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Supplemental Information

Retinoic Acid Is Required for Neural Stem and Progenitor Cell Proliferation in the Adult Hippocampus

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SUPPLEMENTAL MATERIALS AND METHODS

Cell Cycle Analysis. Cell cycle length (T_C), S phase length (T_S) and estimated lengths of cell cycle phases G2/M and G1 were calculated using previously described methods (Brandt et al., 2012). Briefly, to measure S phase length (T_S), IdU was injected ip followed by ip injection of CldU, with an inter-injection interval of 3h. For measurement of T_C, IdU was injected ip followed by CldU with an inter-injection interval of 20h. G1 and G2/M phase lengths were estimated as described (Brandt et al., 2014; Fischer et al., 2014). For cell cycle exit, mice were injected with EdU either 24 hours (stem cells) or 28 hours (progenitor cells) prior to harvest. Brain sections were immunolabeled to detect EdU and Ki67, a cell cycle marker. Exited cells were identified as Edu^+ and $Ki67^-$. Percentage of cell cycle exit was calculated as $Edu^+/Ki67^-$ ÷ total Edu^+ cells. For S phase re-entry, mice were injected with IdU and 24 hours (stem cells) or 28 hours (progenitor cells) later with CldU. Forty-five minutes later, brains were harvested and sections were immunostained for CldU and IdU. Percentage of S phase re-entry was calculated as $CldU^+IdU^+$ ÷ total IdU^+ cells. CldU was detected using rat anti-BrdU (Novus Biologicals) and IdU was detected using mouse anti-BrDU (BD Biosciences). CldU and IdU can be detected simultaneously using these antibodies without cross reactivity (Aten et al., 1992; Vega and Peterson, 2005; Brandt et al., 2012). Each cell cycle parameter was determined for separate stem and progenitor cell types (stem cells: Sox2+/GFAP+; intermediate progenitors: Tbr2+ cells; neuroblasts: DCX+ cells).

ELISA analysis. To analyze VEGFA and HIF1α protein levels *in vitro* or *in vivo*, cells or whole hippocampal tissue were collected in RIPA buffer (#R0278, Sigma) containing a protease inhibitor cocktail tablet. Total protein concentrations were determined using the BCA protein assay. VEGFA and HIF1α protein levels were determined using VEGFA ELISA kit and HIF1α

ELISA kit, respectively according to manufacturer's instructions. All represented proteins levels are normalized to respective total protein concentrations. For animal studies, we analyzed 3 animals (n=3) per treatment condition or genotype for ELISA experiments. For NSC culture studies, three independent experiments (n=3) were analyzed for all treatments.

Quantitative real-time PCR analysis. RNA isolation from cultured cells and whole hippocampal tissue was performed using RNeasy Micro Kit and RNeasy plus Mini Kit, respectively. RNA concentration was determined using UV spectrophotometer and 100ng of RNA was reverse-transcribed using Bio-Rad iScript cDNA synthesis kit according to manufacturer's instructions. cDNA was amplified using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). A 12.5 µl reaction volume containing 6.25 µl of the SYBR Green PCR, 200nM of each primer was run, using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The amplification program consisted of initial denaturation at 95°C for 3 minutes followed by 39 cycles of 95°C for 10 seconds and annealing at 60°C for 30 seconds. For animal studies, 3 animals (n=3) per treatment condition or genotype were analyzed. For NSC culture studies, three independent experiments (n=3) were analyzed for all treatments.

Primer sequences for RT-PCR experiments. Primers used are as follows: *Vegfa*, sense 5′- CAG GCT GCT GTA ACG ATG AA- 3′ and antisense 5′- TTT GAC CCT TTC CCT TTC CT- 3′; *Hif1a*, sense 5′- ACC TTC ATC GGA AAC TCC AAA G-3′ and antisense 5′- CTG TTA GGC TGG GAA AAG TTA -3 ; *Rara*, sense 5′- AGC TCT GCG TTG TGC AGA TCT - 3′ and antisense 5′- AGA GTG TCC AAG CCC TCA GA - 3′; *Rarb*, sense 5′- TTC AAA GCA GGA ATG CAC AG -3′ and antisense 5′- GGC AAA GGT GAA CAC AAG GT-3 ; *Rarg*, sense 5′- CAC AGC CTG CCA GTC TAC AA - 3′ and antisense 5′- CTG GCA GAG TGA GGG AAA AG - 3′; *Rxra*, sense 5′- CTG CCG CTC GAC TTC TCT AC -3′ and antisense 5′- ATA TTT

CCT GAG GGA TGG GC -3 ; *Rxrb*, sense 5′- TGG GGG TGA GAA AAG AGA TG -3′ and antisense 5′- GAG CGA CAC TGT GGA GTT GA -3 ; *Rxrg*, sense 5′- AAT GCT CTT GGC TCT CCG TA -3′ and antisense 5′- TGA AGA AGC CTT TGC AAC CT -3; *Ccdn1* sense 5′- GCG TAC CCT GAC ACC AAT CTC-3′ and antisense 5′- ACT TGA AGT AAG ATA CGG AGG GC-3 ; *Ccdn2* sense 5′- ACC TCC GCG AGT GTT CCT ATT-3′ and antisense 5′-CAC AGA CCT CTA GCA TCC AGG-3 ; *Cdk4* sense 5′- TCA GCA CAG TTC GTG AGG TG-3′ and antisense 5′-TCC ATC AGC CGT ACA ACA TTG-3 ; *Cdk6* sense 5′-TGG ACA TCA TTG GAC TCC CAG-3′ and antisense 5′-TCG ATG GGT TGA GCA GAT TTG-3 ; *Cdkn1a* sense 5′- TCT CTT CGG CCC CGT CAA-3′ and antisense 5′-AAA TTC CAC TTG CGC TGA CTC-3 ; *Cdkn1b* sense 5′-CCT GGT GAT GTC CGA CCT-3′ and antisense 5′-CCA TGA GCG CAT CGC AAT C-3.

Actinomycin D assay. For the transcriptional inhibition assays, rat NSCs were seeded onto 6–well plates and treated with actinomycin D (1μM) (Huang et al., 2010). Inhibitor was applied 30 minutes before treating cells with RA, pan- RAR antagonist or both for 24h. Post treatment, cell lysates were collected for gene and protein analysis. ELISA was performed to detect HIF1α protein levels (see 'Supplementary information' for methods). RNA isolation and quantitative real-time PCR analysis (see 'Supplementary information' for methods and primers).

Hippocampal NSC differentiation into neurons or astrocytes. Neural stem cells were seeded onto poly-l-ornithine and laminin coated 24 well plates. Differentiation of neural stem cells was performed using rodent neuron differentiation kit (#SCR035, Millipore). Briefly, cells were grown in neuron differentiation medium composed of neural stem cell basal medium (#SCM003, Millipore) supplemented with inducers of differentiation, 1μM RA and 5 μM Forksolin (#344282, Millipore) and cultured according to manufacturer's instructions (Millipore). To

confirm neural differentiation, neurons generated were immunostained with neuron specific antibody, rabbit anti-β III tubulin (1:500, #ab18207, Abcam) using immunostaining protocol as described in a previous section.

To generate astrocytes, neural stem cells were seeded onto Geltrex ^{TM} Reduced growth factor basement membrane matrix (# 12760, Thermo Scientific) coated 24 well plates in neural stem cell basal medium (#SCM003, Millipore). After 2 days culture medium was changed into astrocyte differentiation medium composed of neural stem cell basal medium (#SCM003, Millipore) supplemented with astrocyte differentiation factors, 50ng/ml BMP4 (-BP-010, R&D Systems), LIF (#LIF1005, Millipore) and 1% heat-inactivated fetal bovine serum (Lonza). Medium was changed every 3-4 days.

Neurons and astrocytes generated *in vitro* were treated with either vehicle (DMSO), RA or RA and pan-RAR antagonist for 24 hours. Cell lysates were then collected and ELISA was performed to measure HIF1α and VEGFA levels (see ELISA analysis).

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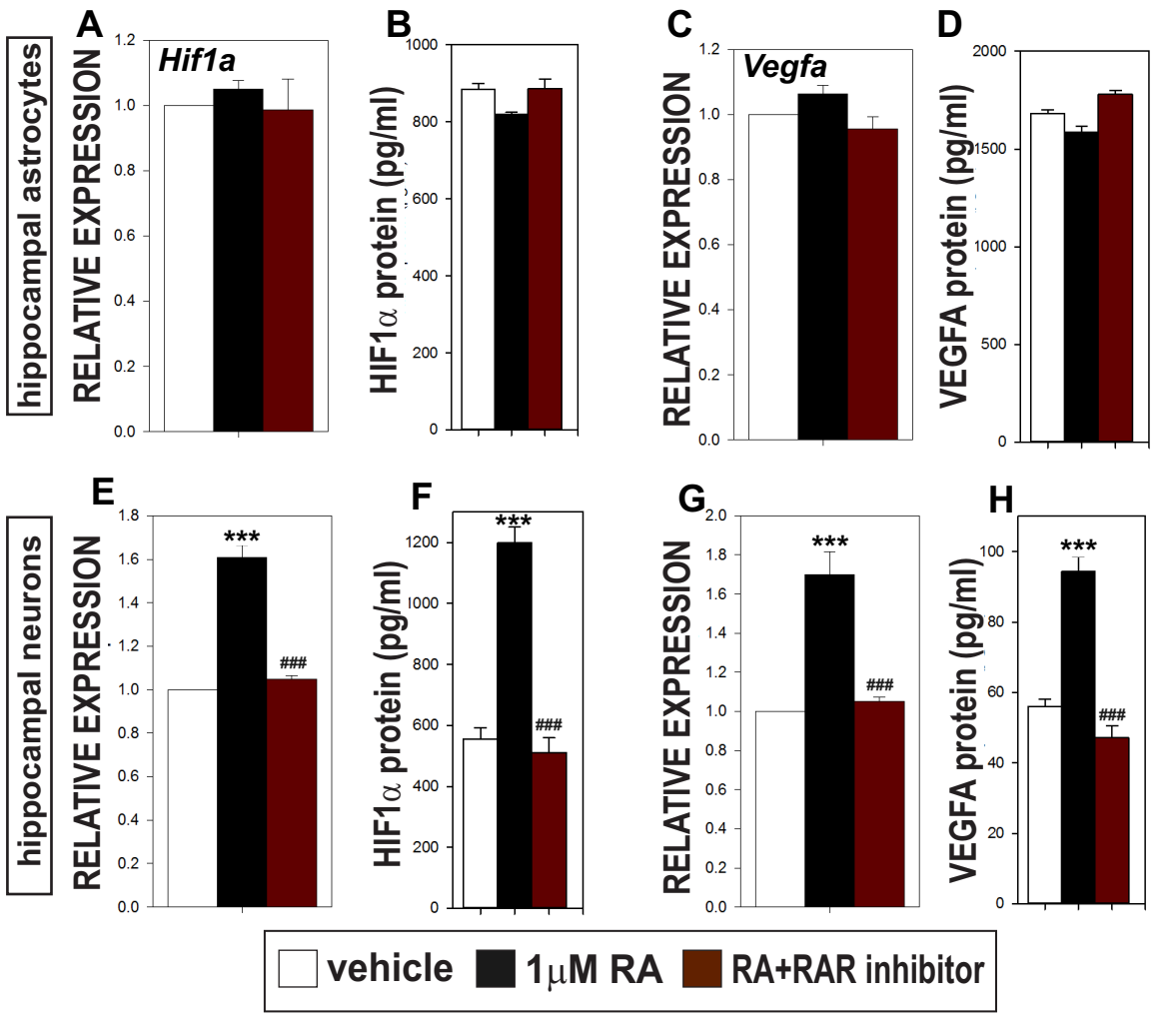

Figure S1 (related to Fig. 2)

RA increases HIF1A and VEGFA expression in neurons but not in astrocytes *in vitro*. (***A-D***) In cultured astrocytes differentiated from adult hippocampal NSPCs, RA does not result in a significant difference in *Hif1a* (**A**) gene or (**B**) protein expression or significantly alter *Vegfa* (**C**) gene or (**D**) protein expression. In neurons differentiated from adult hippocampal NSPCs, RA significantly increases *Hif1a* (**E**) gene and (**F**) protein expression as well as *Vegfa* (**G**) gene or (**H**) protein expression. Data represented as mean±SEM, *p≤0.05, **p≤0.01, ***p≤0.001.

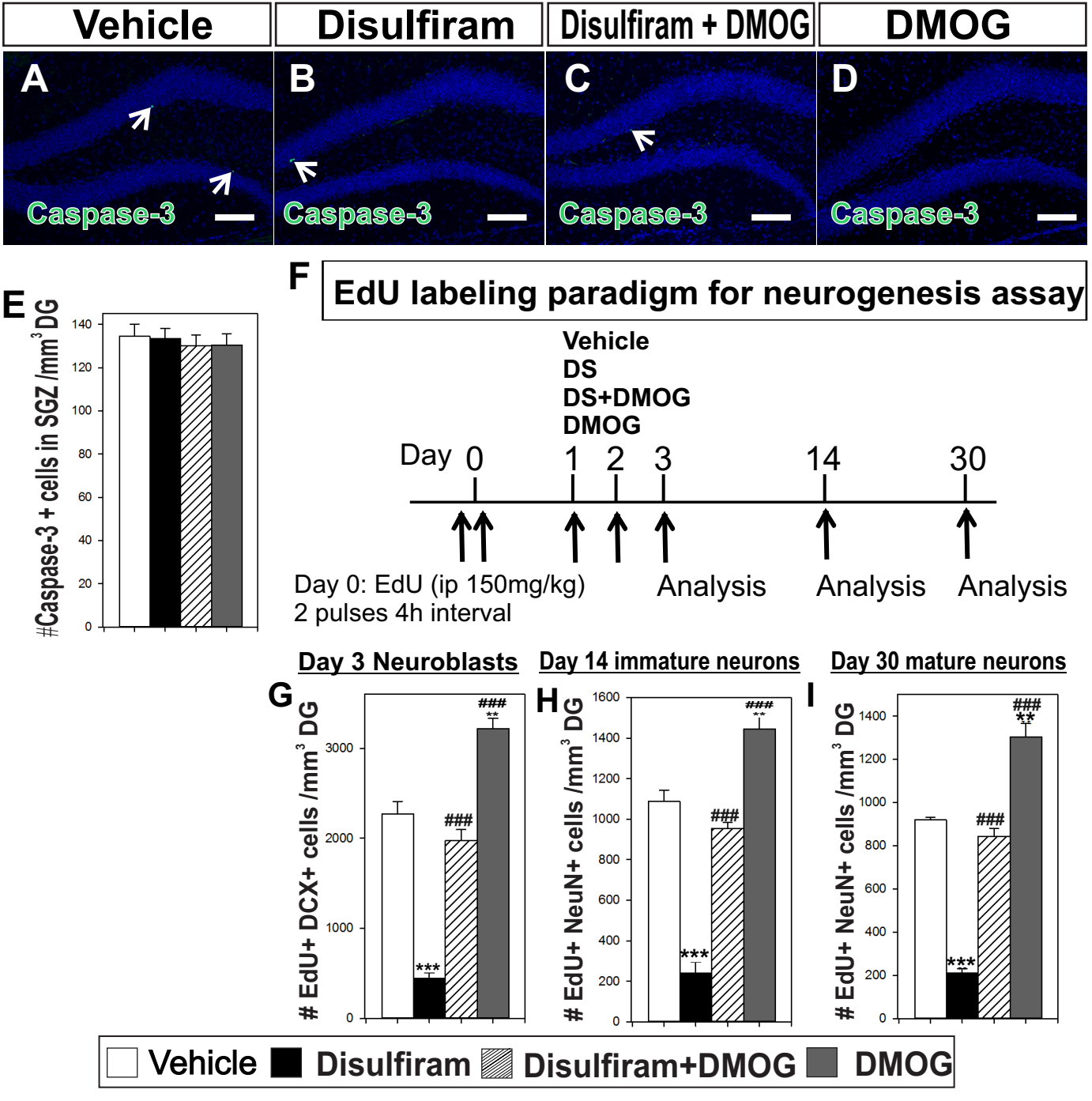

Figure S2. (related to Fig. 4)
A-E. Cell death, as determined by quantification of cleaved-caspase3+ cells in the DG, was unchanged across all treatments. ***F.*** Experimental paradigm describing method to quantify neurogenesis. ***G-I.*** Quantification of new born neuroblasts, immature neurons and mature neurons in the hippocampus across all treatments shows RA-HIF1A pathway regulates adult hippocampal neurogenesis. DG: dentate gyrus, scale bar=100μm, data represented as mean±SEM, *p≤0.05, **p≤0.01, ***p≤0.001)

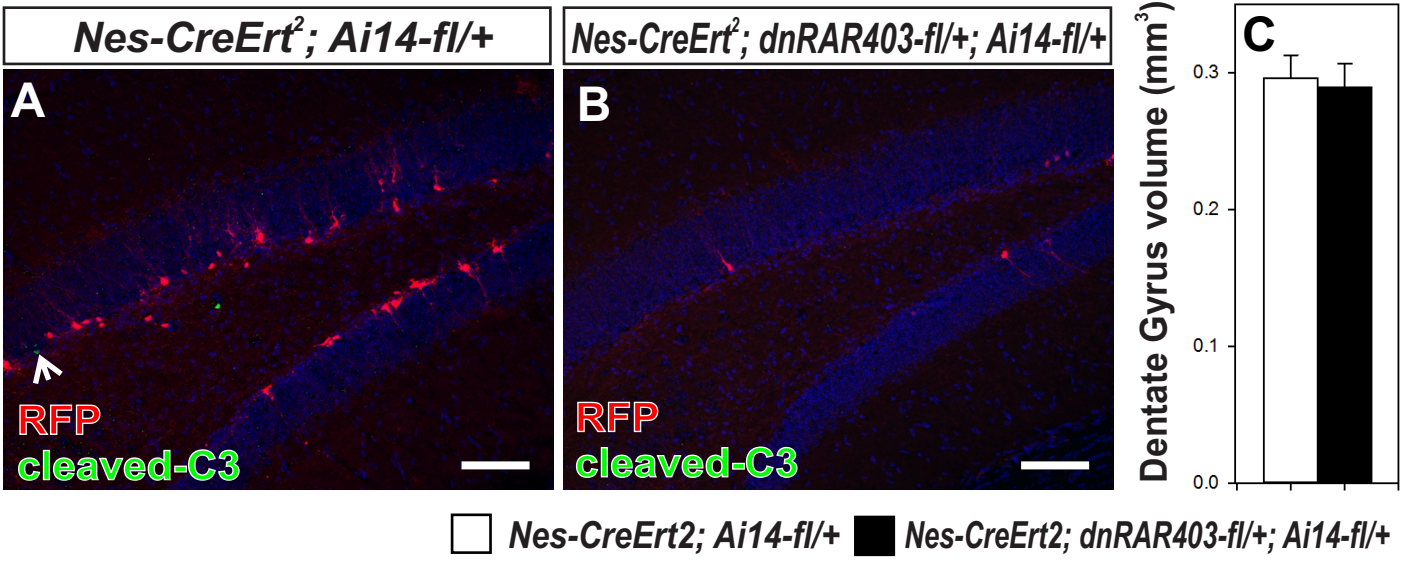

Figure S3. (related to Fig. 6,7)
A-B. Cell death remains unchanged in controls and mutants as evidenced by similar number of RFP+Cleaved caspase3+ cells in the SGZ ***C.*** Dentate gyrus volume remains unchanged in controls and mutants. Scale bar=100μm, data represented as mean±SEM.