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Supplemental Information

Development of Gonadotropin-Releasing Hormone-Secreting Neurons from Human Pluripotent Stem Cells

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Figure S1 (related to Figure 1)

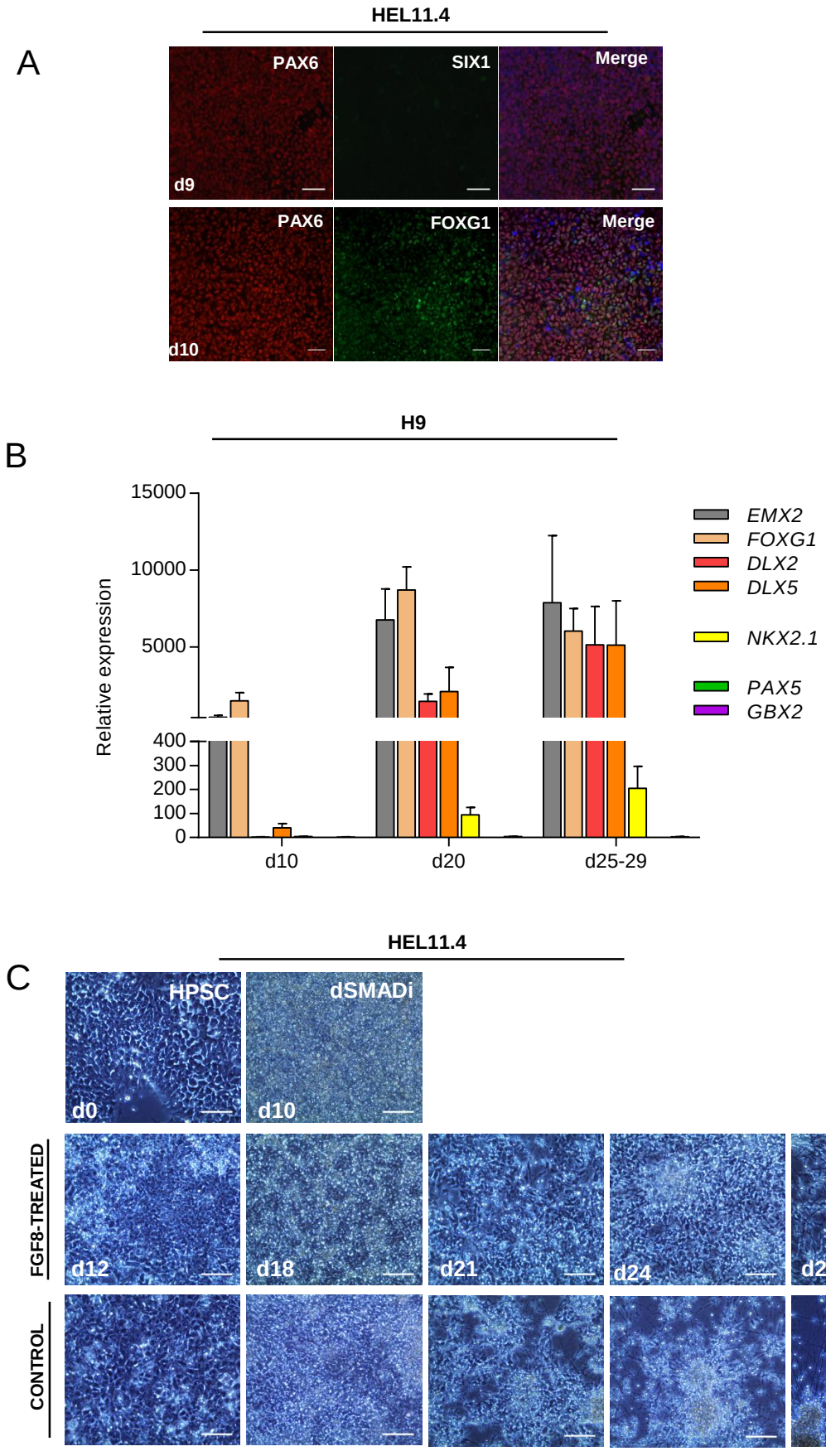
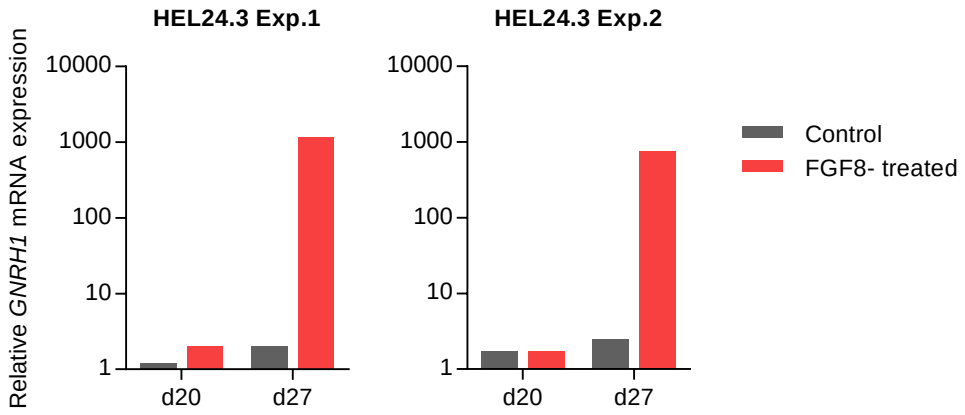


Figure S2 (related to Figure 2)

A



B

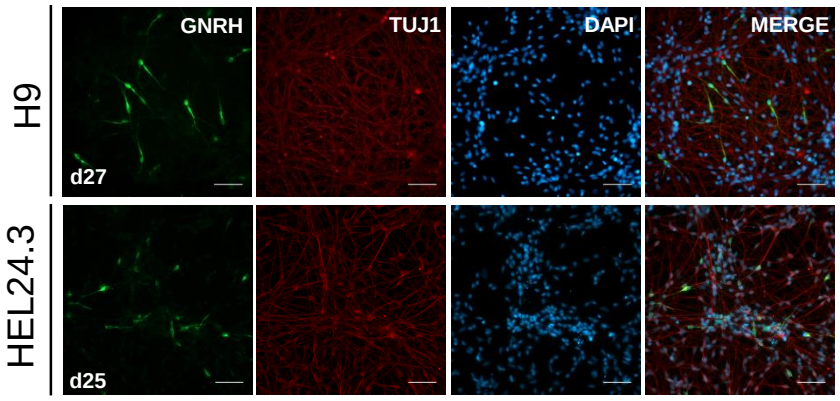


Figure S3 (related to Figure 3)

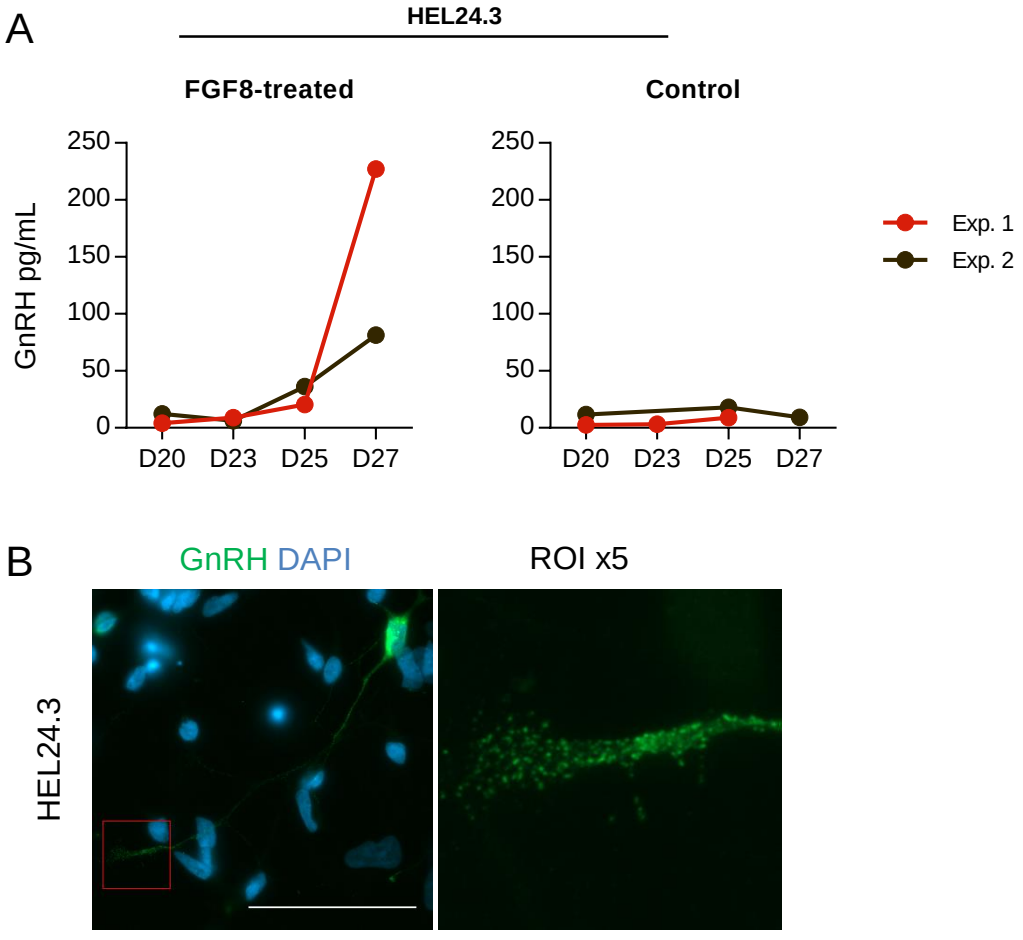


Figure legends

Figure S1. Immunocytochemical characterization after dual SMAD inhibition, and H9-derived cells expression of anterior neural progenitor markers by qPCR during differentiation

A) Immunocytochemistry during the first 10 days of dual SMAD inhibition in HEL11.4-derived cells. PAX6 was detected uniformly throughout the cultures (d9 and 10) and FOXG1 staining was also abundant at d10. In keeping with the qPCR results, expression of preplacodal marker SIX1 was not detected (d9). Scale bar, 50 µm.

B) Relative expression of anterior neural progenitor (*EMX2, FOXG1, DLX2* and *DLX5*), ventral forebrain (*NKX2.1*), and caudal CNS markers (*PAX5* and *GBX2*) at d10, d20 and d25-29 in FGF8-treated H9-derived cells. See Figure 1C for corresponding data from HEL11.4. The relative *FOXG1* expression in H9 cells at d10 appeared higher than in HEL11.4 cells. Bars represent mean + SEM.

C) Representative bright field images (Leica EC3, HiPlan 10x PH objective) of Hel11.4 iPSCs, at specified time points of differentiation. Morphology of FGF8-treated and control cells (CNTRL) are shown. FGF8-treated cells expanded more rapidly as compared to control cells, detectable at days 12, 18 and 24. Scale bar, 100 µm.

Figure S2. GnRH-expressing cells emerge after treatment with FGF8 and DAPT

A) Relative *GNRH1* mRNA expression in HEL24.3 from two individual experiments (See also main Figure 2A).

B) H9-derived, and HEL24.3-derived cells stained by GnRH and TUJ1 at the final stages of differentiation. Scale bar, 50 µm.

Figure S3. GnRH decapeptide secretion in culture medium of FGF8-treated cells.

A) GnRH secretion in HEL24.3 from two experiments, showing increased GnRH secretion in FGF8-treated cells. (See also main Figure 3A).

B) GnRH staining appeared granular, suggesting vesicular packaging of the decapeptide (See also main Figure 3B).

Supplemental Experimental Procedures

Human pluripotent stem cells (hPSCs)

All hPSCs were obtained from intramural Biomedicum Stem Cell Centre core unit (BSCC, University of Helsinki, Finland). HiPSCs HEL11.4 and HEL24.3 were induced at BSCC from healthy donor fibroblasts by Sendai virus cocktail of *OCT4*, *SOX2*, *KLF4* and *C-MYC*. The absence of viral pluripotency factors expression was verified before the onset of differentiation experiments.

All cell culture reagents were purchased from Life Technologies unless stated otherwise. Human embryonic stem cell line H9 (passages 40-50; WiCell) and human induced pluripotent stem cell line HEL11.4 (passages 34-42,(Mikkola et al., 2013)) were maintained on Geltrex®-coated plates with StemPro® (Life technologies) or Essential 8 medium (E8, Life Technologies). hPSCs were passaged by incubating 2-4 minutes in 0.02% EDTA (Sigma) and re-plated at a dilution of 1:4 to 1:6.

Differentiation of hPSCs into neural progenitor cells and GnRH neurons

Cells were maintained throughout the differentiation on Geltrex®-coated plates in N2B27 basal medium (50% DMEM/F12 and 50% Neurobasal medium supplemented with 0.5x N2 and 0.5x B27, 1 mM Glutamax, and 1x Penicillin-Streptomycin (Sigma)). For neural induction, hPSCs were passaged in

StemPro or E8 and re-plated to form a uniform monolayer of cells. When the cells had reached ~95% confluence, medium was replaced with dual SMAD inhibition medium: (N2B27) supplemented with 2 µM Dorsomorphin (BMP inhibitor, Sigma) and 10 µM SB431542 (TGF-β inhibitor, Sigma). On day 10, the cells were dissociated into small clusters by 5 min. incubation with 200 Unit/mL collagenase IV and mechanical scraping, and re-plated at a dilution of 1:2 in dual SMAD inhibition medium supplemented with 10 µM Rock inhibitor Y-27632 (Sigma). At day 11, dual SMAD inhibitors were withdrawn and cells were cultured for 9 days in N2B27 supplemented with 100 ng/mL FGF8 (Peprotech 100-25). On day 20, cells were dissociated with 0.02% EDTA and re-plated at a dilution of 1:6-1:12 in N2B27 with FGF8 to obtain 30-40% confluent layer of small cell clusters. From day 21 onwards, the N2B27 culture medium was supplemented with both 100 ng/mL FGF8 and 20 µM Notch inhibitor DAPT (Sigma). From d0-d21 medium was refreshed daily, and from d21 onwards every 48 hours. Control cells were treated similarly, but without FGF8 supplementation.

RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted using RNA Spin II kit (Macherey-Nagal) according to the manufacturer's instructions, except that DNase treatment was performed separately with RQ1 DNase (Promega), 1U/µg RNA, in the presence of rRNasin RNase inhibitor (Promega), 1U/µL of reaction volume. First-strand cDNA was reverse-transcribed from 1 µg of total RNA using iScript™ cDNA Synthesis Kit (BIO-RAD). Real-time quantitative PCR reactions included 25 ng of first-strand cDNA, HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne), and 0.5 µM forward and reverse primers. Reactions were performed in a LightCycler® 480 (Roche) for 45-50 cycles of 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 20 seconds. The expression levels of target genes were normalized to cyclophilin G. All primer sequences are listed in Table S1.

Immunostainings

Cells were fixed by 4% paraformaldehyde for 10-15 min. at room temperature (RT) and permeabilized in PBS containing 0.5% Triton X-100 (Sigma) for 7 min. Non-specific binding was blocked with UltraVision Protein Block (Thermo scientific) for 10 min. Samples were incubated with primary antibodies overnight at +4°C and with secondary antibodies for 30-45 minutes at RT. Nuclei were counterstained with DAPI (Sigma). Antibodies and DAPI were diluted in PBS containing 0.1% Tween 20 (Sigma). Slides were mounted with Fluoromount^{TM} Aqueous Mounting Medium (Sigma), and images were captured by Zeiss AxioImager.Z1 upright epifluorescence microscopes (Zeiss), or Leica TCS CARS SP8 confocal microscope (Leica) using 20x/NA 0.8, 40x/NA 1.10 and 63x/NA 1.20 HC PL APO CS2 objectives (Biomedicum Imaging Unit), and analyzed with ImageJ (NIH). All antibodies and dilutions used are listed in Table S2.

Quantification of FOXG1 and GnRH positive cells

Quantification of FOXG1 (d21) and GnRH positive cells (d25-29) was based on images taken at 400x magnification (40x objective). Immunopositive cells were counted manually and the relative proportion calculated to the total cell number. Three representative images per experiment were analyzed.

Quantification of secreted GnRH

Cell culture medium was collected concurrently with medium refreshments at indicated time points. Medium was centrifuged shortly and stored in -80°C. Secreted GnRH was quantified by competitive Fluorescent Enzyme Immunoassay (EIA, Phoenix Pharmaceuticals Inc, FEK-040-02) according to manufacturer's instructions, except that undiluted media samples and standard decapeptide (Abcam, ab41769) were diluted in N2B27. GnRH concentrations were determined from the standard curve fitted with four-point parameter logistic nonlinear regression model.

Depolarization by KCl

On d25, the culture medium was refreshed and 16-20 hr later a medium sample was collected. KCl (60 mM) or vehicle (H_2O) was added, the cells were incubated in standard cell culture conditions, and a second medium sample was collected 3 hr later for GnRH analysis with EIA. The experiment was carried out twice in H9 cells and once in HEL11.4 cells. The fold change in GnRH release from KCl-treated over vehicle-treated cells in each experiment was calculated from 2-3 technical replicates.

Gap closure assay

On d20 cells were seeded at high density (1:4 split ratio) onto Geltrex coated 96-well plates containing Oris™ Cell Seeding Stoppers (Platypus Technologies, WI, USA)) to create a 2 mm diameter gap in the middle of the wells. At d25, the stoppers were removed, the cells were washed once with DMEM/F12 to remove unattached cells, and the differentiation media were changed to either N2B27 (Control) or N2B27 and $GABA_A$ receptor agonist muscimol (0.1mM, Sigma) and left to migrate in +37ºC for 50 hrs. At the end of the assay, the cells were fixed and immunostained for GnRH as described above, and the GnRH-positive cells that had entered the uncovered area were counted. Photos of the filled gap area were taken using ZOE™ Fluorescent Cell Imager (BioRad). We counted 2-4 technical replicates (96-wells) per condition per experiment. The assay was carried out four times (twice in H9, and once in HEL11.4 and once in HEL24.3).

Human tissues and immunohistochemistry

A human fetus (8 gestational week) was obtained from voluntarily terminated pregnancy with the parent's written informed consent (Gynecology Hospital Jean de Flandre, Lille, France). Permission to utilize human fetal tissues was obtained from the French agency for biomedical research (Agene de la Biomédecine, Saint-Denis la Plaine, France, protocol n°: PFS16-002). The fetuses were immersion-fixed

in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 1 week, cryoprotected in 30% sucrose in PBS for 48 hours, embedded in Tissue Tek (Miles, Elkhart, IN), and frozen in liquid nitrogen. Human tissues were cryosectioned (Leica cryostat) at 20 μm. Immunohistochemistry was performed as previously reported (Hanchate et al., 2012). Briefly, the sections were rinsed with 0.1 M PBS and incubated at 4°C overnight with the following primary antisera diluted in 0.1 M PBS containing 0.3% Triton X-100 and 10% normal donkey serum: guinea-pig anti-GnRH (1:10000), a generous gift from Dr. Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary), or rabbit anti-GnRH (1:3000), gift from Prof. G. Tramu (Centre Nationale de la Recherche Scientifique, URA 339, Université Bordeaux I, Talence, France)(Beauvillain and Tramu, 1980), and anti-FOXG1. The sections were then washed in PBS and incubated for 1 hour with Alexa-Fluor 488- and 568-conjugated secondary antibodies (Molecular Probes, Invitrogen, San Diego, CA) diluted 1:400 in PBS and counterstained with DAPI nuclear staining (1:10000; Molecular Probes, Invitrogen, San Diego, CA). Sections were mounted using Mowiol (Calbiochem, USA) and analyzed using a LSM 710 confocal microscope (Zeiss).

Statistical analysis

Statistical analyses to compare GnRH gene expression data between FGF8-treated and control cells, and the number of migrated GnRH neurons between muscimol-treated and control cells was performed using paired, two-tailed t-test of log-transformed expression data (ratio t-test) Prism 5.0 (Graphpad). At least 4 independent experiments were included in each analysis.

Table S1. Primers for real-time qPCR

Symbol	Gene name	Primer sequences
PPIG	Peptidylprolyl isomerase G (Cyclophilin G)	ACTCCCAGCCTGCTTCATAC
		TACGTCTGAAACGATCCCTTG
DLX2	Distal-less homeobox 2	GGCGTTTCCAAAAGACTCA**G**
		GCGGTTCTGGAACCAGATTT
DLX5	Distal-less homeobox 5	CGCTAGCTCCTACCACCAGT
		TTTGCCATTCACCATTCTCA
EMX2	Empty spiracles homeobox 2	GCTTCTAAGGCTGGAACACG
		CCAGCTTCTGCCTTTTGAAC
EYA1	EYA transcriptional coactivator and phosphatase 1	TTTCAACTTGGCAGACACACA
		ATGTGCTTAGGTCCTGTCCG
FOXG1	Forkhead box G1	CCGCACCCGTCAATGACTT
		CCGTCGTAAAACTTGGCAAAG
GBX2	Gastrulation brain homeobox 2	GTTCCCGCCGTCGCTGATGAT
		GCCGGTGTAGACGAAATGGCCG
GNRH1	Gonadotropin-releasing hormone 1	GTCAACTGGCAGAAACCCAA
		TGCCCAGTTTCCTCTTCAAT
NKX2.1	NK2 homeobox 1	AACCAAGCGCATCCAATCTCAAGG
		TGTGCCCAGAGTGAAGTTTGGTCT
PAX6	Paired box 6	TTTGCCCGAGAAAGACTAGC
		CATTTGGCCCTTCGATTAGA
PAX5	Paired box 5	GACACCGTGCCTAGCGTCAG
		GAGCCAGTGGACACTATGCTGTG
SIX1	SIX homeobox 1	TAAGAACCGGAGGCAAAGAG
		AGCAGAAGGACCGAGTTCTG
SOX1	SRY (sex determining region Y) box 1	TCTGTTAACTCACCGGGACC
		ACTCCAGGGTACACACAGGG

Table S2. Antibodies for immunocytochemistry

Antigen	supplier	catalog #	species	dilution
FOXG1	Abcam	ab182659	Rabbit	1:1250
TUJ1	Sigma	T8578	Mouse	1:1000
SIX1	Sigma	SAB2106448	Rabbit	1:500
PAX6	DSHB	AB_528427	Mouse	1:50
MAP2	Neuromics	CH22103	Chicken	1:1000
SOX2	Thermo Scientific	MAI-014	Mouse	1:500
Ki67	Novocastra	NCL-Ki67p	Rabbit	1:500
GnRH	Gift from Erik Hrabovszky	1018	Guinea pig	1:16000
Cleaved caspase-3	Cell signaling	9664	Rabbit	1:200
Secondary antibodies				
anti-rabbit IgG Alexa Fluor 488	Life technologies	A21206	Donkey	1:500
anti-mouse IgG Alexa Fluor 594	Life technologies	A21203	Donkey	1:500
anti-chicken IgG Alexa fluor 546	Thermo Scientific	A11040	Goat	1:500
anti-guinea pig Antibody-CF^{TM}594	Sigma	SAB 4600096	Donkey	1:1000
anti-guinea pig Antibody-CF^{TM}488	Sigma	SAB 4600033	Donkey	1:1000

Supplemental references

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