Epigenetic control of hippocampal stem cells: modulation by hyperactivation, glucocorticoids and aging

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Ultradian glucocorticoid oscillations control epigenetic programming of cell quiescence in hippocampal neural stem cells

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Graphical abstract

Schematic depiction of the initial stages of the neurogenic cascade in control animals (left) or after glucocorticoid (GC) induced alterations (right). Phenotypical alterations are: increased quiescence, decreased proliferation and apoptosis. In this chapter we discuss how GC rhythmicity is a neuro-endocrine signal involved in the regulation of neural stem/progenitor cell quiescence and proliferation, possibly through differential methylation of gene promoters.
Abstract

A large body of evidence supports the anti-proliferative effect of stress and glucocorticoids (GCs) on hippocampal neural stem/progenitor cells (NSPCs). However, how GCs exert this action remains unclear. In the absence of known stressors, GCs are released in oscillatory patterns, resulting in cyclic glucocorticoid receptor GR activation. We have shown before that the GR regulates adult hippocampal neurogenesis (Ahn) in vivo, and influences Ahn-dependent learning, suggesting that under basal conditions, GR activation can also be modulated by oscillatory GC patterns. Here, we tested this hypothesis by investigating differences in proliferation, cell cycle progression, gene expression and DNA methylation profiles in NSPCs exposed to either oscillatory or chronically elevated GC levels.

We found that NSPCs actively responded to deviations from pulsatile GC patterns displaying a transient cell cycle exit, possibly reflecting cellular quiescence, changes in the expression of key DNA-methylation enzymes, and a coordinated DNA methylation profile. While the majority of DNA methylation changes were transient, a number of gene promoters remained differentially methylated even after a 24h hormone-free period, sufficient time for NSPCs to undergo at least one cell cycle under these experimental conditions. Specifically, stable DNA methylation was observed in promoter regions of among others, genes linked to negative regulation of the Wnt signaling pathway. Thus, alterations in GC oscillatory patterns can actively and lastingly modify the methylation state of specific gene promoters in NSPCs. We further show that deviations from basal GC patterns and changes in DNA methylation levels were accompanied by desensitization to GC-induced cell cycle exit. Our results allow to compare the coordinated epigenetic alterations induced upon NSPCs by alterations in distinct GC release patterns. As such, they help us understand how GC oscillatory patterns translate into unique molecular profiles controlling NSPC proliferation.
Ultradian CORT oscillations epigenetically control NPC quiescence

Chapter 5

Introduction

Glucocorticoids (GCs), cortisol in humans and corticosterone (CORT) in rodents, are well characterized mediators of the stress response in mammals and other organisms. By acting as ligand-induced transcription factors, GCs modify gene expression and thereby exert numerous long-lasting adaptive effects on body and brain. In rodents and human, GCs are released in a circadian manner with peak levels shortly before the onset of the activity period. This circadian release pattern is thought to be needed for adaptation and to maintain hypothalamus-pituitary-adrenal (HPA) axis responsivity. This circadian GC rhythm is built up by, shorter, discrete hourly pulses that constitute the GC ultradian rhythms. The exact biological functions of these GC ultradian rhythms are not fully resolved, but in vitro observations suggest they serve as a molecular pacemaker of glucocorticoid receptor (GR) -dependent gene expression. As a result, the ultradian oscillatory nature of GC release maintains both homeostasis as well as responsiveness of gene expression to GCs.

Conditions during which GCs rhythms are aberrant, such as aging, hypercortisolemia, sleep deprivation, immune-challenges or chronic stress, are associated with alterations in GC oscillatory patterns, disruption of homeostatic balance and disturbances in feedback regulation. In turn, all these alterations can result in GC overexposure, a major risk factor for the development of disease and psychopathology.

High circulating GC levels activate the GR, which terminates the stress response and helps to re-establish homeostasis. The hippocampus is highly responsive to GC levels and it contains neural stem/progenitor cells (NSPCs) that continue to give rise to new neurons in adult and aged individuals. This process of adult neurogenesis follows different stages during which quiescent NSPCs first become activated and then proliferate, generating proliferative neuroblasts. Subsequently, the newly born neuroblasts migrate and differentiate into fully functional newborn neurons that incorporate into the existing, mature hippocampal network. This process of adult neurogenesis is tightly regulated at the molecular level by epigenetic mechanisms, signaling pathways such as Wnt and its canonical target CCND1, transcription factors like REST/coREST and by environmental factors such as stress.

Indeed, several studies have described stress- or CORT-induced decreases in neurogenesis during which both the proliferation and differentiation/survival of NSPC appear to be affected, ultimately resulting in the generation of less new neurons. Interestingly, while exposure to stress decreased NSPC proliferation, this effect was fully reversible after a recovery period. In addition, GR expression specifically in hippocampal NSPCs influences their fate and functional integration and affects AHN-dependent behavior.

Although stress, acting through various mediators including stress hormones such as GCs, modulates neurogenesis and NSPC turnover, little is known about the molecular changes GCs induce directly in NSPCs. So far, few studies have characterized how persistently elevated corticosterone (CORT) levels impact the molecular profile of NSPCs and how this differs from a “normal” pulsatile pattern of GC exposure. Long-term constant exposure to a synthetic GR agonist like Dexamethasone (DEX), affect global DNA methylation and the expression of DNA methyltransferases (DNMTs), suggesting the occurrence of GC-sensitive epigenetic changes. To the best of our knowledge, no study has compared GC oscillations versus constant exposure, and how this may affect gene expression patterns in NSPC. Moreover, it is unclear whether earlier alterations in GC oscillatory patterns may alter NSPC response to subsequent CORT exposure. We hypothesized that GC oscillatory patterns may establish specific gene expression patterns by affecting promoter specific DNA-methylation, which could translate into either alterations in proliferation (and/or survival) upon a subsequent challenge to high CORT. In this study, we therefore studied whether alterations in GC oscillatory patterns can mediate changes in DNA methylation, and/or sensitize NSPCs to a subsequent exposure to GCs in vitro, thus allowing for the identification of direct CORT-mediated effects on NSPC.

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Confocal microscopy on brain slices was performed using a Zeiss LSM510 confocal laser scanning microscope system as described\textsuperscript{24}. The following antibodies were used: polyclonal chicken anti-GFP (Abcam, 1:500), monoclonal mouse anti-GFAP (Chemicon, 1:1000) and polyclonal rabbit anti-GR (H300 Santa Cruz, 1:100) or polyclonal rabbit anti-Ki67 (Abcam, 1:1000) in combination with goat anti-chicken Alexa488 (Invitrogen, 1:500), goat anti-mouse Alexa647 (Invitrogen, 1:500) and goat anti-rabbit Alexa568 (Invitrogen, 1:500) respectively. Sections were counterstained for DNA using Hoechst (Invitrogen, 1:20000) to detect cell nuclei. Hippocampal NSPC GR expression quantifications were carried out as described before\textsuperscript{24} and were expressed as a percentage of total NSPC. Orthogonal projections and Z-stack were made using ImageJ software.

**Materials and methods**

*Animals, in vivo CORT treatments, immunohistochemistry and confocal microscopy*

For *in vivo* characterization of GR and Ki67 expression, 3-month old Nestin-GFP reporter mice (n=3 per group) were used\textsuperscript{28}. Nestin-GFP mice had ad libitum access to food and water and were kept under a 12-hour dark/light cycle (lights on at 08:00h). As described by others\textsuperscript{7}, albeit with some modifications, CORT levels were clamped at constant levels at 0%, 100% and 200% relative to basal PM CORT levels (vehicle, 10mg/kg/day and 20mg/kg/day, respectively; Figure 1F) using slow release biodegradable carrier-binder pellets (Innovative Research of America). Pellets were implanted subcutaneously under isoflurane anesthesia at 08:00h on experimental day1. To validate flattening of CORT rhythms, at 20:00h on experimental day6 PM blood samples were collected and at 08:00h on experimental day7 AM blood samples were collected as described before\textsuperscript{29}, immediately before PFA perfusion. Plasma CORT concentrations were determined using a radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) as described before\textsuperscript{29}. The committee of Animal Health and Care, University of Amsterdam approved animal experiments (DEC259).

Mice were transcardially perfused with 4% PFA and then brains were extracted, sectioned, immunostained to detect GFP and cell-type specific markers.

Our results indicate that both pulsatile and constant CORT treatment reversibly increases the number of NSPCs arrested in the G0/G1 phase of the cell cycle, suggestive of cell quiescence induction. However, at the molecular level this phenotype was accompanied by specific and lasting changes in DNA methylation and alterations in genes encoding key enzymes responsible for DNA methylation. In agreement with these observations, the rhythmicity of the initial exposure to CORT resulted in differential programming of cell-cycle responsiveness to a second GCs exposure, indicating that the adaptive epigenetic (re)programming of NSPCs to stress may depend on CORT rhythmicity.

**Cell culture, CORT treatments and CORT measurements**

Low passage number (<P20) NSPCs were cultured in culture flasks in DMEM/F-12 medium supplemented with 5% charcoal-stripped fetal bovine serum (FBS, Atlanta Biologicals), N2 supplement, (Invitrogen), Bovine Pituitary Extract (BPE, Invitrogen), recombinant-human-EGF (20ng/mL, Sigma) and recombinant-human-FGF (10ng/mL, Sigma), as described before\textsuperscript{24}. NSPCs were seeded the day before the start of the treatments. CORT (corticosterone, Sigma-Aldrich) was dissolved in ethanol and added freshly to NSPC medium to a final concentration of 10\textsuperscript{-6} M (except stated otherwise) prior to incubation. Ultradian CORT oscillations were modeled *in vitro* as previously described by others\textsuperscript{4}, with some modifications (Figure 2A-C). Pulsatile treatment consisted of 24h-long cycles of exposure to 30min-long pulses of either vehicle (ethanol) or CORT interspaced with 30min-long incubations with hormone-free medium. NSPCs were exposed to this pulsatile treatment for 12h, followed by a 12h-long incubation with hormone-free medium. The constant CORT condition consisted of 30min-long cycles of incubation with CORT at the same concentration for 24h, thus without interspaced hormone-free periods. In both cases, these 24h-long cycles were repeated (48 and 72h) as indicated in the timeline of Figure 2A. Starting after
a 72h initial treatment, the washout period (recovery) consisted of a 24h-long incubation with hormone-free medium. When indicated, NSPCs were treated during the last 6h of the washout period with 10^{-6}M CORT or vehicle, to model the effects on further exposure to CORT. Treatment schemes are depicted in Figure 2B and 2B. Efficient washout and stability of CORT during the experiment (Figure 2C) was analyzed by collecting samples every 30min during both pulsatile and constant treatment and CORT concentrations were determined using a radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) as described before\textsuperscript{29}.

**Immunocytochemistry**

Immunocytochemistry was carried out as described before\textsuperscript{24}. Briefly, cells were rinsed three times with PBS and fixed in 4% PFA in PBS for 30min. The fixative was then removed and cells were rinsed three times for 5min with PBS. For detection of proliferation, cells were blocked in blocking buffer (1X TBS/1% skimmed milk powder) for 60min and incubated for 1h at room temperature and then overnight at 4°C with polyclonal rabbit anti-Ki67 (Abcam, 1:1000) diluted in 0.25% gelatin/0.5% Triton X-100 (Supermix). The day after, cells were rinsed three times for 5min in PBS, incubated with donkey anti-rabbit Alexa488 (Invitrogen, 1:1000) for 1h at room temperature, rinsed three times for 5min in PBS and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories).

To assess GR immunoreactivity in Ki67-expressing NSPCs, blocking buffer was applied for 60min before cells were incubated for 1h at room temperature and then overnight at 4°C with a polyclonal mouse anti-Ki67 (Novocastra, 1:200) and polyclonal rabbit anti-GR (H300 Santa Cruz, 1:200) antibody diluted in Supermix. The day after, cells were rinsed three times for 5min in PBS, incubated with goat anti-mouse Alexa568 (Invitrogen, 1:1000) and donkey anti-rabbit Alexa488 (Invitrogen, 1:1000) for 1h at room temperature, rinsed three times for 5min in PBS and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were acquired using a Leica CTR5500 microscope with the Leica MM AF program (MetaMorph, version 1.6.0).

**Quantitative real time polymerase chain reactions**

RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturers' protocol. For mRNA qPCRs, cDNA was synthesized using a superscript II reverse transcriptase (Life Technologies) according to the manufacturers' protocol. Quantitative real time polymerase chain reactions were performed, as described before\textsuperscript{24}, using SYBR green (Applied Biosystems) and the following primer sequences:

- **α-tubulin** (for normalization): fwd:CCCTCGCCTTCTAACGCGTTGC, rev:TGGTCTTTGACTACTCGCTGGC;
- **GR**: fwd:AGGTGCCAAGGGTCTGGAGAGG, rev:TGGTCCCGTTGCTGTGGAGGA;
- **GILZ**: fwd:GTGCGCGACCCCTGCTACCT, rev:ACGAGGTCCATGGCCTGCTCAA;
- **SGK1**: fwd:TGGTGTCTTGGGGCTGTCCTGT, rev:GCCTTCCAGGAGTGTCCTTGC;
- **GAS5**: fwd:AATGGGTCACCTCAAGTGAAGGCA, rev:TTGAGCCTCCATCCAGGCACCT;
- **DNMT1**: fwd:AGGCGCGTCATGGGTGCTAC, rev:GGCGGCGCTTCATGGCATTC;
- **DNMT3a**: fwd:GCCAAGAAACCCAGAAAGC, rev:GTGACATTGAGGCTCCCACA;
- **DNMT3b**: fwd:GCCAAGAAACCCAGAAAGC, rev:GTGACATTGAGGCTCCCACA;
- **CCND1**: fwd:GCCAAGAAACCCAGAAAGC, rev:GTGACATTGAGGCTCCCACA;
- **GR**: fwd:AGGTGCCAAGGGTCTGGAGAGG, rev:TGGTCCCGTTGCTGTGGAGGA;
- **GILZ**: fwd:GTGCGCGACCCCTGCTACCT, rev:ACGAGGTCCATGGCCTGCTCAA;
- **SGK1**: fwd:TGGTGTCTTGGGGCTGTCCTGT, rev:GCCTTCCAGGAGTGTCCTTGC;
- **GAS5**: fwd:AATGGGTCACCTCAAGTGAAGGCA, rev:TTGAGCCTCCATCCAGGCACCT;
- **DNMT1**: fwd:AGGCGCGTCATGGGTGCTAC, rev:GGCGGCGCTTCATGGCATTC;
- **DNMT3a**: fwd:GCCAAGAAACCCAGAAAGC, rev:GTGACATTGAGGCTCCCACA;
- **DNMT3b**: fwd:GCCAAGAAACCCAGAAAGC, rev:GTGACATTGAGGCTCCCACA;
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- **GR**: fwd:AGGTGCCAAGGGTCTGGAGAGG, rev:TGGTCCCGTTGCTGTGGAGGA;
- **GILZ**: fwd:GTGCGCGACCCCTGCTACCT, rev:ACGAGGTCCATGGCCTGCTCAA;
- **SGK1**: fwd:TGGTGTCTTGGGGCTGTCCTGT, rev:GCCTTCCAGGAGTGTCCTTGC;
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For microRNA qPCRs, to detect mature miR-124-3p (Cat# 4427975, Assay ID 001182, Life Technologies) and miR-124-5p (Cat# 4427975, Assay ID 002197, Life Technologies) were performed using a TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) combined with TaqMan® 2x Universal PCR Master Mix (Life Technologies) and were normalized against...
obtain fragments with an average length of 200bp. The power mode was set to frequency sweeping, temperature 6-8°C and water level 12. A maximum of 3μg DNA was dissolved in 130μl TE and loaded in a microtube with AFA intensifier (Covaris). DNA was then analyzed on the Agilent 2100 Bioanalyzer (Agilent Technologies) and fragment distribution was analyzed on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode). The concentrations of the fragmented and captured DNA was determined on a Fluostar Optima plate reader (BMG Labtech) with the Quant-iTTM Picogreen® dsDNA assay kit (Invitrogen) at 480/520nm. A second quality control was performed after fragmentation on an Agilent 2100 HS DNA chip.

Methylated DNA library preparation, amplification and sequencing

A methylated DNA library was prepared, amplified and sequenced using a modified version of the ‘multiplexed paired end ChIP protocol’ (Illumina), using the DNA Sample Prep Master Mix Set 1 (NEB) in combination with the Multiplexing Sample Preparation Oligo Kit (Illumina). The library was prepared from 250ng of fragmented DNA on an Apollo 324 NGS Library Prep System (IntegenX) with a PrepXDNA Library Kit (Wafergen Biosystems) according to the kit’s protocol. Library amplification was done according to the multiplexed paired end ChIP protocol including the indexes from Multiplexing Sample Preparation Oligo Kit (Illumina). Smaller fragments were removed when necessary using a 2% agarose gel (Low Range Ultra agarose; Biorad) in combination with a 1Kb Plus ladder (Invitrogen). 300bp±50bp fragments were excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen), then eluted in 23 μl EB and 1 μl from there was run on an Agilent 2100 HS DNA chip. DNA concentration was determined using smear analysis on a Agilent 2100 Bioanalyzer (Agilent Technologies) and checked for degradation (Figure S1). Samples (n=3) for each experimental condition were pooled into a single sample for further processing.

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Methylated DNA fragmentation and MBD-capture

DNA Fragmentation was performed on a Covaris S2 Focused ultrasonicator with the following settings: duty cycle 10%, intensity 5, 200 cycles per burst during 190sec to

Flow cytometry analysis of cell cycle using propidium iodide

NSPCs were trypsinized (Trypzean, Lonza) for 5min and fixed by slowly adding cold 70% Ethanol (-20°C) and were then left overnight at 4°C. Subsequently, cells were washed twice with PBS for 5 minutes and treated for 20min with RNase (100 µg/ml; Sigma-Aldrich) and incubated for 20min at room temperature with a mix containing propidium iodide (5ug/ml; Sigma-Aldrich), 0.1% sodium citrate and Triton-X100 (0.1%) in PBS. Cells were sorted using a FACS Aria™ III system (BD) with 488nm excitation and 575/26 bandpass filters. At least 9000 cells were analyzed per sample and only single cells were included in the analysis. FACS histograms were plot-fitted using the G2/G1 fixed method (reference) using Multicycle AV and FCS express (De Novo Software).

Global cytosine methylation analysis

Global DNA methylation was measured as previously described. Briefly, NSPCs were trypsinized (Trypzean, Lonza) for 5min, spun down for 3min at 300 x g and total DNA was extracted using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer’s protocol. Global levels of DNA methylation were measured using a MethyIamp™ Global DNA Methylation Quantification Ultra Kit (Epigentek) according to the manufacturer’s protocol. Data were normalized to global DNA methylation levels of vehicle treated NSPCs, as indicated.

RNU6B (Cat# 4427975, Assay ID 001093, Life Technologies).

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Ultradian CORT oscillations epigenetically control NPC quiescence

**Chapter 5**

**DNA methylation base scaling and mapping**

FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.0. The paired end 51bp sequence reads were mapped using Bowtie software v0.12.7, as described. Only unique paired reads were retained and both fragments must be located within 400bp of each other on the mouse reference genome build NGBi37/mm9. Details on mapped reads percentages for each sample are shown in Table 1. Regions within -2000 and +500 bp from a TSS were considered as gene promoters.

**Bio-informatics and statistics**

Dose response curves were created using Graphpad Prism 5.0 and statistically compared with an F-test. Heatmaps were generated using the unsupervised hierarchical clustering option in MultiExperiment Viewer v4.9 (TM4). Gene ontology (GO) analysis was performed using the Genecodis GO algorithm hypergeometrically testing for significantly overrepresented processes (FDR corrected p<0.05). Functional network predictions were produced using the GeneMANIA algorithm. The H2G2 genome browser (NXT-Dx) was used to explore the mapped MBD2 read density. All comparisons were statistically tested using an unpaired two-tailed Student’s t-test or, one-way analysis of variance (ANOVA) test with Tukey’s post test when more than two groups were compared. Statistical analyses were performed using GraphPad Prism 5.0.

**Results**

**Primary hippocampal NSPCs express the GR and enter a quiescent cellular state after GC treatment.**

In line with previous literature, we found GR immunoreactivity in vivo in Nestin-GFP+/GFAP+ cells of the DG (Figure 1A). These cells have been previously characterized as Type 1, quiescent radial glia-like NSPCs. Notably, Nestin-GFP+/GFAP+/GR+ cells displayed a marked GR immunoreactivity outside of the nucleus as we have observed before in early postnatal NSPCs. Additionally, we found GR immunoreactivity in Nestin-GFP+/GFAP- cells (Figure 1B), previously characterized as Type 2, activated NSPCs, in line with previous observations. Furthermore, we found that 90.7±3.0% of the total NSPC population expressed GR in vitro (Figure 1C), reflecting the 79.2±5.3% of Type-1 and -2 NSPC combined expressing GR in vivo (Figure 1D). These two GR+ NSPC populations could also be distinguished by the expression of the cell cycle marker Ki67 (Figure 1C). These and similar observations suggest that GCs can influence both proliferating and non-proliferating NSPC through GR. In line with our hypothesis, we found that all Ki67+ cells expressed the GR in vitro (Figure 1C), suggesting a functional role in this NSPC population. Moreover, we found that 25.6±4.2% of the GR+ cells did not express Ki67, demonstrating GR expression also in non-proliferative NSPCs, as suggested by our in vivo observations presented in Figure 1A, 1B and 1E.

In vivo, Ki67+ activated Type-2 cells were readily detectable, while quiescent Type-1 cells were predominantly Ki67- (Figure 1E), under normal basal CORT rhythmicity (Figure 1F). Suggesting a functional role of NSPC GR expression during basal CORT rhythmicity, both a 7day “flattening” of the basal CORT rhythmicity at daily peak levels, and exposure of “flattened” basal rhythmicity with increased levels of CORT (100% CORT pellet and 200% CORT pellet respectively, Figure 1E) eliminated NSPC Ki67 expression and increased the quiescent NSPC population in vivo (Figure 1G-I).

Still, CORT has been proposed to have direct and indirect effects on NSPC. Moreover, NSPCs only represent a small fraction of the total hippocampus, and in vivo analysis cannot readily identify specific epigenetic modifications. Therefore, to characterize the direct effects of CORT on the epigenetic programming of NSPC and the consequences on cell cycle progression and gene expression, we used a previously described postnatal mouse hippocampal NSPC primary cultures. To further study the effects of CORT on proliferation, we measured the expression of the proliferation marker Ki67 in Nestin-GFP+ cells (Figure 1J). Based on the predominant expression of the GR in Ki67+, proliferative cells, we decided to test the effect of GR activation on NSPC proliferation in vitro. We found that both CORT and the specific GR agonist DEX dose-dependently reduced the rate of NSPC proliferation, with DEX being approximately 10times more potent in inhibiting NSPC proliferation than CORT (5.8x10^-9 M vs. 6.3x10^-8 M, respectively), in agreement with their relative affinities for the GR.
Figure 1 - Postnatal hippocampal NSPC express the GR and are sensitive to a GR-dependent reversible arrest of cell proliferation.

(A) Representative 20µm Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR+ cell (downward pointing arrows) in the DG. Arrowheads indicate GR immunoreactivity in cellular processes extending from the cell body in Nestin-GFP+/GFAP+/GR+ cell. (B) Representative 20µm Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP-/GR+ cell (downward pointing arrowhead) in the DG. (C) Representative micrographs of NSPC in vitro with examples of nuclearGR+/Ki67- NSPC (upward pointing arrow), nuclearGR+/Ki67+ NSPC (downward pointing arrowheads). (D) Bar graph depicting relative abundances of GR+ NSPC (red bars) and GR- NSPC (blue bars) both in vivo and in vitro.
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Based on this, we decided to study the cell cycle, enter G0 phase and re-enter adult stem cells is that quiescent cells exit exposure to GC. The current view of quiescence in mammalian conditions NSPC sensitivity to a subsequent GC oscillations affect NSPC quiescence and incubation with 10^-6 M CORT (Figure 1L), we found that it was maximal after 72h of was significant already after 48h incubation, we found that it was maximal after 72h of incubation with 10^-6 M CORT (Figure 1L), in line with previous observations made on human embryonic NSPCs. Strikingly, the CORT-induced inhibition of proliferation after 72h incubation was completely recovered after a subsequent 24h of incubation in CORT-free conditions (Figure 1L). These observations demonstrate that CORT can produce a reversible inhibition of cell proliferation compatible with the induction of a quiescent cell state in NSPC.

GC oscillations affect NSPC quiescence and condition NSPC sensitivity to a subsequent exposure to GC.

The current view of quiescence in mammalian adult stem cells is that quiescent cells exit the cell cycle, enter G0 phase and re-enter the cell cycle in response to physiological stimuli. Based on this, we decided to study the effect of CORT using flow cytometric analysis of cell cycle with propidium iodide DNA staining on NSPC.

We compared the effect of NSPC incubation with a constant CORT regimen, as frequently used in literature or with a more naturalistic regimen reflecting the in vitro CORT pulsatility (Figure 2A and 2B). The effectiveness of the two incubation regimens was verified by radioimmunoassay determination of CORT levels in NSPC culture medium collected every 30min during the first 24h (Figure 2C). Both NSPC cultures treated for 72h with 10^-6 M CORT pulsatile or constant regimens showed significant increases in the percentage of cells in the G0/G1 phase as compared to vehicle treatment (p < 0.01 and p < 0.001, respectively, with concomitant reductions in the proportion of cells in other phases of the cell cycle; Figure 2D). However, exposure to constant CORT induced a significantly stronger arrest in the G0/G1 cell cycle phase (58.15±1.35 vs. 78.54±1.34%, p < 0.001 pulsed and constant respectively; Figure 2D), suggesting that CORT pulsatility is an important factor controlling cell cycle progression in NSPC.

In agreement with CORT effects on proliferation measured by Ki67 expression, CORT-mediated effects on cell cycle progression were largely reversible and no significant differences in cell cycle progression were found among experimental groups, other than a marginal but significant difference in the proportion of cells in G2/M phase, after 24h incubation in CORT-free medium (10.68±0.63 vs. 8.67±0.44%, p < 0.05 vehicle and constant respectively; Figure 2E). Then, we studied whether the quiescent cellular states induced by different CORT
Figure 2 - CORT oscillations differentially induce a reversible cell cycle arrest and condition the responsiveness of NSPC to subsequent CORT exposure. 
(A) Schematic time-line depiction of the treatment incubation regimens used to incubate NSPCs. (B) Theoretical depiction of the CORT treatment incubation regimens of 24h. (C) Graph displaying the validation of changes in CORT concentration in the culture medium imposed by the pulsatile (red line and triangles) or constant (blue line and squares) treatment. Radio immuno assay determination of CORT levels in the culture medium during the first 24h of both treatments modeling endogenous CORT oscillations and efficient hormone wash-out during inter-peak periods. (D) Pie-charts showing the effect of 72h pulsatile (pulsed) or constant (constant) treatment with 10^{-6} M CORT on cell cycle progression in NSPC. (E) Pie-charts showing the effect of 72h pulsatile (pulsed + recovery) or constant (constant + recovery) 10^{-6} M CORT treatment with a 24h washout on cell cycle progression in NSPC. (F) Pie-charts showing 72h pulsatile (pulsed + recovery + pulse) or constant (constant + recovery + pulse) 10^{-6} M CORT treatment with a 24h removal of CORT followed by a subsequent 6h 10^{-6} M CORT pulse on cell cycle progression in NSPC.
regimens were truly undistinguishable at the molecular level. To test this, we exposed NSPCs previously incubated with pulsatile or constant CORT regimens to a further exposure to CORT. To do this, NSPCs were incubated with 10^{-6} M CORT for 6h, an experimental condition commonly used to induce GR-dependent gene expression^{39}. Interestingly, we found significant differences between cells previously exposed to pulsatile or constant regimens (Figure 2F). Specifically, significantly more NSPCs were found in G0/G1-phase in the pulsed incubation group compared to those that initially received constant CORT (76.41±1.38 vs. 66.83±1.18%, p < 0.05 pulsed and constant respectively), with concomitant changes in the proportion of cells in the S-phase (Figure 2F). Importantly, we did not detect significant levels of cells with single cell DNA content <2N, thus discarding a possible CORT-induced apoptosis in NSPCs in the conditions tested (Figure 2D-F). These results suggest that although NSPC incubation with both CORT regimens induced a reversible inhibition of proliferation in NSPCs, the lasting consequences of both treatments differ. Specifically, NSPC initially exposed to constant CORT were desensitized to CORT-induced cell cycle arrest as evaluated by the proportion of cells in G0/G1 phase.

GC oscillations differentially condition gene expression after GC removal in NSPC.

In a first effort to characterize a possible programming effect exerted by GC oscillations on cell cycle progression in NSPCs, we focused on changes in gene expression and DNA methylation. For this, we analyzed the expression of groups of genes previously linked to GR activation and DNA methylation after 72h incubation with pulsatile or constant CORT^{40-42}. mRNA levels of the GR and the well-characterized GR-responsive genes GILZ and SGK1 were significantly increased by the constant CORT regimen compared to vehicle treatment, while SGK1 was the only significantly upregulated gene by pulsatile CORT treatment (Figure 3A). Interestingly, the expression of the GR-decoy long non-coding RNA GAS5^{42} was significantly decreased by both pulsatile CORT treatment and constant CORT compared to vehicle, suggesting the possible activation of a feedback-loop involving GAS5 in NSPC exposed to constant CORT (Figure 3A). Conversely, GR mRNA targeting mat-miR-124-3p^{41} and mat-miR-124-5p were significantly more induced by pulsatile CORT. Further, the expression of DNMT1, 3a and 3b enzymes involved in DNA methylation in NSPC^{43}, were all significantly decreased by both CORT regimens. The mRNA levels of DNMT3a, however, were more strongly down-regulated by constant CORT regimen compared to pulsatile CORT, while the levels of DNMT1 and DNMT3b were not further decreased (Figure 3A), suggesting that different CORT regimens can have differential effects on the expression of DNA methylation enzymes, and thus DNA methylation in NSPC.

Next, we analyzed the expression of the same groups of genes 24 h after CORT withdrawal, to understand the molecular changes that may have resulted in the differential desensitization to CORT-induced cell cycle arrest by incubation with constant CORT, modeling a disruption in GC oscillations. Strikingly, while CORT regimens initially induced relatively small differences in GR mRNA expression, we found a significant decrease in GR expression in NSPC initially treated with constant CORT compared to pulsatile CORT after a subsequent 24h incubation in CORT-free medium (Figure 3B). SGK1 expression was differentially affected by the initial CORT incubation. While SGK1 was decreased in NSPCs initially treated with pulsatile CORT, it was significantly increased in NSPC initially treated with constant CORT. On the other hand, GILZ and GAS5 were both significantly decreased by both CORT treatments. Conversely, both CORT treatments significantly increased mat-miR-124-3p and -5p levels, with constant CORT
Figure 3 - CORT oscillations differentially induce changes in gene expression and condition the transcriptional response of NSPC to subsequent CORT exposure.

(A) Bar graphs depicting relative (to pulsed veh; white bars) changes in the expression of genes involved in GC response and genes involved in epigenetic regulation, induced by 72h 10^{-6} M CORT pulsatile (gray bars, pulsed CORT) or constant (black bars, constant CORT) treatments. (B) Bar graphs depicting relative (to pulsed veh + recovery; white bars) changes in the expression of genes involved in GC response and genes involved in epigenetic regulation, induced by 72h 10^{-6} M CORT pulsatile or constant treatments followed by 24h hormone washout (gray bars, pulsed CORT + recovery and black bars, constant CORT + recovery, respectively). (C) Bar graphs depicting relative (to pulsed veh + recovery + pulse; white bars) changes in the expression of genes involved in GC response and genes involved in epigenetic regulation, induced by 72h 10^{-6} M CORT pulsatile or constant treatments followed by 24h hormone washout and subsequent treatment with 10^{-6} M CORT for 6h (gray bars, pulsed CORT + recovery + pulse and black bars, constant CORT + recovery + pulse, respectively). Data are expressed as mean normalized fold change (relative to pulsed veh) ± SEM of three independent experiments performed in triplicates. Statistical analyses were done per time-point using one-way analysis of variance test with Tukey’s post hoc test for multiple comparisons (*p < 0.05, **p < 0.01 and ***p < 0.001 relative to pulsed vehicle; #p < 0.05, ##p < 0.01 and ###p < 0.001 relative to pulsed CORT).
showing significantly further increases. Interestingly, the DNMTs showed a more consistent pattern. DNMT 1, 3a and 3b expression levels were decreased in NSPCs initially exposed to pulsatile CORT, while they were significantly increased in cells initially treated with constant CORT (Figure 3B), suggesting strong differences in DNA methylation induced by different CORT treatments.

Interestingly, a further exposure to CORT for 6h induced a significant reduction in GILZ and SGK1, and a significant increase in GAS5 expression only in cells initially exposed to constant CORT (Figure 3C), while GR expression was significantly reduced in NSPC, irrespective of the initial treatment. A further CORT exposure also differentially affected mat-miR-124-3p and -5p expression levels, and both increased only in cells initially treated with constant CORT. Again, DNMTs showed a more consistent pattern. DNMT 1, 3a and 3b were downregulated by CORT in NSPCs, irrespective of the initial treatment (Figure 3C). These results indicate that gene expression was differentially affected by the initial CORT regimen and that the differences lasted for at least 24h, sufficient time for NSPC to undergo at least one cell division.

GCs induce global and promoter specific changes in DNA methylation in NSPC. Our previous observations suggested that the initial CORT regimen might have a programming effect on NSPC by differentially affecting DNA methylation. To study this, we specifically focused on changes in cytosine methylation (5m-C). We found that both pulsatile and constant CORT regimens induced significant reductions in global cytosine methylation as well as specific cytosine methylation levels in protein-coding gene promoters (Figure 4A and 4B).

As methylation of promoter and other chromosomal regions may be differentially regulated, we focused on promoter methylation. To further address whether the pulsatile and constant CORT induced relative promoter hypomethylation (Figure 4B) was composed of more promoters being hypomethylated or the same promoters being more hypomethylated we next performed frequency analysis. Frequency analysis of promoter hypomethylation revealed that the largest differences in promoter methylation between the initial CORT treatments were present in the 20-40% differential methylation category (pulsatile 9562 vs. constant 11531 methylated promoters), with the second largest differences (pulsatile 2433 vs. constant 3492 differentially methylated promoters) present in the 0-20% category (Figure 4C).

Further, unsupervised hierarchical clustering analysis of all differentially methylated promoters between the two CORT treatments (Figure 4D) revealed that 73% of them were hypomethylated by constant CORT compared to pulsed CORT (3492 hypomethylated promoters out of 4767 total differentially methylated promoters, cut off value MBD read density ≥3; Figure 4C and D). A list of percentages of mapped reads corresponding to this dataset is displayed in Table 1. To understand the possible biological meaning of the broad promoter hypomethylation effect induced by constant CORT regimens, we performed GO analysis on the genes with promoters differentially hypomethylated after constant CORT treatment and found that the most significantly

<table>
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<th>Sample</th>
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<th>Total PE reads</th>
<th>Mapping %</th>
<th>Mapped PE reads</th>
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<td>12,026,629</td>
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<tr>
<td>Pulsed CORT + recovery</td>
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<td>20,889,878</td>
<td>48.63</td>
<td>10,158,748</td>
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<tr>
<td>Constant CORT + recovery</td>
<td>37,034,674</td>
<td>18,517,337</td>
<td>46.23</td>
<td>8,560,565</td>
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</tbody>
</table>

PE: paired ends.
overrepresented biological processes (BPs) within these promoters (constant vs. pulsed; FDR corrected p < 0.05) included regulation of transcription (6 BPs), metabolic processes, development, differentiation and phosphorylation, (Figure 4E). Regarding the 1275 promoters that were hypermethylated, by constant compared to pulsed CORT treatment, GO analysis identified that the most significantly overrepresented BPs included regulation of transcription (5 BPs), transport (2BPs), development, phosphorylation, and cell adhesion (FDR corrected p < 0.05, Figure 4F), in striking similarity with the BPs overrepresented within the hypomethylated gene promoters (Figure 4E). Underscoring the biological relevance of this convergence in BPs to our observations regarding transient inhibition of proliferation and cell cycle progression (Figure 1 and 2), constant CORT treatment was associated with hypomethylation of 55 promoters and hypermethylation of 15 promoters of genes associated with cell cycle regulation (Figure 4E and 4F). Overall, these results suggest that the two distinct CORT treatments induced strong differences in global and promoter-specific DNA methylation. Moreover, the broad promoter hypomethylation associated with the constant CORT treatment was also found at specific previously characterized gene promoters (Figure S3) and exemplify that GC oscillations may play a role in regulating promoter methylation states associated with regulation of the cell cycle and differentiation in NSPC.

Deviations from basal pulsatile GC oscillations induce transient and long-lasting changes in promoter methylation in NSPC.

NSPCs in our primary culture system have an average replication time of 16h and therefore cells had gone at least through one cell division within the 24h recovery after 72h CORT exposure. Accordingly, DNA methylation changes preserved after 24h have been preserved through cell division and therefore conform with the strictest definition of "epigenetic". We found that after 24h CORT withdrawal global levels of 5mC remained reduced in NSPC treated with pulsed CORT, while 5mC levels in NSPC treated with constant CORT were not significantly different from levels found in vehicle-treated cells (Figure 4A). In terms of promoter methylation, the same pattern was observed (Figure 4B). The proportion of differentially hypomethylated promoters in NSPC treated with pulsed CORT remained reduced after 24h of...
CORT withdrawal, while the percentage of promoter methylation in cells treated with constant CORT increased relative to from constant CORT-treated cells (Figure 4B). Furthermore, frequency analysis revealed that this promoter percentage of constant CORT + 24h hormone withdrawal was composed of both less promoters being hypomethylated and more promoters being hypermethylated compared to constant CORT (Figure 4C).

Unsupervised hierarchical clustering with optimized gene leaf order analysis of the differentially methylated promoters between the two experimental groups, 72h initial treatment with pulsed CORT followed by 24h withdrawal (Pulsed + recovery) and 72h initial treatment with constant CORT followed by 24h withdrawal (Constant + recovery), revealed that 18% of the promoters remained in the same differential methylation state 24h after CORT withdrawal (Figure 4D, pink+blue bars vs. green+red bars, 845 stable vs. total 4767 promoters, respectively). Further analysis of these constant CORT induced stably methylated promoters, revealed cluster of hypomethylated promoters (214 promoters; Figure 4D, pink bars) and a larger cluster of stably hypermethylated promoters (631 promoters; Figure 4D, blue bars).

In a larger context, Gene Ontology Enrichment (GO) analysis of the 214 stably hypomethylated promoters revealed the most significantly overrepresented BPs (more than 3 promoters per BP, hypergeometric FDR corrected p < 0.05), included development, transport (4 BPs) or carbohydrate metabolism (Figure 5E). GeneMANIA pathway analysis of the top three overrepresented BPs among the stable hypermethylated gene promoters revealed that networks of genes involved in the Wnt signaling (network node: DKK3; Figure 5F), metal ion transmembrane transporter (network node: Slc24a4; Figure 5G) and organic anion transport (network node: Slc7a5; Figure 5H) pathways were significantly affected (FDR corrected p < 0.05).

In agreement with this last observation, the mRNA of CCND1, a cell cycle activator and target of the Wnt signaling pathway14 was downregulated by 72h treatment with either pulsatile or constant CORT (Figure 6A). However, 24h after CORT withdrawal CCND1 levels remained downregulated in NSPC initially treated with pulsatile CORT, while CCND1 was significantly upregulated in NSPC initially treated with constant CORT (Figure 6B). These alterations in CCND1 gene expression were not mediated through differential methylation of the CCND1 gene promoter (Figure 6D). These dissimilar CCND1 regulation induced by the two CORT treatments correlate with the stable hypermethylation of Wnt inhibitor DKK3 and desensitization to CORT induced cell cycle arrest induced by constant CORT and may thus provide a possible mechanism to understand the long lasting effects of alterations in GC oscillations on NSPCs.

Discussion

Most studies aiming to understand the effects of GCs on NSPC proliferation have used long-term stimulation with constant levels of natural or synthetic GR ligands. As GC are released from the adrenal gland in circadian and high frequency ultradian oscillatory patterns, we compared the effects of exposing NSPCs to two treatment modes mimicking constant or pulsatile GC exposure and investigated their consequences on NSPC proliferation, cell cycle progression, gene expression and in DNA methylation in vitro. Further, we asked whether these two treatment modes had consequences for the sensitivity of NSPCs to a subsequent exposure to GCs, thereby
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Figure 5 - CORT oscillations differentially induce stable methylation of gene promoters from several functional pathways including Wnt signalling.

(A) GO analysis of constant 10^{-6} M CORT induced stable hypermethylated promoters (MBD2 read density difference between constant and pulsatile ≥ 3). BPs were identified with Genocodis modular enrichment GO analysis of top and bar graphs show the number of annotated members per BP (red) and their hypergeometric FDR corrected p-values (blue). (B) GO analysis of constant 10^{-6} M CORT induced stable hypomethylated promoters (MBD2 read density difference between constant and pulsatile 10^{-6} M CORT ≤ 3). BPs were identified with Genocodis modular enrichment GO analysis of top and bar graphs show the number of annotated members per BP (red) and their hypergeometric FDR corrected p-values (blue). (C) Pathway analysis of constant 10^{-6} M CORT induced highest overrepresented BP of stable hypermethylated promoters (Figure 4D). Genenmania identified cell fate commitment (red spheres; FDR corrected \( p = 6.39 \times 10^{-4} \)) and canonical Wnt signaling pathway (orange spheres; FDR corrected \( p = 2.43 \times 10^{-2} \)) as significantly overrepresented functional networks. (D) Pathway analysis of constant 10^{-6} M CORT induced second highest overrepresented BP of stable hypermethylated promoters (Figure 4D). Genenmania identified metal ion transmembrane transporter activity (orange spheres; FDR corrected \( p = 1.67 \times 10^{-4} \)) and anion transmembrane transporter activity (blue spheres; FDR corrected \( p = 1.17 \times 10^{-4} \)) as significantly overrepresented functional networks.
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(E) Pathway analysis of constant 10^-6 M CORT induced third highest overrepresented BP of stable hypermethylated promoters (Figure 4D). Genemania identified organic anion transport (blue spheres; FDR corrected p = 2.63*10^-4) and inorganic cation transmembrane transporter activity (red spheres; FDR corrected p = 4.51*10^-5) as significantly overrepresented functional networks. (F) Pathway analysis of constant 10^-6 M CORT induced highest overrepresented BP of stable hypomethylated promoters (Figure 4D). Genemania identified embryonic organ morphogenesis (red spheres; FDR corrected p = 1.26*10^-4) and stem cell differentiation (orange spheres; FDR corrected p = 4.51*10^-5) as significantly overrepresented functional networks. (G) Pathway analysis of constant 10^-6 M CORT induced second highest overrepresented BP of stable hypomethylated promoters (Figure 4D). Genemania identified inorganic anion transport (red spheres; FDR corrected p = 1.27*10^-2) as significantly overrepresented functional networks. (H) Pathway analysis of constant 10^-6 M CORT induced third highest overrepresented BP of stable hypomethylated promoters (Figure 4D). Genemania identified negative regulation of cell activation (red spheres; FDR corrected p = 6.25*10^-2) and hindbrain development (blue spheres; FDR corrected p = 6.25*10^-2) as significantly overrepresented functional networks.
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Indeed, alterations in HPA axis activity generally correlate with the severity of mood disorder symptoms, e.g. in a large part of the patients that suffer from major depressive disorder. Also, a “flattening” of the pulsatile nature of GC release is commonly observed in MDD, while in Cushing patients, their chronic GC hypersecretion correlates well with their depressive symptoms. Interestingly, one of the rate-limiting steps in neurogenesis, i.e. NSPC proliferation, is regulated by antidepressant treatment and notably requires pulsatility of GCs.

The pharmacological effects of fluoxetine on NSPC proliferation occur through the GR, suggesting a delicate interplay between adult neurogenesis, rhythmic changes in GCs and the GR in terminating aberrant GC regulation.

Garcia et al. have found GR expression in different subsets of NSPC in vivo, indicating that CORT can have a direct effect on NSPCs. Corroborating these findings, our observations indicate that both quiescent (Ki67-) and actively proliferating (Ki67+) NSPCs express the GR both in vivo and in vitro. As previously described, CORT does not only have an anti-mitogenic effect on NSPC, but under

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**Figure 6** - CORT oscillations stably affect Wnt signaling target CCND1 gene expression.

(A) Bar graphs depicting relative (to pulsed veh; white bars) changes in the expression of CCND1, induced by 72h $10^{-6}$ M CORT pulsatile (gray bars, pulsed CORT) or constant (black bars, constant CORT) treatments.

(B) Bar graphs depicting relative (to pulsed veh + recovery; white bars) changes in the expression of CCND1, induced by 72h $10^{-6}$ M CORT pulsatile or constant treatments followed by 24h hormone washout (gray bars, pulsed CORT + recovery) vs. constant CORT + recovery, respectively.

(C) Bar graphs depicting relative (to pulsed veh + recovery + pulse; white bars) changes in the expression of CCND1, induced by 72h $10^{-6}$ M CORT pulsatile or constant treatments followed by 24h hormone washout and subsequent treatment with $10^{-6}$ M CORT for 6h (gray bars, pulsed CORT + recovery + pulse and black bars, constant CORT + recovery + pulse, respectively).

(D) Bp-scale read density map of the CCND1 gene promoter. (A-C) Data are expressed as mean normalized fold change (relative to pulsed veh) ± SEM of three independent experiments performed in triplicates. Statistical analyses were done per time-point using one-way analysis of variance test with Tukey’s post hoc test for multiple comparisons (* p < 0.05, ** p < 0.01 and *** p < 0.001 relative to pulsed vehicle; # p < 0.05, ## p < 0.01 and ### p < 0.001 relative to pulsed CORT).
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non-stressed conditions it can also inhibit various other NSPC phenotypes, including differentiation and apoptosis24. Although these effects are largely mediated through the GR, prolonged exposure of NSPCs to high concentrations of dexamethasone may induce apoptosis65, highlighting GR-ligand dependent differences. Moreover, the physiological GR-ligand action has been shown to be fundamentally different between naturally occurring GCs and synthetic GCs, especially under oscillatory administration6. Accordingly, we chose to use a more accurate assessment of physiological CORT action by comparing pulsatile CORT as a basal condition, to constant CORT.

Prolonged exposure to constant dexamethasone has been found to induce senescence related changes in NSPCs, as characterized e.g. by an irreversible inhibition of proliferation that is accompanied by a long-lasting reduction in DNMTs and global DNA methylation26. Our results, on the contrary, indicate that the effects of constant CORT, on proliferation and DNMT3a induced (gene promoter) DNA hypomethylation are generally reversible when compared to pulsatile CORT. Interestingly, while cell cycle re-entry would normally further dilute the amount of DNA methylation at gene promoters, we found indications of active DNA re-methylation, possibly through increased DNMT1 expression levels. This active DNA re-methylation appeared to be a coordinated NSPC adaptation since sets of promoters involved in stem cell differentiation remained hypomethylated. Conversely, constant CORT also induced lasting DNA hypermethylation of a number of gene promoters involved in cell fate commitment, canonical Wnt signaling and transport. This could imply that the progeny of NSPCs exposed to an attenuated CORT rhythm might display alterations beyond the proliferative stage of AHN. Indeed, we have previously reported that knockdown of GR expression in NSPC and thus eliminating their ability to detect basal pulsatile CORT rhythms, accelerated the aberrant differentiation of their progeny24.

DNA demethylation has been implicated in derepression of silenced gene promoters during embryonic development66. However, the impact of DNA demethylation on gene transcription is largely unknown. Interestingly, transient (de)methylation of target promoters may be linked to nuclear receptor-induced gene transcription67 and may be mediated by DNMTs68. In particular, GC regulate DNA methylation within key enhancers of well-characterized GC-responsive gene, such as the liver-specific tyrosine aminotransferase (Tat) gene, during embryonic development69. In this particular example, GC-induced demethylation occurs within 2-3 days after rapid GR-induced chromatin remodeling, which may suggest that DNA demethylation is part of the well-know chromatin remodeling function of the GR. However, Tat enhancer demethylation remained stable after hormone withdrawal69, similar to the coordinated GC-induced effects we describe here for thousands of loci in hundreds of protein-coding gene promoters. As many of the hyp or hypermethylated were present in daughter cells, which have never been exposed to GC themselves, the GC-induced changes in methylation may provide a cellular memory of the first exposure, and its modality (pulsed vs. constant) that is preserved throughout cell division. Finally, it is important to bear in mind that overall, the changes in DNA methylation induced by both GC modalities induced global hypomethylation, with hypermethylation in some specific gene promoters. Interestingly, global hypomethylation accompanied by gene promoter-specific hypermethylation is associated with aging60.

A number of studies have described cell intrinsic characterizations of the quiescent NSPCs and identified several signaling pathway genes such as e.g. REST/coREST70, EGFR70, Notch/Hes571 and Wnt/β-catenin72. These observations suggest that many cell extrinsic factors can play a role in maintaining (or inducing) NSPC quiescence. Indeed, quiescence can be defined as a heterogeneous phenotype in terms of diversity of anti-mitogenic signals and concomitant molecular programs rather than a homogenous state of reversible arrest in proliferation73.

Suppression of differentiation is linked to multiple quiescence-inducing anti-mitogenic stimuli73. Apart from the functional pathways that displayed a coordinated adaptation to the recovery from constant CORT exposure, such as Wnt signaling, we found that transient constant CORT also induced changes in DNA methylation levels of FGFR2, REST and coREST, all indicative of the induction
of a quiescent NSPC phenotype\textsuperscript{16,70}. These data thus indicate that constant CORT, but not pulsatile CORT, can induce a NSPC quiescence program through alterations in levels of DNA methylation. Interestingly, approximately 4767 altered gene promoters were changed, a number that exceeds the predicted ±700 genomic loci containing GR response elements (GREs), and is indicative of a coordinated quiescence program by constant CORT\textsuperscript{74}. Although speculative at this point, this could imply that the constant CORT induced latency of the GR to release the chromatin\textsuperscript{4}, allowing the tethering of the GR to non-GRE containing loci\textsuperscript{74} and thereby remodeling a large number of loci\textsuperscript{75}, would also allow access to DNA methylation related genes to alter promoter methylation levels. Future studies are needed to assess whether these alterations in DNA methylation are "guided" solely by GRs directly, or through indirect mediators such as downstream targets of the GR.

It has previously been shown that the hippocampus responds to the discrete pulsatile nature of the ultradian CORT rhythm\textsuperscript{76}, and that disruption of this pulsatility attenuates gene responsiveness to CORT\textsuperscript{7}. As previously suggested\textsuperscript{4,77}, we provide further data indicating ultradian CORT pulsatility encodes a distinct and biologically important signal, that is markedly different from a constant exposure. One example is the marginal increase in NSPCs in G0 when treated with pulsatile CORT compared to vehicle. When exposed to a second episodic of CORT, pulsatile treated NSPCs acutely went into G0 arrest while previously vehicle and constant CORT treated NSPCs did not. This supports the notion that CORT pulsatility may provide a means for cells to respond more acutely to stress\textsuperscript{78}. In addition, we here provide new insights indicating that the pulsatile nature of ultradian CORT oscillations also translates into a distinct NSPC DNA methylation pattern that is attenuated by constant CORT exposure. Whether the CORT oscillations specifically change NSPC self-renewal and their epigenetic profile and whether that can contribute to alterations in a neuro-endocrine profile and/or stress/GC induced depressive like behavior remains to be tested. Yet by using the \textit{in vitro} system described herein, the direct effects of CORT oscillations on NSPC could also be studied in the absence of any indirect effects which would be present \textit{in vivo}.

Our results provide a preliminary understanding of the epigenetic changes accumulating in NSPCs upon exposure to changed CORT rhythms, and during inhibition of self-renewal. Specifically, an initial episode during which GC release patterns are changed can transiently decrease NSPC proliferation. Yet, a second episode of CORT exposure revealed NSPCs have acquired a desensitization to CORT in terms of proliferation. Whether there is a causal relationship between the lasting changes in DNA methylation and proliferation remains to be addressed experimentally. We did, however, find constant CORT to induce hypermethylation of some promoters of the Dickkopf family, Wnt signaling pathway inhibitors. Interestingly, the Wnt signaling is one key pathway involved in NSPC self-renewal\textsuperscript{72} through the induction of cell cycle activator CCND1\textsuperscript{14}. Moreover, a recent study described Dickkopf1 to be involved in the age-related decrease in neurogenesis, through inhibition of the Wnt signaling pathway\textsuperscript{79}. Interestingly, we found CORT rhythms to exert a dual role in the regulation of CCND1, with a more direct repression upon CORT exposure yet an induction upon removal of the hormone, possibly indirectly through hypermethylation of Wnt signaling inhibitor Dickkopf3. Taken together, these data suggest a mechanistic link between CORT rhythmicity, lasting attenuated methylation of gene promoters and the cell cycle of NSPCs and contribute to the understanding of GC-related alterations in structural plasticity of the brain.

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Supplementary information
Chapter 5 - Supplementary information

Figure S1 - DNA quality control measurements prior to DNA fragmentation.
DNA concentration is determined with smear analysis and checked for degradation for vehicle (A), pulsed (B), constant (C), pulsed + recovery (D) and constant + recovery (E).
Figure S2 - DNA quality control measurements after DNA fragmentation.
Quality assessment was based on data passing the Illumina Chastity filter. Subsequently, reads containing adaptors and/or Phix control signal were removed. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0. The final quality scores for each base are shown for vehicle (A), pulsed (B), constant (C), pulsed + recovery (D) and constant + recovery (E).
Figure S3 - CORT oscillations differentially induce stable gene promoter methylation. 
(A) Bp-scale read density map (top) and corresponding read density quantification (bottom) of the FGFR2 gene promoter. (B) Bp-scale read density map (top) and corresponding read density quantification (bottom) of the EGFR gene promoter. (C) Bp-scale read density map (top) and corresponding read density quantification (bottom) of the REST gene promoter. (D) Bp-scale read density map (top) and corresponding read density quantification (bottom) of the coREST2 (RCOR2) gene promoter.