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Glucocorticoid Hormones Preserve a Population of Adult Hippocampal Neural Stem Cells in the Aging Brain

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\textit{In revision, Cell Stem Cell}
Chapter 5

Abstract

Adult hippocampal neurogenesis declines with aging. This decrease has been implicated in age-related cognitive impairments and may result from a division-coupled depletion of a selective pool of neural stem/precursor cells (NSPC), however, the mechanisms involved remain poorly characterized. Aging is further associated with changes in circulating glucocorticoid hormones (GCs). GCs cyclically activate the glucocorticoid receptor (GR) and are released from the adrenal glands in ultradian near-hourly secretory pulses that result in circadian GC oscillations. Here, we investigated the hypothesis that GC oscillations prevent NSPC activation and are important to preserve a NSPC pool in the ageing hippocampus. We found that NSPC populations lacking GR expression decayed exponentially with age, while GR-positive populations decayed linearly and predominated in the hippocampus from middle age onwards. Strikingly, GR knockdown reactivated NSPC proliferation in aged mice. Interestingly, both in vivo and in primary NSPC cultures, GC oscillations controlled NSPC quiescence and cell cycle progression. In vivo, we show that GC oscillations induced specific genome-wide DNA methylation profiles. Some of the methylation changes induced by GC oscillations were preserved across NSPC generations in a group of gene promoters associated with the canonical Wnt-signaling pathway. Our results indicate that GC oscillations preserve a subpopulation of GR-expressing NSPC during aging, preventing their activation, possibly by epigenetic programming of the canonical Wnt-signaling by DNA methylation in NSPCs. Together, our observations reveal a novel mechanism controlling NSPC proliferation and indicate that GC oscillations preserve a dormant NSPC pool in the aging brain.

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Introduction

Neural stem/precursor cells (NSPC) in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) continue to generate new neurons in the adult hippocampus across the lifespan of mammals, including humans. Newborn neurons generated by AHN are important for specific hippocampus-dependent memory tasks, anxiety, and behavior, and reduced AHN may contribute to an age-associated cognitive decline. Proliferation of NSPCs is a limiting factor for adult hippocampal neurogenesis (AHN). A strong aging-associated decrease in NSPC proliferation has been consistently reported, indicating that age is one of the most important factors contributing to decrease in neurogenesis in the normal brain.

Adult NSPCs may be limited to a maximum number of divisions, and NSPC quiescence could thus contribute to life-long maintenance of a NSPC pool. Previous observations have suggested a model in which cell proliferation is coupled to depletion of the hippocampus NSPC pool, which may be one of the underlying causes of the age-associated decline in AHN. However, the putative extinction of the hippocampal NSPC pool remains controversial and subject to debate as essential information, especially regarding the molecular mechanisms involved, is still scarce.

NSPCs dynamically and selectively respond to aging and several other physiological and pathological stimuli, including the glucocorticoid hormones (GC), which strongly inhibit NSPC proliferation. In mice, GCs acting through the glucocorticoid receptor (GR) have direct effects on NSPC, controlling their differentiation and functional integration within neuronal circuits. In old rats, adrenalectomy increased NSPC proliferation in the hippocampus, whereas lifelong GC reduction to low constant levels increased AHN and prevented the emergence of age-related memory disorders. The effects of lifelong GC suppression are age-dependent, as GC suppression from early life onwards does not enhance AHN. Importantly, in young adult mice, populations of quiescent NSPC heterogeneously express the GR and differentially respond to GC stimulation. However, the relationship between GCs, NSPC proliferation, and AHN is complex and remains incompletely characterized. Therefore the mechanisms by which GCs regulate NSPC proliferation across age require further characterization.

GCs are released from the adrenal glands in ultradian, near-hourly pulses. Their amplitude gradually increases from the end of the inactive phase of the circadian activity cycle, peaking around the middle of the active phase, to finally reach a trough around the beginning of the inactive phase. This rhythmic release pattern generates circadian oscillations in circulating GC. GC oscillations develop after the third week of life in mice and alterations in GC oscillations are observed in aged mammals, including mice and humans. GC oscillations promote and maintain learning-dependent structural plasticity in the cerebral cortex, but their effects on other forms of structural plasticity, e.g., AHN are unknown. Here, we show for the first time that GC oscillations are associated with the preservation of GR-expressing NSPC populations, revealing a novel mechanism that controls NSPC proliferation in the aging brain. Using hippocampal NSPC cultures, we show that GC oscillations carry qualitatively distinct information controlling NSPC proliferation and expression of members of the Wnt-signaling, possibly by epigenetic mechanisms involving DNA methylation.
Chapter 5

Results

NPSC populations decay with different kinetics during aging

We quantified the expression of NSPCs in the DG of Nestin-GFP mice. As previously observed in this model, Nestin-GFP+ NSPCs were readily detectable in the granular zone (GZ) of the DG of animals from 3 up to 18 months of age (Figure 1A-C and G-K). Based on the expression of Nestin-GFP and GFAP, NSPCs were classified in different subtypes, using a modified version of previously established developmental milestones. Nestin-GFP+/GFAP+ radial glia-like Type-1 cells were detected based on their radial morphology and distinguished from Type-2 cells, which present a horizontal morphology. Based on their GFAP expression, Type-2 cells were classified as Type-2a (Nestin-GFP+/GFAP+) or Type-2b (Nestin-GFP+/GFAP-) cells (Figure 1C-F). Type-1, -2a and -2b cells were observed in animals of all ages (Figure G-K).

Similar to previous reports, we observed a significant age-related decrease in total numbers of NSPC. Using extra-sum-of-squares F-testing for best-fit decay curves, we found that the total Nestin-GFP+ NSPC population decayed following exponential kinetics. Importantly, the GZ volume did not change significantly with age (Figure 1L). Interestingly, Type-1, -2a and -2b cells decayed following different patterns. Both Type-1 and -2a cells followed linear, while Type-2b cells followed exponential decay kinetics (Figure 1M). These data demonstrate that significant numbers of Type-1 and -2a NSPC persist into old age, while Type-2b cells are rapidly depleted from the initial NSPC pool.

GR+ NSPC populations persist into old age and decay with different kinetics

Previous studies have shown heterogeneous GR expression in NSPC populations in young animals (Figure 2A-L, Figure S1A). We here characterized GR expression in Type-1, -2a and -2b cells in mature adult, middle-aged and old Nestin-GFP mice (Figure 2A-Q”, Figure S1A) and we observed an age-related change in the relative abundances of GR+ and GR- populations of Type-1, -2a and -2b cells (Figure 2R). In 3 month-old mice, the majority of Type-1 and -2a cells were GR+, while most Type-2b cells were GR-. Notably, the proportion of GR+ cells was significantly increased in all NSPC populations already in 6-months old mice. This predominance of GR+ NSPC populations persisted throughout middle age and into old age (Figure 2R). Thus, the depletion of GR- populations takes place earlier than anticipated from previous studies. Extra-sum-of-squares F-testing showed that the decay of GR+ Type-1, 2a and 2b cell populations fitted best to a linear model. Conversely, the GR- populations fitted best to an exponential decay with age (Figure 2S-U, Figure S1B-G). These data show that GR expression in NSPC populations is associated with linear decay kinetics and significantly longer $t_{1/2}$.

Age-associated increase in GC oscillation amplitude correlates with a predominance of GR+ NSPC populations during aging

Mouse GC (corticosterone; CORT) concentrations were measured in plasma samples collected at AM (08:00) and PM (20:00), representing the trough and peak of their circadian GC oscillations, respectively (Figure 2V). We found that AM GC levels were stable with age, while PM GC levels increased in all age groups compared to 3-month old mice, indicating an age-associated increase in circadian CORT amplitude, which stabilized at 6 months and older ages (Figure 2W). Interestingly, GR- NSPC populations rapidly decayed, coinciding with the increased circadian CORT amplitude characteristic
GCs preserve hippocampal NSCs in the aging brain

Chapter 5

Figure 1. Different NSPC subtypes decrease with age, but follow different kinetics. Nestin-GFP (green) and PCNA (red) immunoreactivity in the DG of (A) 3 or (B) 18 month-old mice. (C) Representative single Z-plane confocal micrograph showing examples of Nestin-GFP+/GFAP+ (Type-1 and Type-2a) and Nestin-GFP+/GFAP- (Type-2b) NSPC in the SGZ of the DG. (D-F) Fluorescent signal across typical GFP+/GFAP+ and GFP+/GFAP- cells in the SGZ. White lines in (C) show the direction of scanning, and the histograms (D-F) show the strength of the fluorescent signals for GFP (green), GFAP (red) and DNA (Hoechst, blue) across the scanned regions. GFAP signals were restricted to the cell periphery. Nestin-GFP (green) and GFAP (red) immunoreactivity in the DG of (G) 3; (H) 6; (I) 10; (J) 14 or (K) 18 month-old mice. Scale bars=50 (A and B), 10 (C) and 25 (G-K) µm. Best-fit curves and 95% confidence intervals of (L) GZ volume (squares) and total Nestin-GFP+ NSPC (circles) per mm3. GZ volume was not significantly different across ages (p > 0.05, F-test). Nestin-GFP+ NSPC fitted to an exponential decay curve (p<0.05, F-test, calculated $t_{1/2}=5.44$ months); (M) type-1 (circles), type-2a (triangles) and type-2b (squares) NSPC. Datapoints are mean±SEM (n=5, * p < 0.05, ** p < 0.01, *** p < 0.001, vs. 3 month-old mice, one-way ANOVA). Best-fit curves are mean±95% confidence intervals. Type-1 and type-2a NSPC fitted linear decay curves (p < 0.05, F-test, calculated $t_{1/2}=1.05$ months).
GCs preserve hippocampal NSCs in the aging brain

Figure 2 - The preservation of NSPC populations is associated with GR expression and age-related changes in the amplitude of circadian CORT oscillations. (A) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR+ NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC expressing the GR. Boxed area is magnified and Z-stacked channels split in (A') where the arrowhead indicates the cell soma or of a single merged Z-plane (A’’). Dashed black lines in (A’’) show the direction of scanning, and the histograms (B) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (C) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR+ NSPC with a characteristic rounded cell-body in the SGZ of the DG, indicative of a type-2a NSPC expressing the GR. Boxed area is magnified and Z-stacked channels split in (C’) where the arrowhead indicates the cell soma or of a single merged Z-plane (C’’). Dashed black lines in (C’’) show the direction of scanning, and the histograms (D) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (E) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR+ NSPC with a characteristic rounded cell-body in the SGZ of the DG, indicative of a type-2b NSPC expressing the GR. Boxed area is magnified and Z-stacked channels split in (E’) where the arrowhead indicates the cell soma or of a single merged Z-plane (E’’). Dashed black lines in (E’’) show the direction of scanning, and the histograms (F) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (G) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR- NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC not expressing the GR. Boxed area is magnified and Z-stacked channels split in (G’) where the arrow indicates the cell soma or of a single merged Z-plane in (G’’). Dashed black lines in (G’’) show the direction of scanning, and the histograms (H) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (I) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR- NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-2a NSPC not expressing the GR. Boxed area is magnified and Z-stacked channels split in (I’) where the arrow indicates the cell soma or of a single merged Z-plane in (I’’). Dashed black lines in (I’’) show the direction of scanning, and the histograms (J) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (K) Representative Z-stacked confocal micrograph showing a Nestin-GFP+/GFAP+/GR- NSPC (arrow) with a characteristic rounded cell-body in the SGZ of the DG, indicative of a type-2b NSPC not expressing the GR. Boxed area is magnified and Z-stacked channels split in (K’) where the arrow indicates the cell soma or of a single merged Z-plane in (K’’). Dashed black lines in (K’’) show the direction of scanning, and the histograms (L) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and GR (red) across the scanned region. (A-L) The GR grey value intensity threshold for being considered positive was ≥1500 across the nucleus. See Figure S1A for quantification of GR intensity background signal. Scale bars represent 20 µm (A, C, E, G, I and K), 15 µm (A’, C’, E’, G’, I’ and K’) and 10 µm (A”, C”, E”, G”, I” and K”). Nestin-GFP (green), GR (red) and GFAP (white) immunoreactivity in the DG of (M) 5, (N) 6, (O) 10, (P) 14 or (Q) 18 months-old mice. Insets correspond to boxed areas. Arrows: Nestin-GFP+/GR- type-1 NSPC, arrowheads: Nestin-GFP+/GR+ type-1 NSPC. Scale bars represent 40 µm (M-G”). (H) relative numbers of GFAP (full bars and full circles) or GR (dashed bars and open circles) type-1 (red), type-2a (green) or type-2b (blue) NSPC per DG. Bars are mean±SEM (n=5). Differences between GR+/GR- populations are indicated (* p < 0.05, ** p < 0.001) and time-points (** p < 0.05, *** p < 0.001) vs. 3 month-old, one-way ANOVA. Best-fit curves and 95% confidence intervals of (K) type-1/GR+ (solid circles) and type-1/GR- (open circles); (T) type-2a/GR+ (solid triangles) and type-2a/GR- (open triangles) or (U) type-2b/GR+ (solid diamonds) and type-2b/GR- (open diamonds). Data-points are mean±SEM (n=5), * p < 0.05, ** p < 0.01, *** p < 0.001, one-way ANOVA. All GR- populations fitted exponential decay curves (p < 0.05, F-test, calculated t1/2 = 2.2, 3.0 and 0.9 months for type-1, type-2a and 2b NSPC, respectively). All GR+ populations fitted linear decay curves (p < 0.05, F-test, calculated t1/2 = 28, 36 and 27 months for type-1, type-2a and 2b NSPC, respectively). (W) Time-windows of blood collection. (M) AM and PM plasma [CORT] vs. age in mice. Bars are mean of individual data-points (red circles)±SEM (n=5). * p < 0.05, ** p < 0.01, *** p < 0.001, vs. 3 month-old, one-way ANOVA. Calculated circadian CORT amplitude (black line) and (X) GR+ or (Y) GR- type-1 (red lines), type-2a (green lines) and type-2b (blue lines) NSPC population kinetics throughout adult life.
Chapter 5

of adulthood (Figure 2Y).

Conversely, in older mice, the decay kinetics of GR+ NSPC populations were not affected by the age-associated increase in CORT amplitude and these populations were preserved (Figure 2X, S1 and S2). These results, together with the GR expression profiles presented in Figure 2R-U, show that an increase in amplitude of GC circadian oscillations is associated with the preservation of GR+ NSPC populations in the GZ in the aging brain. To isolate the effects of GCs from the age/GC interaction we subsequently correlated GC amplitude to NSPC populations within the different age cohorts Figure S2B-F, S2H-L and S2N-R). This analysis, however, failed to separate the individual effects of age and GC amplitude, possibly due to a low group size within age cohorts (n=5). To further study the potential contribution of GCs to the age-associated NSPC decay kinetics, we sought to manipulate NSPC GC signaling in both young and old animals to identify the potential contribution of GCs to the age-associated NSPC decay kinetics.

GR activation in young mice induces NSPC to enter a reversible quiescent cellular state

GC oscillations can be abolished by subcutaneous implantation of slow-release CORT pellets, thereby exogenously inducing constant GC levels. Indeed, in agreement with previous observations in rats and mice, subcutaneous CORT implants suppressed circadian GC oscillations and proliferation (Figure 3A-I and Figure S3A). Interestingly, Ki67+ Type-1, -2a and 2b NSPC populations were present in 3 month old mice with intact oscillating GC levels, but were undetectable in mice of the same age with suppressed circadian GC oscillations (Figure 3J). These results support the hypothesis that circadian GC oscillations control the proliferation of NSPC in the GZ. Strikingly, the GC-induced inhibition of NSPC proliferation was reversed 2 days after removal of the exogenous source of the GCs (Figure 3J), when CORT levels were constant at basal levels (Figure 3I). Furthermore, the percentage of Type-1/Ki67+ cells was significantly increased, with more asymmetric cell division events (Figure 3K). These data indicate a dynamic response of NSPC proliferation, particularly Type-1 cells, to GC oscillations.

GR knockdown in old mice activates proliferation of quiescent Type-1 NSPC

GC oscillations results in cyclic activation/inactivation of the GR. To study the specific role of the GR in aged mice in mediating the effects of GC oscillations on Type-1 NSPC proliferation in the DG, we used 20 month-old mice, in which GR+ NSPC populations largely predominate, circadian CORT amplitude is maximal (Figure 2W) and proliferation is drastically reduced. We utilized a previously described experimental approach to knockdown GR expression using siRNAs. Interestingly, GR knockdown resulted in a significant increase in the number of Ki67+ Type-1 NSPCs, which were extremely rare in the contralateral control hemispheres (Figure 4D-H). We further found a significant increase in the percentage of Type-1 asymmetric cell division events in the GR knockdown condition (Figure 4I), which were not detected in the Ki67+ Type-1 NSPC population in contralateral control hemispheres (Figure 4H and I). To better understand the effects of GR knockdown, we classified Type-1 cells following a recently described morphological analysis. Using this method, a considerable morphological heterogeneity of Type-1 cells, represented by Type-1α cells with longer radial processes and Type-1β with shorter radial processes, has been demonstrated. This morphological heterogeneity was rapidly lost in middle-aged (6-10 months) and old animals, in which Type-1β (Figure 4I and 4K) and GR+ cells (Figure 2R) prevailed. Specifically, we observed an age-dependent exponential loss of morphological heterogeneity, accounted by a reduction in the numbers of
GCs preserve hippocampal NSCs in the aging brain

Chapter 5

Figure 3 – GR activation in 3 month-old Nestin-GFP induces reversible NSPC quiescence.

(A) Nestin-GFP (green), GFAP (white) and Ki67 (red) immunoreactivity in the DG of a 3 month-old Nestin-GFP animal. Boxed area in (A) containing a cluster of Nestin-GFP+/Ki67+ NSPC is magnified in (B). Boxed area in (B) is further magnified and Z-stacked channels split in (B’) where the arrow indicates the cell soma of a Ki67+ type-1 NSPC or of a single merged Z-plane in (B”). Dashed white line in (B”) show the direction of scanning, and the histograms (C) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), GFAP (black) and Ki67 (red) across the scanned region. Representative Z-stacked confocal micrographs of animals treated with (D) 0 mg/kg/day, (E) 12.5 mg/kg/day, (F) 25 mg/kg/day [CORT] pellets, and animals treated with (G) 12.5 mg/kg/day or (H) 25 mg/kg/day [CORT] pellet which were allowed to recover for 2 days. Type-1 NSPCs are indicated by arrows and type-2a/2b NSPC by arrowheads. Scale bars represent 20 µm (A-B and D-H) 15 µm (B’) and 10 µm (B”). (I) Bar graph depicting AM and PM plasma CORT levels after the treatments. Bars are mean [CORT] (ng/ml) of individual data-points (red circles)±SEM and statistical comparisons were done using one-way analysis of variance test with Tukey’s post hoc test for multiple comparisons (n=4, **p < 0.01, AM vs. PM in 0 mg/kg/day, ns p > 0.05, AM vs. PM in both 12.5 and 25 mg/kg/day; $ p < 0.05, 25 mg/kg/day AM and PM vs 0 mg/kg/day AM and PM; § p < 0.001, 12.5 mg/kg/day AM and PM vs 12.5 mg/kg/day AM and PM). (J) Percentages of Ki67+ (full bars and full circles) or Ki67- (dashed—bars and open circles) of type-1 (red), type-2a (green) and...
Type-1α cells, and a strong predominance of Type-1β cells in 10 month-old and older mice (Figure 4K). In 10 month-old animals these predominating Type-1β cells readily incorporate Cy3 labeled naked negative control siRNA, as demonstrated by cytosolic Cy3+ puncta detection 1 day post injection (dpi; Figure S3B-H). In Type-1 NSPCs, levels of GFP could be knocked down by means of a specific GFP siRNA infusion (Figure S3I-N). Thus, in the aged DG most Type-1 cells are GR+, Type-1β, and permissive for naked siRNA-mediated knockdown (Figure 2R, 4K and S3B-N, respectively). These data indicate that GR knockdown in aged mice activates the proliferation of quiescent NSPCs.

**GR activation in senescence accelerated prone mice induces lasting morphological changes in the progeny of hippocampal NSPCs**

To further assess the consequences of suppressing GC alterations on aging NSPC, we utilized a mouse model of accelerated aging, the senescence accelerated mouse prone 8 (SAMP8)46,47. After a 7 day suppression of GC oscillations and GR over-activation by means of CORT pellet implantation, we labeled newborn hippocampal cells using retroviral vectors expressing GFP 2 days after pellet removal (Figure S3O). 28 days post retrovirus injection (dpi) we found more hippocampal GFP+ cells in the animals treated with CORT pellets compared to control pellets (Figure 4L-N). While the majority of retrovirus-labeled cells were GFP+/NeuN+/GFAP-/Iba1-/NG2-, a scarce number of cells were found to be GFP+/NeuN-/GFAP-/Iba1-/NG2+, which significantly increased after the CORT pellet treatment (Figure 4O-Q). In the labeled neurons (GFP+/NeuN+/GFAP-/Iba1-/NG2-), both the dendritic complexity and spine density were increased after CORT pellet treatment (Figure 4R-Y). While the number of thin and stubby spines increased, the number of mushroom spines was decreased after CORT pellet treatment (Figure 4Y).

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

Hippocampal NSPC cultures have been previously used to examine the direct effects of GCs on NSPC26,48. In these cultures we found GR+ and GR- NSPC populations (Figure 5A-B). Using the proliferation marker Ki67 to characterize the effect of GCs on NSPC proliferation (Figure 5C-E), we observed that both CORT and the specific GR agonist dexamethasone (DEX) reduced the rate of NSPC proliferation in a dose-dependent manner, with DEX being approximately 10 times more potent than CORT (IC50=5.8 x10⁻⁹ M vs. 6.3 x10⁻⁸ M, respectively), in agreement with their relative affinities for the GR49. The inhibitory effect of CORT was maximal after 72h of incubation (Figure 5E). Strikingly, this strong CORT-induced inhibition of NSPC proliferation was reverted 24h after CORT washout (Figure 5E). Our observations in vitro indicate that CORT induces a reversible inhibition of NSPC proliferation, compatible with cellular quiescence50.

GC oscillations regulate NSPC cell cycle progression in vitro

To model GC oscillations in vitro, we adapted a previously described method44, in which NSPC were treated with pulses (30 min each) of 10⁻⁶ M CORT, to model GC ultradian...
GCs preserve hippocampal NSCs in the aging brain

Figure 4 – GR Knockdown in 20 month-old Nestin-GFP mice recovers type-1 NSPC proliferation and GR activation of NSPC changes the morphology of their neuronal progeny in 4 month-old SAMP8 mice. (Legend on next page)
Figure 4 (continued). Legend on next page.
pulsatile release, which induces GR-mediated cyclical gene pulsing in the hippocampus. Further, we applied the pulsatile treatment for intervals of 12h interspaced with 12h-long hormone-free periods to model circadian GC oscillations for 72 hours. Oscillatory treatments were compared with constant treatment with 10⁻⁶ M CORT (see Figure 5F-H, Figure S3P, S3Q, and methods section for details). Initially, incubation with pulsatile CORT resulted in a significantly smaller percentage of NSPCs in the GO/G1 phase of the cell cycle (Figure 5F), suggesting that CORT oscillations maintain cell cycle entry and progression in NSPC cultures. Interestingly, CORT’s inhibitory effects on the cell cycle were largely reversed 24h after CORT washout (Figure 5G), confirming that constant CORT promotes a reversible cell cycle exit in NSPC cultures, as suggested by Ki67 expression in vivo (Figure 3) and in vitro (Figure 5E).

To further evaluate possible lasting consequences of this temporal cell cycle exit, we studied the effects of CORT exposure on daughter cells. NSPC cell cycle length in our experimental conditions was 17.8±0.1h, similar to previous observations in vivo, indicating that 18h after CORT washout NSPC present in our cultures have divided at least once and then cultures are largely composed of daughter cells, which have not been directly exposed to CORT. 18h after washout, when NSPC cultures were incubated with 10⁻⁶ M CORT, we found significant differences between daughter cells derived from either NSPCs exposed to oscillating or constant CORT (Figure 5H). This change was compensated by a concomitant decrease in the proportion of cells in the S phase (Figure 5I). Importantly, we did not detect significant levels of cells with single cell DNA content, highlighting the selective decrease in cell cycle duration in NSPCs.
DNA methylation at cytosines (5-mC) plays an important role in the regulation of NSPC proliferation in the adult DG under activity conditions, potentially providing a basis for long-lasting modulation. In agreement with this, we found that the overall reduction in NSPC proliferation and their relative abundance in the aging GZ (Figure 1) correlated with decreased 5-mC expression in type-1, -2a and 2b cells in 18 month-old mice (Figure 6A-C). As DNA methylation is catalyzed and maintained by DNMTs, we investigated...
the effect of GC oscillations on DNMT expression and DNA methylation in NSPC *in vitro*. Initially, the expression of DNMTs 1, 3a and 3b, were all significantly inhibited by both oscillatory and constant CORT treatments (Figure 6D). However, a 24h recovery period in CORT-free conditions revealed significant differences between both treatments (Figure 6E). While both CORT treatments initially downregulated the expression of the three DNMTs, expression levels were significantly upregulated in NSPCs initially treated with constant CORT, while they remained downregulated in NSPCs initially exposed to oscillating CORT. From the 3 DNMTs tested, DNMT3a was the most strongly upregulated (Figure 6E). Underscoring the differences induced by different CORT treatments, only NSPCs derived from cells that were initially incubated with constant CORT reacted to a second CORT exposure with a downregulation of DNMTs (Figure 6F). These results indicate that DNMT expression in NSPC is differentially affected by different CORT histories. Specifically, an initial exposure to oscillating CORT was associated with a lower response of DNMT expression to CORT in daughter cells.

Figure 6 – Age-associated changes in 5-mC response of DNMT expression to CORT in daughter cells.
Chapter 5

GC oscillations induce global and promoter specific changes in DNA methylation in NSPCs

In NSPC cultures, both oscillating and constant CORT exposure induced significant reductions in global 5-mC as well as in specific 5-mC levels in protein-coding gene promoters. Supporting our hypothesis that GC oscillations have lasting effects on NSPC, 5-mC levels remained reduced 24h after CORT washout only in cells that were initially exposed to oscillating CORT levels (Figure 6G-H). Specifically, unsupervised hierarchical clustering (UHC) analysis of all differentially methylated promoters between the two CORT regimens (Figure 6I) revealed that 73% of them were differentially hypermethylated by oscillatory CORT. To identify biological processes that may be regulated by GC oscillations in NSPC cultures, we performed gene ontology enrichment (GO) analysis of gene sets whose promoters were differentially hypermethylated after exposure to oscillatory CORT (Figure 6J). We found that the most significantly overrepresented biological processes (BPs) were regulation of transcription (6 BPs), metabolic processes, development,
GCs preserve hippocampal NSCs in the aging brain

differentiation and phosphorylation. In striking similarity with the BPs overrepresented within hypermethylated gene promoters, GO analysis of the hypomethylated promoters after oscillating CORT identified the most significantly overrepresented BPs as: regulation of transcription (5 BPs), transport (2BPs), development, phosphorylation, and cell adhesion (Figure 6K). Underscoring the biological relevance of this convergence on BPs, oscillating CORT treatment was associated with hypermethylation of 55 and hypomethylation of 15 gene promoters associated with cell cycle regulation. These results indicate a convergence on cell cycle regulation, in agreement with the observations described in Figure 5F-H, overall suggesting that GC oscillations are involved in the control of methylation states in gene promoters associated, among other functions, with cell cycle regulation in NSPCs.

**GC oscillations induce lasting changes in promoter methylation in NSPCs**

We characterized changes in DNA methylation in NSPCs derived from cells with different CORT histories. UHC analysis of promoters differentially methylated in NSPC that were derived from cells initially exposed to oscillating or constant CORT, revealed that 18% of the promoters remained in the same methylation state (845 stably methylated promoters; Figure 6I, pink and blue bars). Further analysis of these stably methylated promoters, revealed clusters of stably hypermethylated (214 promoters; Figure 6I, pink bars) and hypomethylated promoters (631 promoters; Figure 6I, blue bars).

GO analysis of the 214 stably hypermethylated promoters revealed that the most significantly overrepresented BPs were regulation of transcription, cell differentiation and organismal development (Figure 7A). GeneMANIA pathway analysis of the highest overrepresented BPs among the stable hypermethylated promoters, revealed a gene network involved in stem cell differentiation (network node: Tbx3; Figure 7B). Further analysis of overrepresented BPs revealed gene networks involved in regulation of glial cell differentiation (network node: Gsx2; Figure S4) and regulation of (stem) cell activation (network node: Ptpn6; Figure S4). GO analysis of the 631 stably hypomethylated promoters showed that the most significantly overrepresented BPs were organismal development, transport, regulation of transcription and carbohydrate metabolism (Figure 7C). GeneMANIA pathway analysis of the highest overrepresented BPs revealed a gene network involved in Wnt-signaling (network node: DKK3; Figure 7D). Further analysis of overrepresented BPs revealed also gene networks involved in metal ion transmembrane transport (network node: Slc24a4; Figure S4) and organic anion transport (network node: Slc7a5; Figure S4). In agreement with the modulation of Wnt-signaling suggested by pathway analysis, we found that the expression of four components of this pathway was differentially affected by CORT treatments (Figure 7E and 7F). Initially, the expression of GSK3β and β-catenin were downregulated by oscillating CORT and were unaffected by constant CORT, and CCND1 was downregulated by both treatments. Conversely, DKK3 was upregulated by oscillating CORT, in agreement with its promoter hypomethylation, and was unaffected by constant CORT (Figure 7E). The levels of DKK3, GSK3β, CCND1, and β-catenin were significantly upregulated in cells originated from NSPC treated with constant CORT, while GSK3β, β-catenin and CCND1 expression remained downregulated after washout in cells derived from NSPC initially exposed to oscillating CORT (Figure 7F). Conversely, DKK3, which was initially upregulated by oscillating CORT, remained upregulated after washout (Figure 7E-F). These results indicate, together with observations presented in previous sections, that GC oscillations induce different patterns of DNA methylation in NSPCs, which may have programming effects on gene expression in NSPCs, affecting members of the Wnt-signaling and some of its downstream targets.
Chapter 5

Figure 7 - Oscillating CORT induces stable changes in gene promoter methylation in NSPC cultures. GO analysis of stably (A) hyper- or (C) hypo-methylated gene promoters (oscillating vs. constant CORT; MBD2 read density difference ≥3). Bar graphs show number of annotated members per BP (red) and their hypergeometric FDR corrected p-values (blue). Pathway analysis of stably (B) hyper- or (D) hypo-methylated gene promoters induced by oscillating CORT. Embryonic organ morphogenesis (FDR corrected p = 1.26*10^-8), stem cell differentiation (FDR corrected p = 4.51*10^-10), cell fate commitment (FDR corrected p = 6.39*10^-8) and canonical Wnt-signaling pathway (FDR corrected p = 2.43*10^-7) were significantly overrepresented networks. Changes in DKK3, GSK3β, CCND1 and β-catenin expression induced by (B) 72h or (F) 72h followed by 24h washout, oscillating (gray bars) or constant CORT (black bars). Data are mean normalized fold change expression (relative to vehicle) of individual data-points (red circles) ±SEM (n=4, * p < 0.05, ** p < 0.01 and *** p < 0.001 relative to vehicle; # p < 0.05, ## p < 0.01 and ### p < 0.001 relative to oscillating CORT; one-way ANOVA with Tukey’s post hoc test). See also Figure S3 and S4.
GCs preserve hippocampal NSCs in the aging brain

Chapter 5

Discussion

The existence of an aging-associated decline in AHN has been consistently documented. However, the underlying causes and mechanisms involved remain poorly characterized. Here, using Nestin-GFP mice as an in vivo NSPC reporter system, we show that: 1) NSPC populations expressing the GR are preserved across aging in the DG. Specifically, we show that GR+ Type-1, 2a and 2b NSPC populations had a significantly longer calculated $t_{1/2}$ than their GR- counterparts and were still present in significant numbers in old mice. 2) The preponderance of GR+ populations was first detected in 6 month-old mice and coincided with the stabilization of the amplitude of circadian GC oscillations at this age. 3) In old mice, in which the amplitude of GC oscillations is maximal, GR knockdown resulted in a strong activation of Type-1 cells, which was scarce in control animals of the same age. This finding is consistent with previous studies in young animals, showing that a systemic treatment with a GR antagonist reverts the inhibition of AHN induced by stress and other studies in older animals, in which adrenalectomy restored the rate of cell proliferation in the DG.

We have previously demonstrated that GC have direct effects on hippocampal NSPC mediated through the GR. In agreement with this, GR knockdown experiments herein indicate that the GR is involved in the control of Type-1 cell activation by GC oscillations in old mice. Although naked siRNAs are charged molecules, previous studies have demonstrated that local transduction of neuronal cells in vivo does not require a transfection reagent and produces no detectable interferon response upon injection into mice, significantly reducing off-target effects. Here we show that, in the aged DG, Type-1 cells are permissive for nakes siRNA-mediated gene knockdown. However, our RNA interference-based approach lacks cell type specificity and therefore the effects on Type-1 cell proliferation could be due to GR downregulation in other cell-types of the DG.

Recent observations have identified morphological heterogeneity in Type-1 cells in the adult hippocampus, which can be divided into Type-1α and β following specific morphological parameters. However, the relation between these morphological phenotypes and GR expression has not been characterized. We found that Type-1β cells strongly predominated over Type-1α cells in old mice, in which most Type-1 cells are GR+. Interestingly, Type-1β cells could be activated by GR knockdown. Importantly, Type-1β cells frequently generate astrocytes, rather than neurons, in young adult mice. Thus, our observations suggest that although GR inhibition may promote NSPC proliferation in the aging brain, it could result in astro- rather than neurogenesis. In agreement with this, suppression of GC oscillations and GR over-activation in senescence-prone mice resulted in an increase in the number of GFP-labeled NG2+ cells, which may belong to the oligodendrocyte progeny. This observation is consistent with previous ones indicating that GCs induce oligodendrocyte generation from adult NSPCs. However, this is a hypothesis that escapes the aim of this work and requires further experimental validation.

Using hippocampal NSPC cultures, in which the direct effects of GCs can be better characterized, we show that GC oscillations exert lasting effects on NSPC proliferation. Daughter cells derived from NSPCs exposed to GC oscillations maintained their sensitivity to GC-induced inhibition of cell proliferation, suggesting a bona-fide epigenetic
mechanism that may program NSPC proliferation. In addition, daughter cells derived from NSPCs exposed to constant GCs were desensitized to GC-induced inhibition of cell proliferation and proliferated more than cells derived from NSPC exposed to oscillating GC. These observations suggest that periods of prolonged exposure to constant GCs may result in later enhancements of NSPC proliferation rate and eventually a decay of the NSPC pool, with possible negative consequences for long-term hippocampal plasticity\(^\text{18}\). Such periods could take place in e.g. patients of chronic inflammatory diseases, commonly medicated with high GC concentrations for long periods of time, or in clinical cases of hypercortisolism\(^\text{31}\). In some psychosocial stress models, GC levels escape circadian regulation and remain elevated for prolonged periods of time, a condition that resembles human hypercortisolism, and which has strong inhibitory effects on AHN and may be linked to aging-associated mild cognitive impairment\(^\text{64,65}\). Therefore, the preservation of GR+ NSPC we report here may be important to understand mechanisms associated with aging-associated AHN decline.

Further, we found that GC oscillations resulted in a lower response to GCs in terms of DNA methyl transferases expression in daughter cells. This finding suggests that GC oscillations maintain DNMT expression levels within a controlled range in NSPCs. Our findings provide a possible functional implication for previous studies showing that DNMT expression is GR-responsive in NSPC\(^\text{66,67}\), and are consistent with the concept that GC oscillations function to optimize steady state gene expression, stabilizing GC-responsive genes\(^\text{51,68}\). Oscillatory and constant GC treatments carry qualitatively different information and regulate gene expression at different levels\(^\text{68}\). In agreement with this concept, we show that oscillatory and constant GC treatments induced different effects on genome-wide DNA methylation profiles \textit{in vitro}. GC oscillations induced strong hypermethylation effects, with 73% of the differentially methylated promoters being hypermethylated, suggesting that GC oscillations maintain specific DNA methylation states in NSPCs. Indeed, we found that GC oscillations induce specific gene promoter methylation patterns. 70 cell cycle-related gene promoters were differentially affected by GC oscillations, indicating that their genome-wide promoter methylation effects may converge on the regulation of cell cycle in NSPCs.

We show that some of the genome-wide changes on DNA methylation were persistent across NSPC generations. Within the persistently hypermethylated promoters, we identified a gene network involved in stem cell differentiation and within the persistently hypomethylated ones, a network of genes involved in Wnt-signaling, a principal regulatory pathway in AHN\(^\text{69,70}\). Indeed, GC oscillations regulated the expression of 4 components of the Wnt-signaling pathway (DKK3, GSK3β, CCND1 and β-catenin) resulting, in general, in a smaller change in gene expression as compared to incubation with constant GC, in agreement again with the concept that GC oscillations stabilize the expression of GC-responsive genes\(^\text{51,68}\), indicating that GC oscillations may serve to maintain the Wnt-signaling pathway within a controlled range in NSPC. Therefore, an exhaustive functional characterization of the regulation of Wnt-signaling by GC oscillations warrants further investigation.

In summary, we show that GR expression and GC oscillations are associated with the preservation of specific NSPC populations during aging and with the regulation of NSPC proliferation and Wnt-signaling \textit{in vitro}. Future use of experimental approaches aimed to activate or inactivate GR-mediated pathways specifically in NSPC will help to further understand the underlying mechanisms.
Experimental Procedures

All animal procedures were monitored by the Commission for Animal Welfare, University of Amsterdam (protocol # 259 and 314), Ikerbasque or CSIC Madrid. Male 3, 6, 10, 14 and 18 month-old Nestin-GFP transgenic mice\(^{36}\) (n=5 per group) were used. These time points were selected based on start- and end-points of previously defined life-phases (mature adult, middle age and old) in mice\(^{71}\). Mice were housed under standard laboratory cage conditions and kept under 12 hour light/dark cycles (lights on at 08:00, lights off at 20:00) with ad libitum access to food and water. Population decay curves were fitted from numbers of GR+ and GR- NSPCs present in Nestin-GFP mice of different ages, half-lives (t\(_{1/2}\)) were calculated and differences were tested using an extra-sum-of-squares F-test. Tail blood was collected in a stress-free manner at 20:00 (PM) the night before and at 08:00 (PM) on the morning of perfusion, as described before\(^{72}\). Ultradian CORT oscillations were modeled in vitro as previously described\(^{51}\), with some modifications. NSPC cultures were exposed to pulsatile treatment consisting of cycles of exposure to 30 min-long pulses of either vehicle (ethanol) or 10\(^{-6}\)M (except stated otherwise) CORT interspaced with 30 min-long incubations with hormone-free medium for 12 h, followed by a 12 h-long incubation with hormone-free medium to model circadian CORT oscillations. Constant CORT treatment consisted of 30 min-long cycles of incubation with CORT for 24h, without interspaced hormone-free periods. The effectiveness of the incubation regimens was verified by determination of CORT levels in culture medium samples every 30 minutes (Figure S3). 72h after the start of the initial treatment, a 24 h-long washout culture period (recovery) in hormone-free medium was performed. When indicated, NSPC were treated after recovery for 6h with CORT or vehicle. Treatment schemes are depicted in Figure S3. Flow cytometry analysis of cell cycle using propidium iodide was done using standard procedures. Global DNA methylation was measured using MBD2-isolated Genome Sequencing, essentially as described\(^{73}\). Immunohistochemistry, confocal microscopy, stereotaxic injections to the DG, cell culture, QRT-PCRs and bioinformatic analyses were all performed as described before\(^{26}\). Detailed descriptions are provided as Supplemental Information.

Author Contributions

MS, PB, LGC, SG, EGG, TJ performed experiments and analyzed data; PJL, HEV, SWS, NT, HM and JME participated in experimental design and result discussion and interpretation, MS, PB, and CPF conceived the study, designed experiments, analyzed and interpreted results and wrote the manuscript.

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Chapter 5
Supplementary Information

(Patho)physiological Regulation of Adult Hippocampal Neurogenesis by Seizures, Glucocorticoids, and microRNAs

Pascal Bielefeld
Figure S1. Related to Figure 2 - GR expression threshold determination in NSPC subpopulations and modeling of their decay kinetics.

(A) Bar graph displaying GR staining intensities in non-cellular regions of the hilus as a background (BG; white bar and red circles) and of type-1/GR+ (red solid bar and full circles), type-1/GR- (red dashed bar and open circles), type-2a/GR+ (green solid bar and full circles), type-2a/GR- (green dashed bar and open circles), type-2b/GR+ (blue solid bar and full circles) and type-2b/GR- (blue dashed bar and open circles) NSPC. Data are mean±SEM (n=5). Differences between GR+/GR- NSPC and hilar BG are indicated (*** p < 0.001, one-way ANOVA).

(B) Plots displaying best-fit curve comparison of age-related type-1/GR+ decay kinetics. The red line indicates a exponential curve fitting and the black dotted line a linear curve fitting.

(C) Plots displaying best-fit curve comparison of age-related type-2a/GR+ decay kinetics. The green line indicates a exponential curve fitting and the black dotted line a linear curve fitting.

(D) Plots displaying best-fit curve comparison of age-related type-2b/GR+ decay kinetics. The blue line indicates an exponential curve fitting and the black dotted line a linear curve fitting.

(E) Plots displaying best-fit curve comparison of age-related type-1/GR- decay kinetics. The red line indicates an exponential curve fitting and the black dotted line a linear curve fitting.

(F) Plots displaying best-fit curve comparison of age-related type-2a/GR- decay kinetics. The green line indicates an exponential curve fitting and the black dotted line a linear curve fitting.

(G) Plots displaying best-fit curve comparison of age-related type-2b/GR- decay kinetics. The blue line indicates an exponential curve fitting and the black dotted line a linear curve fitting.

Data point values (polygons) are expressed as mean±SEM of five animals. Best-fit curve values are expressed as mean±95% confidence interval. Linear or exponential curve fitting preference was tested using a sum of squares F-test (an exponential model preference is indicated with a p < 0.05, p-values are shown in plots).
GCs preserve hippocampal NSCs in the aging brain

Chapter 5

Figure S2, Related to Figure 2 - Linear decay of GR+ NSPC correlates with age-associated increases in circadian CORT amplitude.
(A) Plots displaying linear regression between the circadian amplitude of CORT and the number of total type-1 (red line and circles), GR+ type-1 (orange line and squares) and GR- type-1 (pink line and diamonds) of the animals across all ages and specifically in the 3 (B), 6 (C), 10 (D), 14 (E) and 18 (F) month cohorts.
(G) Plots displaying linear regression between the circadian amplitude of CORT and the number of total type-2a (dark green line and squares), GR+ type-2a (intermediate green line and triangles) and GR- type-2a (light green line and asterisks) of the animals across all ages and specifically in the 3 (B), 6 (C), 10 (D), 14 (E) and 18 (F) month cohorts.
(M) Plots displaying linear regression between the circadian amplitude of CORT and the number of total type-2b (dark blue line and upward triangles), GR+ type-2b (intermediate blue line and downward triangles) and GR- type-2b (light blue line and stars) of the animals across all ages and specifically in the 3 (B), 6 (D), 10 (P), 14 (Q) and 18 (R) month cohorts.
Pearson analysis p-values are indicated in the legends, significant (p < 0.05) deviations from a slope of 0 indicate circadian CORT amplitude level-related association with numbers of NSPC.
Chapter 5

- Figure S3 Schouten et al.

A

Time in experiment (days)

0 1 2 3 4 5 6 7 8 9 10

Veh, 12.5 and 25mg/kg/day CORT slow release pellet implantation

PM blood collection Pellet removal

3 month old Nestin-GFP ♂

B

Time in experiment (days)

0 1 2

10 month old Nestin-GFP ♂

CORT slow release pellet implantation

AM blood collection PM blood collection

10 month old Nestin-GFP ♂

C

Time in experiment (days)

0 1 2

Seed NSPC Start treatment (1st hit)

SAMP8 s.c. veh/CORT (12.5 mg/kg/day) pellet implantation

Stop treatment Collect samples

P

Time in experiment (hours)

-24 0 24 48 72 96

CORT pulse (2nd hit)

Q

Average Type-1 NSPC GFP intensity (gray value)

Distance (μm) Intensity (gray value)

DNA/Nestin-GFP/siNC Cy3

Nestin-GFP+ NSPC with small (<1μm) siNC Cy3 + puncta 1dpi

D

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC with large (>1μm) siNC Cy3 + puncta 1dpi

E

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. GR

F

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. GFP

G

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. NC

H

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. NC

I

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. GFP

J

Distance (μm) Intensity (gray value)

Nestin-GFP+ NSPC s.i. NC
GCs preserve hippocampal NSCs in the aging brain

Figure S3, Related to Figure 3-7 - Experimental design used to model GC oscillations and experimental validation. See also Supplemental Experimental Procedures.

(A) Schematic depiction of the experimental setup used to obtain data described in Figure 3
(B) Representative Z-stacked confocal micrograph of a type-1 NSPC of a 10month old Nestin-GFP animal 1dpi of negative control siRNA labeled with Cy3 (siNCCy3) showing a Nestin-GFP+/siNCCy3+ NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC expressing the Cy3 labeled siNC. Boxed area is magnified and Z-stacked channels split in (C) or of a single merged Z-plane (D), the arrows indicate small (<1µm) siNCCy3+ puncta. Dashed white line in (D) shows the direction of scanning, and the histograms (D’) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), and siNCCy3 (red) across the scanned region. (E) Orthogonal views of the same type-1 NSPC as (C-D’).

(F) Representative Z-stacked confocal micrograph of another type-1 NSPC of a 10month old Nestin-GFP animal 1dpi of siNCCy3 showing a Nestin-GFP+/siNCCy3+ NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC expressing the Cy3 labeled siNC. Boxed area is magnified and Z-stacked channels split in (F) or of a single merged Z-plane (G), where the arrows indicate large (>1µm) siNCCy3+ puncta. Dashed white line in (G) shows the direction of scanning, and the histograms (G’) show the strength of the fluorescent signals for DNA (Hoechst, blue), GFP (green), and siNCCy3 (red) across the scanned region. (H) Orthogonal views of the same type-1 NSPC as (F-G’).

Scale bars represent 12 µm (C-H).

(I) Schematic depiction of the experimental setup used to obtain data below (J-N) and described in Figure 4A-I.
(J) Representative Z-stacked confocal micrograph of a type-1 NSPC of a 10month old Nestin-GFP animal 3dpi of siNC showing a Nestin-GFP+/siNC siNC showing a Nestin-GFP+ NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC. Boxed area is magnified and a single Z-plane is shown (K). Dashed white line in (K) shows the direction of scanning, and the histogram (K’) shows the strength of the fluorescent signals for GFP (green).

(L) Representative Z-stacked confocal micrograph of a type-1 NSPC of a 10month old Nestin-GFP animal 3dpi of siGFP showing a Nestin-GFP+ NSPC with a characteristic vertical process and triangular cell-body in the SGZ of the DG, indicative of a type-1 NSPC. Boxed area is magnified and a single Z-plane is shown (M). Dashed white line in (M) shows the direction of scanning, and the histogram (M’) shows the strength of the fluorescent signals for GFP (green).

(N) GFP expression in type-1 cells 3 dpi. Data are average GFP intensity (gray value) of individual data-points (open circles) ±SEM (n=3, *** p < 0.001, siNC vs. siGFP, Student’s t-test). Scale bars represent 12 µm (I-M).

(O) Schematic depiction of the experimental setup used to obtain data described in Figure 4I-Z.
(P) Schematic depiction of the experimental setup used to obtain the in vitro data described in Figure 5-7.
(Q) Plots displaying the experimental validation of the different CORT incubation regimen used in vitro described in Figure 5-7.
Chapter 5

Figure S4, Related to Figure 7 - CORT oscillations induced stable alterations in gene promoter methylation and associated functional pathways. (continues on next page)

(A) Pathway analysis of oscillating CORT-induced second highest overrepresented BP of stable hyper-methylated promoters (Figure 7A). GeneMANIA identified inorganic anion transport (red spheres; FDR corrected p = 4.51*10^{-19}) as significantly overrepresented functional networks.

(B) Pathway analysis of oscillating CORT-induced third highest overrepresented BP of stable hypo-methylated promoters (Figure 7C). 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 = 6.25*10^{-8}) as significantly overrepresented functional networks.
GCs preserve hippocampal NSCs in the aging brain
Chapter 5

Supplemental Experimental Procedures

Aging animal cohorts, CORT measurements, immunohistochemistry, and confocal microscopy

3, 6, 10, 14 and 18 month-old male Nestin-GFP transgenic mice\(^1\) (n=5 per group) were used for experiments. Mice were housed under standard laboratory cage conditions and kept under 12 hour light/dark cycles (lights on at 08:00, lights off at 20:00) with ad libitum access to food and water. At the indicated ages, tail blood was collected in a stress-free manner in ice-cold EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands) at 20:00 (PM) the night before and at 08:00 (PM) on the morning of perfusion, as described before\(^2\). Samples were kept on ice and subsequently centrifuged at 13,000 rpm for 15 min, blood plasma was stored at −20°C. AM and PM plasma CORT levels were measured using a commercial radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) as described before\(^3\). Animals were transcardially perfused at the indicated ages at 08:00±0.3hours (Figure 2V) with 4% paraformaldehyde in PBS and brains were extracted, sectioned in 8 series of 40µm and immunostained as described before\(^3\), using the following antibodies: 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. PCNA and 5-mC stainings required antigen retrieval, which was performed by heating brain sections in 0.1 M citrate buffer (pH 6.0) in a standard microwave (Samsung M6235) to a temperature of approximately 95°C for 15 minutes (5 minutes at 800 Watt, 5 minutes at 400 Watt and 5 minutes at 200 Watt). Antibodies used were monoclonal mouse anti-PCNA (DAKO, 1:400) and monoclonal mouse anti-5-mC (Eurogentec, 1:500) in combination with goat anti-mouse Alexa647 (Invitrogen, 1:500) and if applicable combined with rabbit anti-GFAP (DAKO, 1:500) 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. Confocal microscopy was performed as described before using a Zeiss LSM510 laser scanning microscope\(^3\). Hippocampal NSPC populations were quantified in the subgranular zone and granule cell layer (granular zone; GZ) and were either expressed in absolute numbers per mm\(^3\) GZ or in relative percentages of the total NSPC subpopulation.

Generation of best-fit curves, population half-life calculations, correlations, and statistical analysis

Non-linear (exponential decay) best-fit curves \(N(t) = N_0 \cdot e^{-\lambda t} + N\) with \(N\) as number of cells in cells/mm\(^3\) GZ, \(t\) as time in months and \(\lambda\) as the decay rate constant as a decimal) or linear (first order polynomial) decay curves \(N(t) = N_0 - \lambda t\) with \(N\) as number of cells in cells/mm\(^3\) GZ, \(t\) as time in months and \(\lambda\) as the slope in cells/mm\(^3\)•month\(^{-1}\) including their 95% confidence intervals were plotted on the numbers of (GR+ and GR-) Type-1 and Type-2 NSPCs using Graphpad Prism 5.0 software. Non-linear (exponential) decay curves were tested for a significantly better fit than linear (first order polynomial) decay curves.
using an extra-sum-of-squares F-test and were considered significantly different if the F-test reached a p < 0.05. Subsequently, depending on the aforementioned extra-sum-of-squares F-test results, half-lives (or $t_{1/2}$) were calculated for the either exponential ($t_{1/2} = \ln(t_{1/2})/\kappa$) or linear ($t_{1/2} = N_0/2/\lambda$) curves from GR+ and GR- Type-1 and Type-2 NSPC. For CORT concentrations versus NSPC population correlations a Pearson correlation analysis was used and was subsequently tested for significant deviation from a slope of 0 and were considered significantly different if $p < 0.05$. All other comparisons were statistically tested using one-way analysis of variance (ANOVA) test with a Tukey’s post-hoc test and were considered significantly different if $p < 0.05$. Graphpad Prism 5 software was used for the generation of best-fit curves and statistical analyses.

**Subcutaneous pellet implantation experiments**

CORT levels were manipulated using slow release biodegradable carrier-binder pellets to various daily concentrations (vehicle, 12.5 mg/kg/day and 25 mg/kg/day, n=4 per experimental group; Innovative Research of America), as described by others\(^4\), albeit with some modifications. Pellets were implanted subcutaneously between the shoulder blades of Nestin-GFP animals under isoflurane anesthesia at 08:00h on experimental day 1. When indicated, pellets were removed under isoflurane anesthesia at 08:00h on experimental day 8. AM and PM plasma CORT concentrations were determined on day 7/8 or 3 days after pellet removal (recovery group) on day 9/10 (see Figure S3A). Immunohistochemical analysis was performed on 4 animals per experimental group, as described in the corresponding section.

**Stereotaxic siRNA injections**

For GR knockdown experiments, 20 month old male Nestin-GFP mice underwent stereotaxic surgery, delivering 1µl of a 40µM mixture of 4 previously validated\(^3\) siRNAs (FlexiTube GeneSolution, Qiagen, CAGACTCAGCATGGGAATTA, AAGCGTGATGGACTTGTATAA, CAGTGGTGCGATAGCAACAAA, AAGGAAGGTCTGAAGAGCCAA) against the mouse GR (Nr3c1, Entrez gene ID: 14815) into the left DG or negative control siRNA (target sequence: AATTCTCCGAACGTGTCACGT; Qiagen) into the contralateral DG (anterior-posterior: -2.0, medial-lateral: ± 1.5, dorsalventral: -2.0). 72 hours after siRNA infusion, 6 animals were sacrificed by transcardial perfusion-fixation, brains were extracted and processed for immunohistochemistry as described in the corresponding section. Similarly, for naked siRNA uptake verification, 10 month-old male Nestin-GFP mice (n=3) underwent the same procedure delivering negative control Cy3-labeled siRNA (siNCCy3; Allstars negative control siRNA; Cat. No. SI03650318, Qiagen). These animals were sacrificed 24 hours after (1dpi) siRNA infusion as described above. In addition for naked siRNA knockdown validation, 10 month-old male Nestin-GFP mice (n=3) were injected with siNC (Allstars negative control siRNA; Cat. No. SI03650318, Qiagen) and siRNA directed against GFP (positive silencing control GFP-22 siRNA, Cat. No. 0001022064, Qiagen). Naked siRNA knockdown validation animals were sacrificed 3dpi. Brain slices were obtained and stained for DNA using Hoechst and native GFP and Cy3 colocalization or GFP intensity levels were measured using the Zeiss LSM510 confocal as described in the corresponding section.

**SAMP8 animal experiments**

4 month old male senescence accelerated prone 8 (SAMP8)\(^5,6\) mice received subcutaneous pellets as described above for 7 days. Subsequently, pellets were removed and the
animals were allowed to recover for 2 days before they underwent stereotaxic delivery
of retrovirus as described before\(^3\). 28 dpi of the retrovirus 4 animals per group were
sacrificed by transcardial perfusion-fixation, brains were extracted and processed for
immunohistochemistry as described in the corresponding section. The GFP signal from
RV-GFP+/NeuN+/Iba1-/NG2- newborn cells was traced using ImageJ and sholl analyses
were performed as described before\(^3\). Furthermore, from RV-GFP+/NeuN+/Iba1-/NG2-
newborn cells the spine density and morphology analyses were performed in
NeuronStudio on secondary/tertiary dendritic segments, as described before\(^3\).

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

Primary hippocampal NSPC cultures were prepared and maintained 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\(^3\). NSPC 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\(^{-6}\)M (except stated otherwise) prior
to incubation. Ultradian CORT oscillations were modeled in vitro as previously described\(^7\),
with some modifications. Briefly, pulsatile treatment consisted of cycles of exposure to
30 min-long pulses of either vehicle (ethanol) or CORT interspaced with 30 min-long
incubations with hormone-free medium. NSPCs were exposed to this pulsatile treatment
for 12 h, followed by a 12 h-long incubation with hormone-free medium. The constant
CORT condition consisted of 30 min-long cycles of incubation with CORT at the same
concentration for 24h, without interspaced hormone-free periods. Starting after a 72h
initial treatment, the washout period (recovery) consisted of a 24 h-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 Figure S3. Efficient washout and stability of CORT
during the experiment (Figure S3L) was analyzed by collecting samples every 30 min
during both pulsatile and constant treatment and CORT concentrations were determined
using a commercial radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands)
as described above.

**Immunocytochemistry**

Immunocytochemistry was carried out as described before\(^3\). Briefly, cells were rinsed
three times with PBS and fixed in 4% PFA in PBS for 30 min. The fixative was then removed
and cells were rinsed three times for 5 min with PBS. For detection of proliferation,
cells were blocked in blocking buffer (1X TBS/1% skimmed milk powder) for 60 min
and incubated for 1 h 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 5 min in PBS, incubated with donkey
anti-rabbit Alexa488 (Invitrogen, 1:1000) for 1 hour at room temperature, rinsed three
times for 5 min 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
60 min before cells were incubated for 1 h 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 5 min in PBS, incubated with goat anti-mouse Alexa568 (Invitrogen, 1:1000) and donkey anti-rabbit Alexa488 (Invitrogen, 1:1000) for 1 hour at room temperature, rinsed three times for 5 min 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, using SYBR green (Applied Biosystems) and the following primer sequences: α-tubulin (for normalization) forward: CCCCCGTCTCTTACCCGGTTGC, reverse: TGGTCTTGTCACTTGGCATCTGGC; DNMT1 forward: AGGCGCGTCACTGGTCTAC, reverse: GGGGGGCCTCATTGCAGTCC; DNMT3a forward: GCCAAGAAACCCAGAAGAGC, reverse: GTGACATTGAGGGTCCACA; DNMT3b forward: GCCTGGATCTTACCATGTT, reverse: ATCTTTCCCACAGGAGTC; DKK3 forward: CACAAATACTGGCACGAGTA, reverse: GTGACATTGAGGGTCCACA; CCND1 forward: GCCATGACTCCCCACGATTT, reverse: CTACCATGGAGGGTCCACA; β-catenin forward: GAACAGGGTGCTATTCCACGA, reverse: TGGAGAGCTCCAGTACCC.

Flow cytometry analysis of cell cycle using propidium iodide

NSPCs were trypsinized (Trypzean, Lonza) for 5 minutes 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 20 min with RNase (100 µg/ml; Sigma-Aldrich) and incubated for 20 min 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 FACSaria™ III system (BD) with a 488nm excitation laser. Propidium iodide was detected within the PE/Texas Red channel with a 610/10 bandpass filter. 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 using Multicycle AV and FCS express (De Novo Software).

Global cytosine methylation analysis

Global DNA methylation was measured using MBD-isolated Genome Sequencing, essentially as described. Briefly, NSPCs were trypsinized (Trypzean, Lonza) for 5 minutes, spun down for 3 minutes at 300x 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 Methylamp™ Global DNA Methylation Quantification Ultra Kit (Epigentek) according to the manufacturer’s protocol. Data were normalized to global DNA methylation levels of vehicle treated NSPC, as indicated.

Methylated DNA sample preparation and quality control

DNA was isolated from NSPCs as described above. DNA concentration was determined on a Fluostar Optima plate reader (BMG Labtech) with the Quant-iTTM Picogreen® dsDNA assay kit (Invitrogen) at 480/520nm. Concentration was determined using smear analysis
on a Agilent 2100 Bioanalyzer (Agilent Technologies) and checked for degradation. Samples (n=3) for each experimental condition were pooled into a single sample for further processing.

**Methylated DNA fragmentation and MBD2-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 190 sec to 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 3 μg DNA was dissolved in 130 μl 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 a 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)\(^9\), 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 250 ng 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). 300 bp +/- 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 an Agilent 2100 Bioanalyzer and samples were diluted to 10 nM. DNA fragments were sequenced using the Hi-Seq 2000 Massive Parallel Sequencer system (Illumina) with 2x51 + 7(index) sequencing cycles. Initial quality assessment was based on data passing the Illumina Chastity filter control. Subsequently, the reads containing adaptors and/or Phix control signal were removed. A second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0.

**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\(^9\). The Bowtie parameters were set to 0 mismatches in the seed (first 28 nucleotides). Only unique paired reads were retained and both fragments must be located within 400bp of each other on the mouse reference genome build NCBI37/mm\(^9\). 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
GCs preserve hippocampal NSCs in the aging brain

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) as described and 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 other 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.

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