogen receptors. The existence of a membrane-bound receptor for corticosteroids (herein referred to as mCR) was postulated by Willmer in 1961 [64]. Willmer's suggestion that steroid hormones interdigitate with, and alter the permeability of, lipids in the plasma membrane, lost currency as evidence that steroids bind to intracellular proteins (nuclear receptors) and stimulate protein synthesis began to accumulate from 1961 onwards [65,66]. However, in 1974 Satre and Vignais described corticosterone binding to mitochondrial preparations from the adrenal and kidney [67], a finding that eventually extended to other cell types [68]. A series of authors provided evidence for membrane-bound steroid recognition sites in the brain [69-71]; among these, Towle and Sze demonstrated specific corticosterone binding to plasma membrane preparations from rat brain synapses [72]. These membrane binding sites had a relatively high affinity for corticosterone (K_D 10^{-7} M *vs.* 10^{-9} M in the case of cytosolic binding sites) and treatment with phospholipase A2 or phospholipase C led to complete dissociation of membrane-bound corticosterone. Similarly, Orchinik et al. described the presence of mCR in brain synaptosomal fractions obtained from the amphibian *Taricha granulosa* (rough-skinned newt) [69]. These receptors showed pharmacological specificity for corticosterone and cortisol (K_D 10^{-9} M), and lesser affinities for aldosterone and other natural and synthetic steroids (such as dexamethasone and RU 38486). Importantly, Orchinik et al. reported a linear relationship between the potencies of various compounds (corticosterone being the most potent) in inhibiting male reproductive behavior (inhibition by corticosterone within 8 minutes of application) and their ability to bind the putative mCR [69]. In subsequent studies, these authors described similar neuronal mCR in mammalian [73] and bird [74] brains and suggested a role for guanine nucleotide-binding proteins in the formation of a ternary complex of corticosterone and the putative neuronal mCR, i.e. the mCR appears to be coupled to G proteins [75]. Additional evidence for the existence of a mCR was eventually provided by Orchinik's colleagues who solubilized and partially purified membrane-bound corticosterone binding sites from the amphibian brain [76]; the assumed mCR had a molecular weight of about 63 kDa, as compared to 97 kDa and 110 kDa in the case of the nGR and nMR, respectively. More recently, studies by Johnson et al. [77] provided anatomical evidence for the existence of nGR within the postsynaptic density of neurons in the rodent amygdala. At present it is unclear as to whether there are any homologies between the mCR and either the nGR or nMR.

Ultrastructural studies with an antibody against purified rat nGR revealed immunoreactivity associated with the plasma membrane of rat hippocampal and hypothalamic neurons [78]. Notably, membrane-associated immunoreactive nGR sites were observed in or near membranes covering the dendrites and somata of pyramidal neurons; nGR immunoreactivity was also seen in the vicinity of the Golgi complex. With regard to the plasma membrane, Liposits and Bohn [78] noted that nGR immunoreactivity was associated with coated vesicles which, together with their localization along the membrane, suggested that nGR might either be transported and inserted into the plasma membrane, or coupled to mediators of transduced signals. In this respect, parallels may be drawn with what was reported above with respect to the membrane-bound mediators of estrogen actions. Palmitoylation of the nER has been suggested as a mechanism that facilitates integration of the nuclear receptor into (or the proximity of) the cell membrane, thus providing access to BSA-conjugated steroids and interactions of the receptor with membrane-associated signaling proteins [79]. While it remains to be shown that classical corticosteroid receptors can be palmitoylated and trafficked to the plasma membrane, recent studies have identified a highly conserved 9-amino acid motif in the ligand binding domain of estrogen, progesterone, androgen and glucocorticoid receptors that could serve as a substrate for palmitoylation [80]; these observations suggest that palmitoylation may be a general mechanism that allows nuclear receptors to double up as *bona fide* membrane receptors.

Supporting the plausibility of this view, Matthews et al. have shown that nGR interacts with caveolin [81].

Many unliganded nuclear receptors (e.g. nGR), are tethered in the cytoplasm through their association with chaperone proteins such as heat shock protein 90 (hsp90); this complex is dissociated upon arrival of the ligand [24]. Interestingly, hsp90 is known to interact with src kinase [82], a membrane-proximal kinase thought to mediate the rapid activation of the MAPK pathway by corticosteroids. In addition, hsp90 interactions with MEK2, another kinase upstream of MAPK, has been shown to mediate MAPK pathway activation by estradiol [83]. In fact, nGR itself reportedly interacts with Raf-1, a downstream effector of Ras, and upstream regulator of the MAPK pathway [84].

Receptors for several neurotransmitters (some of which are ion channels) have been shown to bind CS [76,85,86]. Although it remains unclear as to whether these interactions serve as a conduit of the rapid actions of CS, the latter seems plausible given the evidence that neurosteroids can modulate chloride flux and thereby, neuronal excitability, by binding to an allosteric site on the $GABA_A$ receptor [87].

In summary, there is growing support for the view that CS can initiate signaling at the plasma membrane through one or more of the following mediatory mechanisms: (i) G protein-coupled membrane-bound CS receptors, (ii) steroid modulatory sites on plasma-bound neurotransmitter receptors, (iii) interactions between cytoplasmic CS receptors and kinase family-interacting chaperone molecules, and/or (iv) palmitoylation. Elucidation of the mechanisms underlying the rapid actions of CS will require a stepwise analysis of the contributions of each member of this 'interactome' - a major challenge.

From the sightlines - peeping on a rapidly changing stage

This section will focus on the cellular endpoints that can be used to support the view that corticosteroids rapidly influence neuronal activity, focusing on alterations in membrane excitability and signaling cascades that originate at or close to the plasma membrane. However, attempts to summarize the existing literature are confronted with the fact that the results derive from disparate protocols and experimental models in different laboratories. For example, a wide range of corticosteroid doses and exposure times have been applied to studying synaptic transmission in either rat or mouse dissociated hippocampal neurons or hippocampal slices. We will, however, first consider early studies on hypothalamic neurons by Kasai and colleagues and Saphier and Feldman, using *in vitro* ionotophoresis. Kasai and colleagues showed that cortisol excited tuberoinfundibular neurons in the paraventricular nucleus (PVN) which project to the median eminence from where their neurosecretory products reach the anterior pituitary; however, these authors also reported inhibitory effects of cortisol in the PVN, suggesting this to result from inhibition of noradrenergic inputs [88-90]. Saphier and Feldman, observed a significant reduction in the spontaneous firing rates of similar hypothalamic neurons after the application of corticosterone [91,92]; these changes had a rapid onset and were maintained even after iontophoresis of the hormone was stopped. Further, they reported on a subset of neurons whose activity was not altered by corticosterone; glutamate-induced excitation of these neurons was however suppressed in the presence of corticosterone.

Together, the studies described above represent a hypothalamic electrophysiological correlate of the negative feedback control of adrenocortical secretion, and illustrate that corticosteroids can elicit different responses from different brain areas or neuronal populations within an anatomical region or specific neuronal phenotypes within a given subfield; moreover, the responses depend on neural inputs to the particular set of neurons under investigation [91,93]. Given the suggested importance of the hippocampus in mediating glucocorticoid negative feedback (see above), it is surprising that Barak [94] failed to observe any changes in the activity of hippocampal neurons upon applying corticosterone. As will become evident below, despite a large number of studies that focussed on the CA1 subfield of the hippocampus, it is difficult to compile a consensus view of how corticosteroids impact on the activity of this region.

Examining spike accommodation in hippocampal neurons, Vidal et al. reported that corticosterone (1 μM) decreases spike numbers [95], whereas Joëls and de Kloet [96] and Beck et al. [97], using 1 nM, observed the steroid to increase spike numbers and decrease the after-hyperpolarisation (AHP) amplitude; these effects were abolished in the presence of spironolactone (nMR antagonist). Importantly, 30 nM of corticosterone, which activates nGR (as well as nMR), decreased spike numbers and increased AHP amplitude, leading the authors to conclude that the bifurcating actions of low and high doses of corticosterone reflect the activation of nMR and nGR, respectively [96]. Further, given the gradual rise in corticosterone levels upon arrival of a stimulus (e.g. stress), they proposed a concentration-dependent biphasic cellular response to corticosterone, i.e. an initial increase in neuronal excitability, followed by suppression of neuronal excitability. Similar findings were reported earlier by Rey et al. (effects observed between 0.2 and 10 nM corticosterone; peak increase in spike amplitude at 2 nM corticosterone) [98].

Given that the amplitude of the AHP is determined by Ca^{2+} and Ca^{2+}-dependent K^+ transients [99,100], it is interesting that Landfield and colleagues reported that high doses of the synthetic GR agonist RU28362 (7 μM) enhance the amplitudes of voltage-dependent calcium channel (VDCC)- mediated Ca^{2+} spikes in a protein synthesis-dependent manner [101]. In contrast, Tian et al. suggested that the increase in the slow after-hyperpolarization amplitude seen after exposure to high doses of corticosterone may involve cAMP-dependent phosphorylation and Ca^{2+}-activated K^+ channels [102]: dexamethasone (1 μM), a synthetic glucocorticoid with high selectivity for the nGR, blocked PKA-mediated inhibition of Ca^{2+}-activated K^+ channels without influencing VDCC-mediated Ca^{2+} currents in a mouse pituitary cell line (AtT20). It should be noted that Tian et al. treated their cells with dexamethasone for 2 h and that these effects required *de novo* protein synthesis for their manifestation [102,103]. Because activation of NMDA receptors results in an influx of Ca^{2+} and, as mentioned above, Ca^{2+} determines the AHP amplitude [99], corticosteroid-NMDA receptor interactions have been analyzed in a number of studies using electrophysiological recordings as the endpoint. For example, Wiegert et al. showed that exposure of mouse hippocampal slices to corticosterone (100 nM) for 20 min resulted in NMDA receptor-mediated suppression of primed-burst potentiation and synaptic potentiation [104] (induced by stimulation at 10 Hz, in contrast to the more commonly-used 100 Hz LTP regimen). In contrast, theta-burst potentiation (see Additional File 2 for information on different stimulation protocols), which requires activation of both NMDA receptors and voltage-dependent Ca^{2+}-channels was not affected by corticosterone treatment. The same authors also described a role for L-type Ca^{2+} channels in the synaptic actions of corticosterone [105]. In the context of the question of whether corticosterone can rapidly alter synaptic function, it is important to note, however, that Wiegert et al. [104] and Chameau et al. [105] made their electrophysiological recordings between 1 and 6 h after initial exposure to the steroid. On the other hand, Chameau et al. [105] found by quantitative PCR that corticosterone did not change the mRNA expression of the pore-forming Ca_v1 subunit of the L-type Ca^{2+} channel, and ruled out transcriptional mechanisms in the effects they observed.

Wiegert et al. [104] showed that RU 38486 blocks corticosterone-induced impairments of synaptic plasticity, implying mediation of the effects by nGR. A similar conclusion was drawn from their previous work on $GR^{dim/dim}$ mice, a strain carrying a point mutation of the DNA binding domain of the nGR which precludes transcriptional effects; briefly corticosterone did not influence VDCC-mediated Ca^{2+} currents in hippocampal slices from $GR^{dim/dim}$ mice [106]. To address the question of how glucocorticoids enhance Ca^{2+} currents on the one hand, and reduce synaptic efficacy on the other, Joëls' laboratory examined synaptic efficacy 1-4 h after a brief exposure to corticosterone (1 μM CORT for 20 min) [107]. Their investigations revealed that synaptic transmission was potentiated when VDCCs were activated, and impaired only when NMDA receptors were activated; moreover, they found that these effects were RU 38486-sensitive, indicating their mediation by nGR. Together, these observations point to the importance of considering all of the individual components that contribute to the overall response in field recordings. In this respect, it is worth recalling that the magnitude of LTP and LTD is a function of the number of AMPA receptors that are present at the synaptic surface (see Additional File 2). Miniature excitatory postsynaptic currents (mEPSCs, which represent the spontaneous release of neurotransmitter quanta from presynaptic terminals) are mediated by AMPA receptors and changes in the mEPSC amplitude represent postsynaptic changes in AMPA receptor properties and/or numbers. Indeed, Martin et al. observed that corticosterone increases the amplitude (but not frequency) of miniature excitatory postsynaptic currents and demonstrated that corticosterone increases trafficking of the GluR1 and GluR2 subunits of the AMPA receptor to the synaptic surface, apparently through an nGR-dependent mechanism [108]. This last study is in good agreement with that by Karst and Joëls, who also reported nGR-mediated increases in mEPSC amplitude [109].

Despite the overwhelming amount of data implying a role for nGR and/or nMR in mediating the effects of corticosterone on synaptic transmission, other evidence indicates that the rapid actions of corticosterone are mediated by mCR. For example, corticosterone was shown to dose-dependently (0.1, 1, 10, 100 μM) inhibit inward NMDA receptor-mediated currents, within seconds, in primary hippocampal cultures [110]. This effect faded upon wash-out of the hormone and was not reversible with RU 38486; assuming that RU 38486 binds specifically to nGR, the latter finding precludes mediation through nGR. The latter interpretation is supported by the finding that the effects of corticosterone were reproducible with membrane-impermeable BSA-conjugated corticosterone. Results from Takahashi et al. also dismissed a mediatory role for nGR or nMR in the mediation of corticosterone effects; however, they reported that the steroid prolongs the elevation of NMDAR-mediated Ca^{2+} influx in dissociated hippocampal neurons independently of VDCC and mobilization of intracellular Ca^{2+} stores [111]. In contrast, other authors reported that corticosterone and BSA-corticosterone (30 min) inhibit the peak amplitude of NMDA

receptor-mediated Ca^{2+} currents in the CA1 subfield of the mouse hippocampus [93], that bath application of corticosterone to hippocampal slices inhibits VDCC-mediated Ca^{2+} currents within minutes [112], and that corticosterone increases synaptosomal uptake of Ca^{2+} upon K^+-induced depolarization [113].

At this stage, it is important to note that some of the discrepant reports on corticosterone-induced changes in NMDAR-mediated Ca^{2+} currents may reflect the different durations of exposure to the steroid used by different groups. In fact, Wiegert et al. defined a narrow time window (10 min before high frequency stimulation) during which corticosterone facilitates synaptic potentiation; longer bath applications of the hormone were found to impair synaptic potentiation [114].

Most of the evidence reviewed above presumes postsynaptic sites of corticosterone action. New studies of CA1 neurons also report changes in the frequency of mEPSCs, thus implying presynaptic sites of action. Thus, Karst et al. [50] and Olijslagers et al. [51] showed that corticosterone increases the frequency of AMPA receptor-mediated mEPSCs. Both studies show that application of BSA-conjugated corticosterone produced similar effects to those obtained with corticosterone, and interestingly, that *de novo* protein synthesis was not essential for their manifestation. Together, these results hint at the involvement of receptors other than nGR and nMR; nevertheless, nMR antagonism by spironolactone resulted in a blockade of the corticosterone-induced increases in mEPSC frequency. [50,51] [but see [114]]. On the other hand, since RU 28362, a synthetic nGR agonist, did not reproduce the effects of corticosterone, and because the effects were not antagonizable with RU 38486, Karst et al. [50] and Olijslagers et al. [51] proposed that the putative mCR might share identity with the nMR. The latter suggestion is supported by experiments in mice with targeted mutations of nGR and nMR [50,106] and work by Groc et al. [115]. Using dissociated hippocampal cells to visualize AMPA receptor trafficking, the latter authors observed increased synaptic surface expression of GluR2 subunits of the AMPA receptor within minutes of exposure to corticosterone, BSA-conjugated corticosterone or aldosterone (the prototypic nMR agonist).

Related to the electrophysiological measures summarized in the last few paragraphs, Olijslagers et al. demonstrated that activation of the MAP kinase ERK1/2 is crucial for the corticosterone-induced increase in mEPSC frequency [51]. Interestingly, their experiments showed non-dependence on postsynaptic G protein activity on mEPSC frequency. Rather, by using the H-Ras G12V strain of mouse which displays strong presynaptic activation of ERK1/2 due to constitutively high expression of the H-Ras transgene, they suggested that the actions of corticosterone are initiated at presynaptic sites, increasing the probability of presynaptic neurotransmitter release [50,51]. Moreover, in agreement with other studies [111], Olijslagers et al., reported that intracellular Ca^{2+} stores do not influence mEPSC frequency upon exposure to corticosterone [51]. Lastly, it should be noted that although the involvement of G proteins in corticosterone-induced changes in mEPSC frequency were excluded [51], direct infusion of GDPβS into the postsynaptic cell prevented the decrease of the peak amplitude of $I_A$currents (postsynaptic K^+ conductance) by corticosterone [51]; this finding points to mediation through a postsynaptic mCR-dependent mechanism.

A number of studies suggest a role of G proteins in the mediation of the rapid actions of corticosterone. For example, ffrench-Mullen showed that the inhibition of Ca^{2+} currents by cortisol in guinea pig CA1 neurons depends on pertussis toxin-sensitive G-proteins [112]. The same author also showed that the effects of cortisol are significantly diminished in the presence of PKC inhibitors (BIS and PKCI 19-31), and ruled out a role for PKA in the mediation of the actions of cortisol [112]. Similarly, Chen and Qiu showed that corticosterone rapidly inhibits VDCC-mediated Ca^{2+} currents in a phaeochromocytoma cell line of neural origin (PC12 cells), and that inhibition of G proteins by application of either pertussis toxin or GDPβS significantly attenuates the ability of either corticosterone or BSA-corticosterone to stimulate the influx of Ca^{2+} [116]. They also demonstrated that activation of PKC with phorbol 12-myristate 13-acetate results in an inhibition of Ca^{2+} entry though VDCC after depolarization with K^+, and that the application of corticosterone activates PKC within 5-15 minutes. Lastly, like Qi et al. [117] who obtained similar results in primary hippocampal neurons, Chen and Qiu [116] showed that both, corticosterone and BSA-conjugated corticosterone trigger the activation of PKC and a series of MAP kinases (ERK1/2, p38MAPK and c-Jun) in PC-12 cells; maximum kinase activation occurred within 15 min of application of the hormone and the effects could not be attenuated by RU 38486.

Reality

Blood (and brain) corticosteroid levels rise and fall in a pulsatile manner under basal (unstimulated) conditions, and the circadian and stress-induced rises in corticosterone secretion occur gradually, taking minutes or even hours to reach peak levels. This raises the question of whether corticosteroid levels above a certain threshold have an impact on physiology and behavior and provokes curiosity about the mechanisms that could underpin the rapid biological actions of corticosteroids. Original interest in the fast actions of corticosteroids was awakened by attempts to understand the 'fast' and

'slow' negative feedback actions of corticosteroids at the level of the pituitary and the brain. Pioneering research by Mary Dallman used ingenious experimental designs which eventually provided evidence for the rapid actions of corticosteroids in reducing their own secretion [118] and, as already mentioned, the search for electrophysiological correlates was pursued in the hypothalamus in parallel. Today, predominantly based on work from the laboratories of Stafford Lightman and colleagues [4], it would appear that the ultradian rhythmic secretion of relatively high-amplitude corticosterone may serve to ensure low levels of adrenocortical activity during the organism's resting phases; these brief pulses presumably act rapidly to suppress brain-pituitary drive of adrenal secretion.

At the behavioral level, Orchinik et al. [69] elegantly demonstrated the potency of corticosterone in inhibiting male reproductive behaviour in newts, within 8 min of application. In mammals, Jozsef Haller and colleagues have shown that corticosterone injections elicit aggressive and anxiety-related behavior (latency of 7 min) in rats whose endogenous adrenocortical activity is suppressed by inhibition of 11β-hydroxylase activity with metyrapone [119-121]. Several authors have also described the ability of corticosterone to rapidly alter locomotor behavior in rodents; for example, acute systemic injections of corticosterone to rats (placed in a novel environment) were shown to stimulate locomotion within 7.5 minutes of administration [122].

Rhythms in the secretion of corticosteroids and other neuromodulatory molecules can influence experimental outcomes, even in *in vitro* settings. For instance, Ca^{2+} currents into hippocampal CA3 neurons in *in vitro* preparations are highest during the subjective night, when corticosterone levels are highest [123]. Similarly, Brunel and de Montigny [124] reported that the firing rate and pharmacological responsiveness of CA3 neurons is highest during the nocturnal peak in corticosterone secretion *in vivo*. Importantly, using hippocampal slice cultures, Chaudhury et al. demonstrated that the amplitude of LTP is greatest during the subjective night [125]. Additionally, Eckel-Mahan and colleagues reported circadian dependency in the efficiency of consolidation of long term memory [126].

Many studies support the idea that stress, a large part of whose actions are mediated by corticosteroids, influences learning and memory. Besides the quality and intensity of the stressor, the context in which the stressful stimulus is perceived, is an important determinant of the behavioral outcome. The latter is more easily explained in terms of 'intrinsic' and 'extrinsic stress [127]; 'intrinsic stress' refers to situations in which stress is either elicited by, or directly associated with, the cognitive experience (e.g. spatial learning), whereas 'extrinsic stress' describes situations in which the stress occurs outside the context of the momentary stress situation (e.g. foot shock stress *before* spatial learning). According to a model developed by Sandi and Pinelo-Nava [127], learning and memory will be facilitated by stressors that activate the same (or similar) neural circuitries that are required for interpreting and responding to a particular cognitive challenge. Supporting this view, Cahill and McGaugh [128] and Sandi [129] reported that emotionally arousing experiences are better remembered than neutral ones. In fear conditioning experiments, Cordero et al. noted that post-training corticosterone levels correlate with the strength of stimulus required to encode memories [130,131]. Moreover, the importance of corticosterone in information acquisition and consolidation of memory is well known, even if still poorly understood [132-135]. The relative importance of nMR and nGR in these processes are elegantly discussed by Schwabe et al. [136], and Revest et al. [134] have demonstrated a mediatory role of the MAPK pathway in the facilitation of hippocampus-dependent contextual fear conditioning by corticosteroids. In the previously-cited work on long-term contextual fear memory by Eckel-Mahan and colleagues [126], rhythms of MAPK (ERK1/2) activation were shown to coincide temporally with the degree of persistence of memory. Given that corticosterone acutely increases ERK1/2 phosphorylation [51,116,117,134], the results presented by Eckel-Mahan and colleagues [126] should be considered in the context of the hypothesis proposed by Sandi and Pinelo-Nava [127] and the pioneering work by Oitzl and de Kloet [137]; in addition, since the amygdala plays a major part in the regulation of fear and has reciprocal interactions with the hippocampus and other cognition-regulating brain areas, future interpretations of the work by Eckel-Mahan and colleagues [126] should embrace the idea that corticosteroids can exert actions on a network of interconnected brain structures, whose individual responses will determine the ultimate behavioral output.

Besides the acute behavioral and physiological actions of corticosteroids, much research has been focused on understanding the influence of chronically elevated corticosteroid secretion. Notwithstanding the above-mentioned fact that corticosteroids may exert acute effects during the rising phase of the endocrine response to stress, it is important to note that the latter is, generally, a protracted one. Thus, while the acute rises in corticosteroid secretion may shape the overall long-term response, the longer duration of corticosteroid exposure after stress allows recruitment of an array of intracellular responses (including nuclear receptor-mediated events) and cellular, physiological and behavioral adaptations. It is important to note that, although the

adrenocortical response to stress primarily serves an adaptive purpose, in certain circumstances, it may switch to being maladaptive, marked by transient or chronic pathology, as discussed earlier in this article.

The physiological and behavioral responses to stress depend on myriad molecules and processes, with an important contribution by corticosteroids; effects of the latter are often studied in isolation at the cost of other contributory factors and the neural networks which regulate, or may be regulated by, corticosteroids. This can be exemplified by considering our earlier discussion of corticosteroid interactions with glutamatergic transmission and reports that the direction and/or magnitude of LTP and LTD are influenced by the intensity and emotional value of a given stressor; for example, LTP is only reduced in animals exposed to uncontrollable stress [138], but not in animals that can escape from the stressor [139]. Using the paradigm of foot-shock stress, Wang et al. reported that stress induces a shift in synaptic plasticity; thus, whereas stress facilitates LTD induction, it impairs LTP induction [140]. Besides showing that these effects of stress can be blocked by RU 38486, these last authors showed that blockade of the NMDA receptor restores LTP inducibility in stressed animals; further they demonstrated that stress-induced changes in synaptic efficacy can be abolished by prior administration of Ro25-6981, a specific antagonist of the NR2B subunit of the NMDA receptor. A role for the NR2B subunit in the synaptic plasticity thought to be essential for the orchestration of the behavioral response to stress was also suggested by Wong et al. who showed that Ro25-6981 reverses elevated platform stress-induced deficits in spatial learning and memory, as tested in the Morris water maze (MWM) [141].

The NR2B subunit is predominantly associated with extrasynaptic NMDA receptors whose activation depends on glutamate "spill-over", a phenomenon that can be mimicked with *threo*-β-benzyloxyaspartate (TBOA), a blocker of glutamate re-uptake. Wong et al. [141] found that TBOA application to animals 5 min before low frequency stimulation resulted in the successful induction of LTD, indicating that stress leads to glutamate "spill-over". Linking LTD with stress-induced memory impairment, the authors showed that preventing LTD induction by infusion of a GluR2 peptide analogue that cannot be internalized abolished the ability of stress to cause memory deficits in the MWM test; these findings add to the evidence that acute stress results in the internalization of AMPA receptors, followed by synaptic depression and learning and memory deficits.

We previously discussed how the MAPK signaling pathways may be linked with LTP and LTD (and learning and memory). In this respect, it is interesting to note that this pathway is concomitantly activated by stress, presumably due to activation of nGR [142,143], believed to be essential for the phosphorylation of ERK1/2 [134]. Moreover, the observation that tail shock and restraint stress robustly activate ERK1/2 and impair synaptic potentiation in the CA1 subfield suggests a major role for the MAPK pathway in mediating the actions of stress [144]. In addition to inducing the phosphorylation of ERK1/2, stress activates other kinases (e. g. p38 MAPK, CaMKII) and pCREB within 2 min of swim stress [145]. Surprisingly, however, the latter responses are accompanied by a reinforcement (rather than impairment) of LTP in the dentate gyrus of the hippocampus. This finding indicates that different stressors may elicit quite different electrophysiological responses and/or, that the synaptic effects of stress differ from one hippocampal subfield to another. Since the effects of stress on biochemical and electrophysiological signalling in the dentate gyrus were found to be subject to modulation by serotonin [145], it is plausible that differential monoaminergic innervation of the different hippocampal subfields defines the ultimate cellular response.

We summarize some potential mechanisms that may account for the rapid and slower effects of corticosteroids on neuronal physiology, with a focus on synaptic events, in Figure 4. An attempt is made to show how signals originating at the neuronal surface are integrated both at the synaptic and transcriptional levels.

Critique

From the preceding, it appears safe to assume that, irrespective of the behavioural or physiological outcomes, acute and chronic elevations of corticosteroid secretion initiate common mechanisms and biochemical processes; convergence of these events will depend on parameters such as exposure dosage and time, as well as the context in which they occur. Given the potential for convergence (as well as potentiation), improved knowledge of the initial stages of corticosteroid signalling, whether membrane- or nuclear receptor-mediated, is clearly desirable. Studies on the rapid neural actions of corticosteroids are likely to gain further interest, especially as newer analytical tools become available and knowledge about the fast actions of other steroid hormones grows. It therefore seems appropriate to list some critical issues and needs, the consideration of which may foster progress through cautious reflection:

- **definition of the terms "rapid" or "fast" actions** of corticosteroids in terms of the timeframe within which a clearly defined (electro)physiological, biochemical and/or behavioural response is elicited in animals or neuronal cell and brain slice preparations;

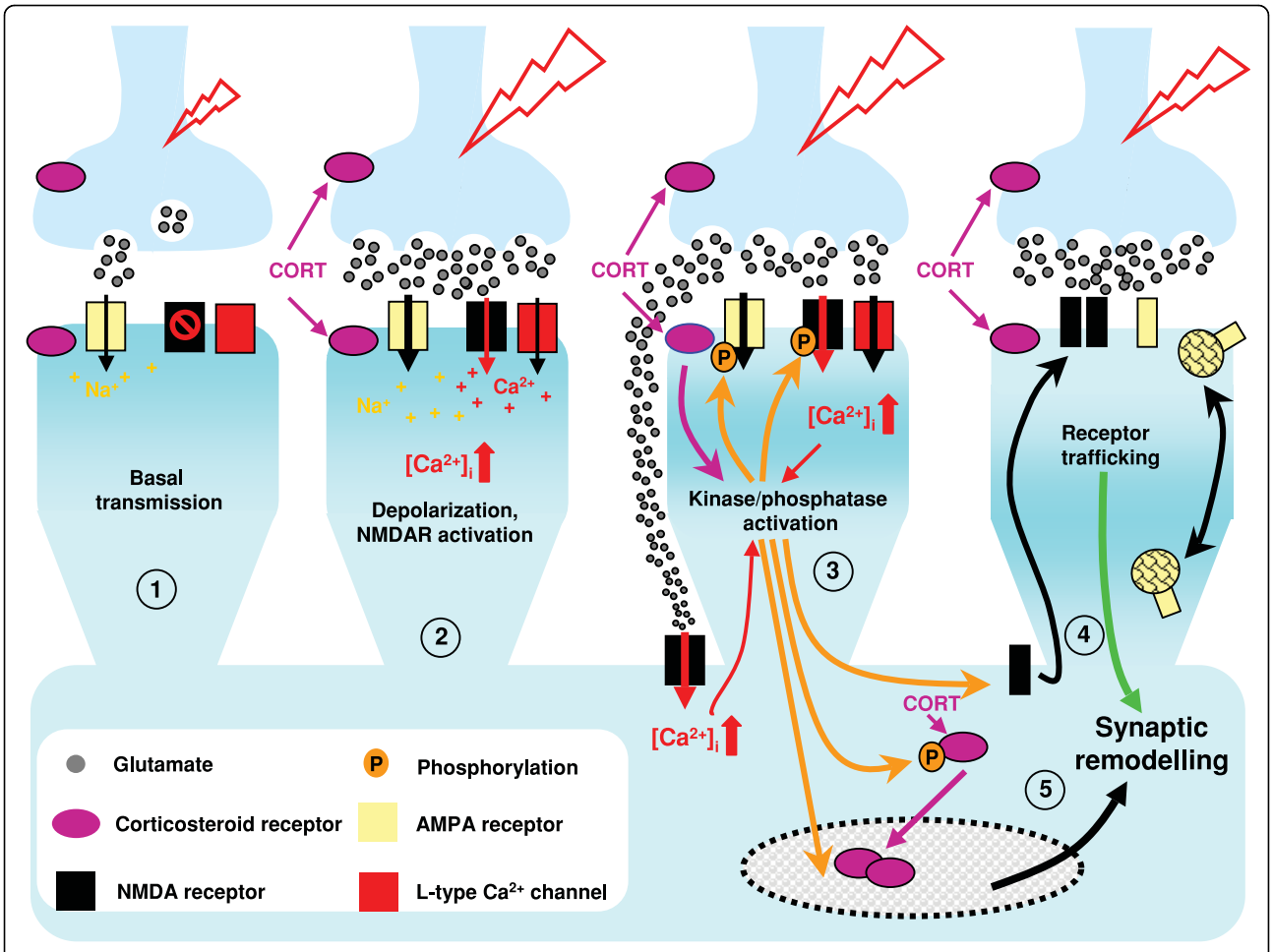

Figure 4 Working model of sequential corticosteroid influences on synaptic physiology. Corticosterone-mediated changes in synaptic transmission occur at different levels and in different sequential steps. ① depicts synaptic transmission under basal conditions. Neuronal excitation results in glutamate secretion from synaptic vesicles at presynaptic sites into the synaptic cleft. Glutamate binds to postsynaptic glutamate-gated ion channels (in particular, AMPA receptors), which open to permit ion fluxes (Na^+ influx, K^+ efflux) across the AMPA receptor, resulting in a depolarization of the postsynaptic cell. Due to a voltage-dependent Mg^{2+} block in its membrane domain, the NMDA receptor remains inactive under basal conditions, and is activated when a certain transmission threshold is reached. ② Exposure to corticosteroids (e.g. during stress) may lead to activation of ERK1/2 in the presynaptic terminal (possibly through membrane corticosteroid receptors [51]); increased glutamatergic stimulation of postsynaptic AMPA receptors results in an increase in the frequency of AMPA receptor-mediated miniature postsynaptic currents (mEPSCs). ③ Enhanced activation of AMPA receptors in the previous step further depolarizes the postsynaptic membrane and activates NMDA receptors. Activated NMDA receptors (Na^+ and Ca^{2+} influx, K^+ efflux) lead to further depolarization of the postsynaptic cell, resulting in the opening of voltage-dependent Ca^{2+} channels (VDCC) and high postsynaptic concentrations of Ca^{2+}. Corticosteroids may stimulate glutamate secretion so strongly, causing glutamate "spill-over" which activates not only synaptic, but also extrasynaptic, glutamate receptors [141]; the latter are mainly NMDA receptors of the NR2B subtype. The increased intracellular levels of Ca^{2+} trigger a cascade of Ca^{2+}-dependent signaling pathways in the postsynaptic cell, which may, in turn, induce the phosphorylation and de-phosphorylation of postsynaptic glutamatergic receptors and of nuclear corticosteroid receptors (nMR and nGR). Activation of extrasynaptic NMDA receptors is thought to trigger NR2B-dependent kinases, which might initiate trafficking of extrasynaptic NR2B receptors into the postsynaptic surface. Furthermore, Ca^{2+}-dependent signaling pathways in the postsynaptic cell participate in the regulation of AMPA receptor trafficking to and from the synaptic surface, as indicated in ④. Phosphorylation of nuclear corticosteroid receptors, influences their translocation to the nucleus and therefore, their transcriptional activity [32], as indicated in ⑤.

• standardized test protocols (**steroid dose, animal or cellular models, and sex[c]of animals**); in *in vitro* studies, **drug diffusion times and active concentrations** achieved at target cells should be controlled; similarly, in *in vivo* research, **pharmacokinetic factors**, including solvent and route of administration, should be considered; **age** of animals, but also of material used for *in vitro* testing, is important because of dynamic age-related changes in the expression of key partners such as glutamate receptor subunits [146]; since corticosteroids are secreted according to a strict **circadian** rhythm, both the

availability of endogenous corticosteroids as well as of primary and secondary downstream effectors will vary over the day - this demands **testing at a given circadian time** to ensure comparable measurements [123-125].

• while **surgical adrenalectomy** is a useful approach to ensure that only the actions of exogenously-administered steroids are being recorded, the operation requires anaesthesia and may involve potentially confounding post-operative pain; **chemical adrenalectomy** is a good alternative (e.g. blockade of corticosteroid synthesis with metyrapone), but it may have (indirect) non-selective effects on the production of other steroids; adrenalectomy, in general, induces massive apoptosis and stimulates neurogenesis in the dentate gyrus within just a few hours, changes that probably result in **reorganized neuronal circuits and measurable outputs** [147].

• attention to the fact that **acute and chronic corticosteroid exposures** differ significantly, and that **administration of corticosteroids only mimics an intermediate phase of the organism's response to stress**;

• clear **exclusion of transcriptional and translational events** initiated by activation of cognate nuclear receptors;

The show must (will) go on

While the nuclear receptor-mediated actions of corticosteroids are well established, those that appear to be mediated through non-classical, possibly membrane-bound receptors, have perhaps not received sufficient appreciation. The lack of consistent results (see need for standardization in previous section), compounded by the relatively fruitless hunt for putative membrane receptors, accounts for the scepticism that haunts this area of research. Increased respectability might be gained by initially seeking answers to some of the following questions:

• How can the neural actions ascribed to peripherally-produced corticosteroids be distinguished from those that result from those elicited by corticosteroids thought to be produced in neural tissue?

• Can the rapid actions of corticosteroids observed predominantly in the CA1 subfield of the hippocampus be generalized to other hippocampal subfields, or indeed other brain regions?

• Do the endpoints assessed after application of corticosteroids reflect actions exclusively at the hippocampus? *In vitro*, do we get only a partial (or perhaps, false) picture? *In vivo*, are we monitoring responses from a network of corticosteroid-sensitive brain regions? How are the outputs modulated by other neurochemical states and inputs?

• Do corticosteroids directly interact with membrane proteins? What is the chemical identity of these molecules? Are they distinct from the known nuclear receptors and if not,

  ◦ Do they represent post-translational modifications (e.g. palmitoylated versions of the nuclear receptors, as suggested for the mER)?

  ◦ Is there biochemical evidence for interactions with other known membrane receptors (e.g. glutamate receptors); do these receptors have allosteric binding sites for corticosteroids as well as for pharmacological antagonists of nMR and nGR? (cf. estrogens, progestins)

• How do events that are triggered by corticosteroids at the membrane funnel into long-term cellular and organismic adaptations (e.g. by positive or negative priming of the gene machinery regulated by nMR and nGR)?

• How do the rapid actions of corticosteroids contribute to their longer-lasting actions (e.g. 'priming' of nuclear receptor-mediated events?)

• Is it possible to define corticosteroid actions - fast and slow - in terms of spatio-temporal maps, keeping in mind that damage induced in a relatively short time in one area may take longer to spread to other interconnected areas [cf. [13]]?

• Is it feasible to generate genetic or pharmacological tools that will facilitate acceptance and further study of mCR?

Appendix

a) Corticosteroids: way upstream - the title of this article is adapted from Alan Ayckbourne's stage play *Way Upstream* in which two couples on a boating holiday run into some strange happenings.

b) A painted cloth in front of which a short scene is played while the main stage set is changed.

c) Research on the rapid actions of corticosteroids has mainly exploited male rodents or tissues derived from them. Corticosteroid secretion is strongly influenced by sex, as are physiology and behaviour. Many of the physiological and behavioural readouts monitored in such studies reflect the prevailing sex steroid *milieu*; in females, sex steroids are secreted in a cyclical fashion.

Additional file 1: Summary of rapid effects of corticosteroids and estrogens on the central nervous system [148-181].
Click here for file
[http://www.biomedcentral.com/content/supplementary/1756-6606-3-2-S1.PDF]

Additional file 2: Synaptic plasticity and learning and memory [182-221].
Click here for file
[http://www.biomedcentral.com/content/supplementary/1756-6606-3-2-S2.PDF]

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Author details
[1]Max-Planck-Institute of Psychiatry, Kraepelin Str. 2-10, 80804 Munich, Germany. [2]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Faculty of Medicine and Dentistry, University of Bristol, Bristol, UK.

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TR, AP and OFX wrote the manuscript; KC critically reviewed the manuscript and suggested improvements. All authors read and approved the final form of the manuscript.

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