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The stress-induced transcription factor NR4A1 adjusts mitochondrial function and synapse number in prefrontal cortex

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The stress-induced transcription factor NR4A1 adjusts mitochondrial function and synapse number in prefrontal cortex

Abbreviated title: Stress pathway to dendritic spine loss via NR4A1

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The energetic costs of behavioral chronic stress are unlikely to be sustainable without neuronal plasticity. Mitochondria have the capacity to handle synaptic activity up to a limit before energetic depletion occurs. Protective mechanisms driven by the induction of neuronal genes likely evolved to buffer the consequences of chronic stress on excitatory neurons in prefrontal cortex (PFC), as this circuitry is vulnerable to excitotoxic insults. Little is known about the genes involved in mitochondrial adaptation to the build up of chronic stress. Using combinations of genetic manipulations and stress for analyzing structural, transcriptional, mitochondrial and behavioral outcomes, we characterized NR4A1 as a stress-inducible modifier of mitochondrial energetic competence and dendritic spine number in PFC. NR4A1 acted as transcription factor for changing the expression of target genes previously involved in mitochondrial uncoupling, AMPK activation and synaptic growth. Maintenance of NR4A1 activity by chronic stress played a critical role in the regressive synaptic organization in PFC of mouse models of stress (male only). Knockdown, dominant negative and knockout of NR4A1 in mice and rats (male only) protected pyramidal neurons against the adverse effects of chronic stress. In human PFC tissues of men and women, high levels of the transcriptionally-active NR4A1 correlated with measures of synaptic loss and cognitive impairment. In the context of chronic stress, prolonged expression and activity of NR4A1 may lead to responses of mitochondria and synaptic connectivity that do not match environmental demand, resulting in circuit malfunction between PFC and other brain regions constituting a pathological feature across disorders.
SIGNIFICANCE STATEMENT

The bioenergetics cost of chronic stress is too high to be sustainable by pyramidal prefrontal neurons. Cellular checkpoints have evolved to adjust responses of mitochondria and synapses to the build up of chronic stress. NR4A1 plays such role by controlling mitochondria energetic competence with respect to synapse number. As an immediate-early gene, NR4A1 promotes neuronal plasticity but sustained expression or activity can be detrimental. NR4A1 expression and activity is sustained by chronic stress in animal models and in human studies of neuropathologies sensitive to the build up of chronic stress. Therefore, antagonism of NR4A1 is a promising avenue for preventing the regressive synaptic reorganization in cortical systems in the context of chronic stress.
Stress, especially when it is chronic and uncontrollable, produces depressive-like phenotypes and cognitive impairment in animal models (Krishnan and Nestler, 2011; McEwen and Morrison, 2013; McKlveen et al., 2013). Chronic stress impacts neuronal plasticity throughout the brain in part through excessive levels of glutamate and corticosterone (CORT) secretion that remodel dendritic territories in a manner that alters their functional properties (Pittenger and Duman, 2008; Popoli et al., 2012; Myers et al., 2014). Excessive levels of CORT impair neuronal sensitivity to serotonin and neurotrophins, resulting in reduced synapse number and neurotransmission (van Riel et al., 2003; Arango-Lievano et al., 2015). In hippocampus and cortex, chronic stress and CORT inhibit long-term potentiation (Kim and Diamond, 2002; Goldwater et al., 2009), enhance long-term depression (Xu et al., 1997), and produce dendritic atrophy, spine loss and eventually cell death (Sapolsky et al., 1985; Radley et al., 2006; Liston et al., 2013; McEwen and Morrison, 2013).

Responses to acute and chronic stress are both considered adaptive to prepare for current and future demands, as it is paramount for survival (Karatsoreos and McEwen, 2011; Myers et al., 2014). The typical inverted U-shaped relationship between stress/CORT inputs and functional outputs (mitochondrial/synaptic/behavioral) indicates that neuronal perceptions and responses to external signals are flexible to fit with network demands (Picard and McEwen, 2014; Sapolsky, 2015; Jeanneteau and Arango-Lievano, 2016). Neurons use their genomic and epigenomic arsenal to adapt their metabolism in accordance with their connectivity and network activity (Attwell and Laughlin, 2001; Gray et al., 2017). For instance, repetitive trains of excitation during seizures cause mitochondrial dysfunction leading, eventually, to neuronal death (Tang and Zucker, 1997). Modeling studies indicate that
synaptic depotentiation is desirable to support neuronal survival when energetic stores are limited. Synaptic scaling changes mitochondrial functions in response to excessive/depressed glutamatergic excitation (Bindokas et al., 1998). Negative feedback mechanisms may have evolved to suppress synapse potentiation that would drain energetic stores upon glutamatergic over-excitation.

Checkpoints could lie in the genes that control signaling loops between mitochondria and synapses (Li et al., 2004; Jeanneteau and Arango-Lievano, 2016). Hundreds of genes are induced/repressed in response to neuronal activity and stress, and, remarkably, each gene has a fine homeostatic pattern of expression (Valles et al., 2011; Datson et al., 2012; McEwen et al., 2015). These newly transcribed genes play important roles in many aspects of the brain’s adaptive abilities through the regulation of neuronal metabolism, dendritic growth and synapse remodeling. We focused on \textit{NR4A1} (\textit{NUR77/NGFI-B}) because it shuffles between mitochondria, cytosol and nucleus to modify metabolism (Zhao and Bruemmer, 2010; Pawlak et al., 2015), synaptic plasticity (Bridi and Abel, 2013; Chen et al., 2014) and cognition (Hawk and Abel, 2011; McNulty et al., 2012). \textit{NR4A1} has the necessary attributes to adapt mitochondrial functions and synaptic activity in the context of stress, as it is an activity-dependent immediate early-gene responding to a variety of stressors and sensory stimuli (Chen et al., 2014; Helbling et al., 2014). Using complementary genetic approaches, we provide evidence for a causal role of \textit{NR4A1} in mediating stress/CORT-elicited dendritic spine loss in prefrontal cortex (PFC). \textit{NR4A1} acted as transcription factor to change the expression of target genes previously involved in wasting the mitochondrial energetic budget and activating the AMPK catabolic pathway (Steinberg and Kemp, 2009; Weisova et al., 2012). Inappropriate processing of this pathway during chronic stress, as opposed to acute stress, may lead to
responses of mitochondrial function and synaptic connectivity that do not match environmental
demand. As a result, stress-induced circuit malfunction between PFC and other brain regions
may be a pathological feature across disorders (Sampath et al., 2017). This prompted us to
validate results from animal studies to human studies of major depressive disorders (MDD) and
Alzheimer’s disease (AD), as neuropathology in both diseases is aggravated by stress involving
synaptic loss in PFC, disrupted CORT levels and cognitive impairment (Lupien et al., 2009;
Sotiropoulos et al., 2011; Heim and Binder, 2012).

MATERIALS AND METHODS

All experiments were carried out in accordance with the Directive by the Council of the
European Communities (86/609/EEC) and approved protocols (00651.01) following
institutional guideline for the care and use of laboratory animals.

Reagents. Corticosterone, STO609 (CAMKK inhibitor), A769662 (AMPK activator),
glutamate, oligomycin, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) are
from Sigma Aldrich S.A.R.L, Saint-Quentin, France. Doxycycline is from Clontech
laboratories, Saint-Germain-en-Laye, France. BDNF is from Abnova France.
Tetramethylrhodamine methyl ester (TMRM) is from Santa Cruz Biotechnology, Dallas, USA.
Antibodies used were as follows: cyto-NR4A1 (ABIN460855 from antibodies-online GmbH,
Atlanta GA), pan-NR4A1 (E6 from Santa Cruz Biotechnology, Dallas, USA), anti-human
NR4A1 (D63C5), phospho-NR4A1 (S350-P), ubiquitin (P4D1), P190A, phospho-ACC (S79-
P), AMPK and phospho-AMPK (T172-P), HDAC2, S6 are from Cell Signaling Technology,
Ozyme France. Actin (Sigma Aldrich S.A.R.L, Saint-Quentin, France), FKBP51 and HSP90
(BD Biosciences-Europe), GAPDH (ThermoFisher Scientific, Waltham, MA), GFP and Drebrin (M2F6) are from Abcam, Cambridge, UK. RFP (Rockland Immunochemicals, Limerick, PA), PSD95 (NeuroMab Antibodies Inc, Davis, CA), Synaptophysin (Life Technologies Applied Bioscience, Carlsbad, CA), NR1 (ab1516) and NR2 (ab1548) are from Merck Millipore, St Quentin en Yvelines, France.

Rodent models. Thy1-YFP transgenic mice (B6.Cg-Tg(Thy1-YFP)HJrs/J (Feng et al., 2000)) and NR4A1 knockout mice (B6;129S2-Nr4a1tm1Jmi/J (Lee et al., 1995)) were grown in pure C57Bl6 background. Time-pregnant CD1 mice (Janvier Labs, Genest Saint Isle, France) were utilized for in utero electroporation experiments. Frozen brain tissues from Nr4a1 deficient rats (Nr4a1tm1Mciwi) grown in Fawn-Hooded Hypertensive (FHH) background (Transposagen Biopharmaceutical Inc. and the NIH Rat Knockout Consortium Program http://www.transposagenbio.com/knock-out-rat-consortium). All animals were allowed ad libitum access to food, water, and maintained on a 12-h light-dark cycle. Males were used in all protocols. Chronic unpredictable stress includes one of the following daily random stressors (wet bedding, no bedding, food deprivation, crowded cage, 2h or 6h restrain, forced swim, shaking, 24-hr light cycle, tail suspension) for 10 consecutive days from PND21. Doxycycline (2 mg/ml) was administered via the drinking water and refreshed every 3 days.

Tail suspension test. One-month-old mice were subjected to a single tail suspension test on the day before sacrifice. For habituation, the mouse-tail was taped 5 minutes before suspension to a hook located in a 56-cm x 40-cm x 33-cm dark box. Frontal visual inspection of immobility postures was measured using a timer. Slight movements of hind limbs only were
also considered immobility as described (Can et al., 2012). Immobility in the tail suspension test (TST) is a typical measure of behavioral despair modified by long-term administration of CORT and chronic stress (Krishnan and Nestler, 2011).

**In-utero electroporation.** One μg of DNA was injected in the ventricle at E15 on CD1 mouse embryos and electroporated (NEPA21, Nepagene, 30V, pON 50 msec, pOFF 950 msec, 5 pulses) as described (Arango-Lievano et al., 2015). Mice were anesthetized with 4% isoflurane/oxygen and maintained at 1.5–2% isoflurane (Abbott laboratories, Chicago, IL) throughout surgery using TEC3N (Anesteo, Lunel, France). Mice received preemptive analgesia with Lidocaïne (Xylovet®, 3.5 mg/kg at incision site). A subcutaneous injection of the analgesic buprenorphine (Buprecare®, 0.05 mg/kg) was administered post-surgery and the next day. Expression of transgenes in experimental animals generated by in utero electroporation was stable in adolescents but faded before reaching adulthood (Arango-Lievano et al., 2016). Due to this technical reason, adolescent mice were used throughout the study.

**Microdissection and lysate preparation.** Animals were anesthetized with pentobarbital (for mice 50 mg/kg; for rats 100 mg/kg, i.p., Ceva santé Animale, Libourne, France) prior to decapitation. Cortical biopsies were punched out of fresh mouse brain sections (200 μm) on ice using a punch set and snap frozen in liquid nitrogen. Tissue homogenates were lysed in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 1% NP40, 0.1% SDS, 0.1% triton X-100 complemented with protease inhibitors, 1 mM Na3VO4, 10 mM NaF and 10 nM
calyculin A. Protein samples for Western blot analysis were cleared from debris by centrifugation (14,000 rpm for 10 minutes).

**Cell culture and lysis.** Time-pregnant Sprague Dawley rats (Janvier Labs, Genest Saint Isle, France) were sacrificed by narcosis to prepare primary E18 cortical neurons cultured on glass coverslips coated with poly-D-lysine, and maintained in Neurobasal medium containing B27 supplement, 0.5 mM L-glutamine, 5-fluorouridine and uridine (10 μM each) (Life Technologies Applied Bioscience, Carlsbad, CA) as previously (Lambert et al., 2013). HEK 293 cells were grown in DMEM containing 10% FBS. Cells lysates were prepared in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP40, 0.1% SDS plus protease inhibitors, 1 mM Na2VO4, 10 nM calyculin A and 10 mM NaF and cleared (14,000 rpm for 10 min). Primary cortical cultures were grown for 3 weeks (DIV21) before analysis of spine morphology and biochemistry.

**DNA and transfections.** Luciferase-reporter constructs consist of a minimal POMC inactive promoter used as inactive control and 3x tandem NurRE-POMC fusion (gift from J. Drouin, IRCM, Montreal). Short hairpin (sh)RNA plasmids against murin nr4a1 and scramble sequences are from GeneCopoeia (Tebu-Bio, Le Perray-en-Yvelines, France). NR4A1 cDNA (gift from P. Tontonoz, UCLA, USA) was subcloned in pSLIK-venus under the control of a doxycycline-inducible promoter (ATCC repository, USA). *In vitro* electroporation of plasmid constructs of primary cortical neurons were performed with the AMAXA system according to manufacturer’s instructions (Lonza, Levallois-Perret, France). HEK 293 cells were transfected with Lipofectamine 2000 (Life Technologies Applied Bioscience, Carlsbad, CA, USA).
Mutagenesis. NR4A1 mutants (R337A, ΔNLS, S340A, S350A) were made by site-directed mutagenesis using Quickchange (Agilent Technologies, Les Ulis, France). Deletion mutant ΔAF1 was generated by PCR and subcloned into pSLIK-venus. All constructs were verified by sequencing.

NR4A1 transcriptional activity. NurRE-luc together with renilla plasmids (10:1 ratio) were electroporated in primary neurons or in layer II/III cortical neurons in vivo together with GFP (10:1:2 ratio). Luciferase/ Renilla activities were assessed with Dual luciferase reporter assay according to manufacturer’s instructions (Promega France) from extracts of primary neurons or GFP-positive biopsies of cortex microdissected under a Leica MZ16F fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland) from PND31 mice anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused at a rate of 3 ml/min through the ascending aorta with 30 ml of ice-cold 0.9% NaCl prior to decapitation.

Nuclear Fractionation. To purify nuclear extracts, neurons were rinsed in ice cold PBS and incubated for 15 min on ice with buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT with protease and phosphatase inhibitors) before harvesting. 0.5% NP40 was added to the cells for 3 min on ice and lysates were centrifuged for 1 min at 13000 rpm. The resulting supernatant was stored as the cytoplasmic fraction and the pellet was further rinsed in 1 ml of buffer A. The pellet was vortexed for 20 min in 50ml buffer B (20 mM HEPES-NaOH, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA with protease and phosphatase inhibitors) and cleared by centrifugation for 10 min at 13,000 rpm. The
resulting supernatant was collected as the nuclear fraction and purity assessed using markers HSP90 and HDAC2.

**Immunoprecipitation.** Protein concentrations were measured with Bradford assay against BSA standards (ThermoFisher Scientific, Villebon sur Yvette, France). Polyclonal antibodies against pan-NR4A1 (E6 from Santa Cruz Biotechnology, Dallas, USA) were used for immunoprecipitation with protein A-conjugated magnetic beads (ThermoFisher Scientific, Villebon sur Yvette, France) and Western blot for detecting Ubiquitin (P4D1 from Cell Signaling Technology, Ozyme France), cyto-NR4A1 (ABIN460855 from antibodies-online GmbH, Atlanta GA) and pan-NR4A1 by chemiluminescence (ECL, GE Healthcare Life Science, Velizy-Villacoublay, France). Densitometric analysis of grayscale images was performed with NIH ImageJ.

**TMRM Fluorescence Live Imaging.** Imaging of TMRM (50 μM), quenching mode (in 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH2PO4, 15 mM glucose, 1.2 mM CaCl2, 5 mM NaHCO3, 1.2 mM Na2SO4, 20 mM HEPES, pH = 7.4) with an inverted fluorescence microscope (IX70, Olympus France) coupled with a Coolsnap HQ camera (Roper scientific SARL, Lisses, France) from primary cortical neurons electroporated with the indicated construct and seeded on poly-D-lysine coated Ibidi bottom dishes (Biovalley, Nanterre, France). GFP-positive cells were identified prior recording changes of TMRM fluorescence overtime (AF = F-F0/F0x100, where F = fluorescence intensity at any time point, F0 = baseline fluorescence) by illumination at 555 nm and detection at 570 nm (Metaflour software, Molecular devices, Sunnyvale, California). Background intensity was subtracted from whole-cell intensity and F0 was
measured as average normalized fluorescence emitted during the 120 s prior to glutamate
treatment. Images were captured every 5 seconds. For quantification, we extracted the average
of the last 3 minutes of each epoch (100 μM glutamate, washout, 1 μM oligomycin, 10 μM
FCCP) due to the lag of time during perfusion of drugs or rinse with culture medium.
Statistical comparisons between groups reported differences during epochs (glutamate N= 16
GFP, 27 NR4A1, 7 ΔAF1; washout N= 16 GFP, 27 NR4A1, 7 ΔAF1; oligomycin, N= 10 GFP,
10 NR4A1, 7 ΔAF1; FCCP, N= 10 GFP, 10 NR4A1, 7 ΔAF1).

ATP concentrations. Whole cell ATP levels were monitored using the ATP Bioluminescence
assay kit HSII according to manufacturer’s instruction (Sigma Aldrich S.A.R.L, Saint-Quentin,
France). ATP levels were normalized to the total amount of proteins dosed with Bradford assay
against BSA standards (ThermoFisher Scientific, Villebon sur Yvette, France).

GOLGI staining and dendritic Spine Studies. Animals were anesthetized with pentobarbital
(50 mg/kg, i.p.) and perfused at a rate of 3 ml/min through the ascending aorta with 30 ml of
0.9% NaCl prior decapitation. The FD-Rapid GolgiStain Kit was used according to
manufacturer’s instruction (FD-Neurotechnologies, GENTEAUR France) and labelled brain
slices imaged with transmitted light on AxioImager-Z1 (Carl Zeiss, Iena, Germany) equipped
with x100 oil-immersion objective. Fluorescence images from blinded groups were taken on
LSM510 laser-scanning confocal microscope (pinhole set to 1 airy unit) (Carl Zeiss, Iena,
Germany) equipped with x63 Plan-Neofluor NA1.3 oil-immersion objective and digital zoom-
8. Z-stack images were processed using ImageJ (NIH). Laser excitation, fluorescence emission
capture, pinhole were held constant throughout the study. Dendritic segments included in the
analysis met the following criteria: (i) be parallel or at acute angles relative to the coronal surface of sections to allow unambiguous identification of spines, (ii) segments had no overlap with other branches, (iii) dendritic segments from apical tuft were imaged in cortical layer 1 distant from about 100 μm from pyramidal neuronal soma in layer 2, and (iv) dendritic segments from apical tuft were imaged in cortical layer 1 distant from about 200 μm from pyramidal neuronal soma in layer 3. Total length of dendrites analyzed for dendritic spine density exceeded 100 μm per mice, which exceed 500 μm per group depending on the number of animals. No more than 2 dendritic segments per cell (which branch order was not recorded) were scored in a total of 10-15 cells per group. Thin, mushroom and stubby spines were counted as one unique category and filopodia disregarded, as they were extremely rare at PND31. A total exceeding 1000 dendritic spines from at least 20-30 dendritic segments were counted per group, which depended on the number of animals per group (N is indicated in figures legends). The number of dendritic spines enumerated at pyramidal cortical neurons depends on methodology, species and age as previously reported (Cerqueira et al., 2007; Radley et al., 2008; Bloss et al., 2011; Anderson et al., 2016). For example, the range is 15-30 spines/10 μm at apical tuft dendrites of PFC in adolescent mice monitored by fluorescence confocal microscopy in figure 1 and as described (Radley et al., 2008; Swanson et al., 2013) whereas it is 6-13 spines/10 μm at apical tuft dendrites of PFC in adolescent mice monitored in Golgi-Cox impregnated neurons imaged by transmitted light microscopy in figure 2 and as described (Cerqueira et al., 2007). In vitro after 3 weeks in culture, the range is 3-7 dendritic spines/10 μm in primary cortical neurons in figure 6F and as described (Arango-Lievano et al., 2015).
Histochemistry. Animals were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused at a rate of 3 ml/min through the ascending aorta with 30 ml of 0.9% NaCl, followed by 30 ml of 4% ice-cold PFA. Brains were harvested and post-fixed for 2 h and equilibrated in 30% sucrose (Sigma Aldrich S.A.R.L, Saint-Quentin, France). Free-floating coronal sections rinsed in PBS were blocked in 5% normal goat serum, 5% normal horse serum, PBS, 0.1% triton X-100 for 2 h at 25 °C. Primary antibodies (GFP 1:3,000; RFP 1:2,000; pan-NR4A1 1:400) were incubated for 2 days at 4 °C and secondary antibodies (ThermoFisher Scientific, Villebon sur Yvette, France 1:2,000) for 2 h at 25 °C. Cultured cells were fixed in 4% PFA, 20% sucrose in PBS for 10 min at 25 °C. After quenching the fixative with 50 mM NH₄Cl in PBS, cells were permeabilized with 0.1% triton X-100 in PBS for 3 min, blocked with 10% goat serum, 2% BSA and 0.25% fish skin gelatin in TBS for 30 min and then incubated with antibodies for 3 h in blocking solution at room temperature. Cells were washed in TBS, 0.25% fish skin gelatin and mounted in mowiol (Sigma Aldrich S.A.R.L, Saint-Quentin, France). Antibodies against p-NR4A1 and cyto-NR4A1 did not work for immunostaining. This is why quantitative analyses were performed by Western blot. NR4A1 antibodies recognized two majors bands at 60 and 75 Kda, which disappear in KO cultures (Chen et al., 2014). Post-translational modifications like phosphorylation, ubiquitination, sumoylation explain, in part, the shift of molecular weights (Hazel et al., 1991; Chen et al., 2014; Zhang et al., 2017).

RNA extraction. Total RNA from primary neurons was extracted with TRIzol (ThermoFisher Scientific, Villebon sur Yvette, France) and cDNA was synthesized from 1 μg of RNA using the First-Strand cDNA Synthesis Kit for Real-Time PCR (USB, ThermoFisher Scientific, Villebon sur Yvette, France) and random primer mix (USB, ThermoFisher Scientific, Villebon...
Quantitative PCR. Hot-start SYBR Green PCR kit (Qiagen France SAS) was used in 10 μL reactions containing 2.5 μL of cDNA, and 100 nM primer mixture. Rodent specific primers are as follow: *drp1* 5'-TGATGGGAAGGGTTATTCA-3' and 5'-TGGCCAGATGGGTACTTC-3'; *mfn1* 5'-TCGCAGTCAGCAGTGAAAC-3' and 5'-TGCCACGTTTACTGAGTCCA-3'; *mfn2* 5'-AGGAAATTGCTGCCATGAAC-3' and 5'-TGTTGAGTTCGCTGTCCAAC-3'; *opa1* 5'-GGACCCAAGACAGTGTGTT-3' and 5'-GTTTCCTCCGGACTGTGGA-3'; *fis1* 5'-GCCTGGTTCGAAGCAAATAC-3' and 5'-CACGGCCAGGTAGAAGACAT-3'; *ucp2* 5'-ACAAGACCATTGCACGAGAG-3' and 5'-ATGAGGTTGGCTTTCAGGAG-3'; *ucp4* 5'-TCTCACAAAAACCGACTCC-3' and 5'-ACCATCCGACCTCCAGAGTA-3'; *ucp5* 5'-GGAATGCTGGGAGACACAAT-3' and 5'-GTCCCACTATTGCCCTCTGA-3'; *gapdh* 5'-cctgcaccaccaacctccct-3' and 5'-ctgtggtcatgagcccttcc-3'; human *nr4a1.3* 5'-tttagttctcgttctgtgg-3' and 5'-attatecggtactccggtca-3'; human *tubb* 5'-cagggcttgccagtccactc-3' and 5'-gtgagggcactgaaggttg-3'. Q-PCR was performed with ABI 7900 instrument (Applied Biosystems, ThermoFisher Scientific, France), followed by melt-curve analysis. Fold changes in gene expression were calculated using ΔΔCt (Ct = cycle number at threshold) analytical method that includes normalization against house-keeping gene human *TUBB* (encoding β-tubulin) or rodent *GAPDH*.

Human Studies. AD: Frozen tissues corresponding to Brodmann areas 9-10 (medial anterior prefrontal cortex) from 24 cases diagnosed with AD (12 moderate: 7 females/ 5 males + 12 severe: 8 females/ 4 males) and 17 healthy cognitively normal controls (6 females/ 11 males).
were obtained from the Rush Religious Orders Study, the University of Pennsylvania Brain
Bank (Center for Neurodegenerative Disease Research), the Harvard Brain Bank (Harvard
Brain Tissue Resource Center), and the Emory Brain Bank (Center for Neurodegenerative
Disease) as previously described (Ginsberg et al., 2010b; Ginsberg et al., 2010a; Arango-
Lievano et al., 2016) with a pre-mortem clinical diagnosis and cognitive assessment scores
collected within one year prior to death using the Mini-Mental State Exam (Folstein et al.,
1975). A board certified neuropathologist blinded to the clinical diagnosis performed a
neuropathological diagnosis based on established criteria (Braak and Braak, 1991; Hyman and
Trojanowski, 1997). Exclusion criteria included argyrophilic grain disease, frontotemporal
dementia, Lewy body disease, mixed dementias, Parkinson's disease, and stroke. Subject
groups matched as closely as possible for age and postmortem interval (PMI) (for detail see
Figure 9-1). Medications of persons during life were not accessible as per HIPAA guidelines
for a pathological study. Informed consent was obtained for all subjects. This study is
performed under the guidelines of the Nathan Kline Institute and the New York University
Langone Medical Center. Tissue samples were processed as described (Ginsberg et al., 2000;
Counts et al., 2004) and Western blots repeated 5 times for each sample to ascertain
reproducibility and minimize experimental bias. Experimentalists were blind to the groups.
Digitalized data were measured by optical densitometric analysis with NIH imageJ, subtracted
of background and normalized to GAPDH levels.

MDD: Frozen tissues corresponding to Brodmann area 9 (medial prefrontal cortex) from 27
depressed subjects (14 females/ 13 males) and 27 psychiatrically healthy control subjects (14
females/ 13 males) matched for sex and as closely as possible for age and PMI were obtained
from the University of Pittsburgh Brain Tissue Donation Program. All tissue samples were
obtained at autopsy following consent from the next of kin. All depressed subjects met diagnostic criteria for MDD according to the *Diagnostic and Statistical Manual of Mental Disorders IV* (1994). Control subjects did not meet criteria for an axis I disorder at any time in their lives. Subject groups did not differ in mean age, PMI, brain pH, RNA integrity number (RIN), or tissue storage time (for details see Figure 8-1). Informed consent was obtained for all subjects. Details of medications of persons during life were not accessible as per HIPAA guidelines for a pathological study. This study is performed under the guidelines of Yale University School of Medicine. Total RNA (500 ng) extracted from human PFC was reverse-transcribed into cDNA using random hexamer primer mix and SuperScriptIII qRT-PCR kit (Life Technologies Applied Bioscience, Carlsbad, CA, USA) (Duric et al., 2010). Experimentalists were blinded to the subject groups. A combination of factors accounted for the reduced number of samples (N= 25 controls and 24 MDD) in the qPCR analysis (e.g., low signal strength or statistical outliers based on standard deviation).

**Statistics.** Data were compared using 2-tailed Student’s *t*-test, correlated with Pearson r for comparing 2 sets of variables. Cumulative distribution comparing 2 variables was performed with kolomogorov-Smirnov (K-S) test. We used a factorial ANOVA to compare multiple groups, using stress status, CORT treatment, genotypes, NR4A1 constructs, doxycycline treatment serving as independent factors, followed by *post-hoc* pairwise comparison with appropriate tests (Sidak’s, Dunnett and Bonferroni) conducted with Prism 6.0 (GraphPad Software, La Jolla, CA). All data are shown as means ± standard error of the mean (SEM). Statistical significance was set at *p* < 0.05. Statistical outliers 2-fold higher than standard deviation were removed from analysis of *Nr4a1* mRNA in MDD cohort. No data from animal
studies were removed from analyses. For in vivo experiments, the number of dendritic spines was averaged per animal, and per group (e.g., 1-2 dendritic segments/neuron, 5-7 neurons/mouse, N= 5-7 mice as per group conditions). For in vitro experiments, the number of culture dishes per group was equivalent to the number of independent experiments. Estimates of sample size were calculated by power analysis based on preliminary data. Sample size was chosen to ensure 80% power to detect the pre-specified effect size. Animals and culture dishes were attributed to various experimental groups in a random fashion. All data collected in animals were from littermate controls and averaged per experimental groups. Knockout animals were bred as heterozygotes to generate homozygote littermates. All data collected in vitro were replicates from independent experiments and averaged per experimental groups.

Pre-established criteria for stopping data collection included: (i) mice electroporated in the wrong cortical regions; (ii) mice reaching ethical endpoint limits; (iii) unexpected mortality (e.g. 1 mouse NR4A1 KO injected with CORT died); (iv) brains badly perfused and unusable for histological studies.

RESULTS

NR4A1 caused dendritic spine attrition in vivo via a genomic mechanism.

To test if NR4A1 regulates dendritic spine number post-development in vivo when it is most expressed (Figure 1-1 and (Chan et al., 1993; Davis and Puhl, 2011)), we used a doxycycline-inducible construct to achieve temporal control of NR4A1 expression between postnatal day (PND) 21 and PND31 (Figure 1A). Analysis of the apical tuft dendrites in NR4A1 overexpressing pyramidal neurons in PFC and S1 at PND31 indicated that spine number was reduced upon exposure to doxycycline (2-way ANOVA for effect of doxycycline: $F_{(2,24)} =$
31.12, \( p < 0.0001 \), post hoc Bonferroni’s test comparing 0d with 3d \( p = 0.0168 \) and 0d with 10d \( p < 0.0001 \), Figure 1B). This was not an artifact of doxycycline given that neurons expressing GFP alone maintained similar spine density in the presence or absence of treatment (unpaired t-test, \( t(9) = 0.66, p = 0.52 \), Figure 1C). These spines contain PSD95 puncta juxtaposed with synaptophysin puncta suggestive of functional connectivity (Figure 1-2).

To determine if NR4A1 used a genomic mechanism to reduce dendritic spine number in vivo, we electroporated doxycycline-inducible NR4A1 mutants: (i) \( \Delta AF1 \) lacks the transactivation domain (Calnan et al., 1995) and (ii) R337A cannot bind to DNA (Meinke and Sigler, 1999). Both mutants induced from PND21 to PND31 failed to diminish dendritic spine coverage in pyramidal neurons contrary to the wildtype (main effect of NR4A1 constructs by 2-way ANOVA \( F_{(3,36)} = 9.82, p < 0.0001 \); effect of interaction of doxycycline with NR4A1 constructs \( F_{(3,36)} = 5.95, p = 0.0021 \), post hoc Bonferroni’s test for the effect of WT: \( p = 0.0031 \), \( \Delta AF1: p = 0.12 \), and R337A: \( p = 0.97 \), Figure 1C). These data indicated that NR4A1 required its transactivation domain for dendritic spine attrition in vivo.

**Stress and CORT-mediated dendritic spine loss in cortex required NR4A1.**

NR4A1 expression in the adult brain is very sensitive to stress and CORT (Helbling et al., 2014), implying that it could contribute to dendritic spines attrition in such contexts. To address this possibility, we employed \( \Delta AF1 \) as dominant negative to block dendritic spine loss caused by chronic unpredictable and uncontrollable stress (main effect of stress compared to controls: 2-way ANOVA \( F_{(1,19)} = 39.89, p < 0.0001 \), post hoc Bonferroni’s test \( p = 0.0015 \), Figure 1C). Induction of \( \Delta AF1 \) in mouse cortex with doxycycline supplied during the entire stress paradigm from PND21 to PND31 preserved dendritic spines compared to induction of
GFP (main effect of ΔAF1 by 2-way ANOVA $F_{(1,22)} = 7.1, \ p = 0.0014$; interaction of
doxycycline with ΔAF1 $F_{(1,22)} = 9.426, \ p = 0.0056$, post hoc Bonferroni’s test for the effect of
ΔAF1: $p = 0.0018$ and GFP: $p > 0.99$, Figure 1C). To confirm this result, we aimed at silencing
the endogenous NR4A1 expression with shRNA sequences that are efficient at downregulating
recombinant NR4A1 in vitro (Figure 1D). The most efficient sequence, sh#1 was introduced in
cortex by in utero electroporation during embryogenesis and compared to its scramble (sc)
sequence as control. Sh#1 prevented stress-induced spine loss (main effect of sh#1 by 2-way
ANOVA $F_{(1,21)} = 52.3, \ p < 0.0001$; main effect of stress $F_{(1,21)} = 14.53, \ p = 0.001$; interaction of
sh#1 with stress $F_{(1,21)} = 4.72, \ p = 0.04$, post hoc Bonferroni’s test on scramble group $p =
0.0018$ and sh#1 group $p > 0.99$, Figure 1E), uniformly at the apical tuft dendrites (Figure 1-
3A). Sh#1 also prevented CORT-mediated spine loss (main effect of sh#1 by 2-way ANOVA
$F_{(1,21)} = 36.02, \ p < 0.0001$; main effect of CORT $F_{(1,21)} = 13.15, \ p = 0.0016$; interaction of sh#1
and CORT $F_{(1,21)} = 2.3$ post hoc Bonferroni’s test on scramble group $p = 0.0017$ and sh#1
group $p = 0.4$, Figure 1F), uniformly at the apical tuft dendrites (Figure 1-3B). These results
indicate that dendritic spine plasticity to stress and CORT depended on NR4A1 expression.

Knockout of NR4A1 protected against adverse effects of chronic CORT exposure.

We sought confirmation in NR4A1 knockout mice using the chronic CORT exposure model.
Morphological analysis of Golgi-Cox impregnated pyramidal neurons (Figure 2A) showed that
the distribution of dendritic spines (Fig 2B) and overall density in PFC was denser in +/- than
+/+ mice after chronic CORT exposure (main effect of genotype by 2-way ANOVA $F_{(1,16)} =
26.37, \ p < 0.0001$; main effect of CORT $F_{(1,16)} = 105.6, \ p < 0.0001$; interaction of genotype and
CORT $F_{(1,16)} = 19.2, \ p = 0.0005$, post hoc Bonferroni’s test $p < 0.0001$ and effect of CORT
injection $F(1,16) = 105.6$ post hoc Bonferroni’s test $p < 0.0001$, Figure 2C). An other typical adverse effect of chronic CORT exposure is behavioral despair in the tail suspension test (Nestler and Hyman, 2010) in which -/- mice performed better than +/+ mice (main effect of genotype by 2-way ANOVA $F(1, 91) = 26.34$, $p < 0.0001$, post hoc Sidak’s $t$-test comparing total time immobile between genotypes $p < 0.05$, Figure 2B). These data indicated that knockout of NR4A1 protected against some adverse effects of chronic CORT exposure.

Chronic stress and CORT exposure increased NR4A1 activity in mouse cortex.

Knockdown, knockout and dominant negative data, all suggested that NR4A1 could be more active upon stimulation of the glucocorticoid stress pathway than at rest. To monitor NR4A1 activity in pyramidal neurons of PFC, we established a transcription-based luciferase assay in vivo. A synthetic promoter harboring 3x tandem NR4A1-responsive elements (NurRE) upstream of luciferase showed endogenous activity in mouse cortex at rest at PND31 compared to cortical tissue electroporated with the minimal promoter lacking NurRE (unpaired $t$-test, $t(8) = 5.63$, $p = 0.0005$, Figure 3A). Using this assay, we found that chronic CORT exposure and stress increased activity of neuronal NR4A1 in mouse cortex compared to controls at rest (acute stress: unpaired $t$-test, $t(10) = 2.46$, $p = 0.033$; chronic stress: unpaired $t$-test, $t(10) = 3.53$, $p = 0.005$; and chronic CORT: unpaired $t$-test, $t(10) = 3.46$, $p = 0.006$). Remarkably, chronic stress had a stronger effect than acute stress for activating NR4A1 in PFC (unpaired $t$-test, $t(10) = 2.45$, $p = 0.033$).

To confirm this result, we used Western blot to detect NR4A1 total protein levels in PFC lysates (Figure 3B,C). Acute stress increased NR4A1 levels compared to controls (unpaired $t$-test, $t(12) = 4.06$, $p = 0.0016$) whereas chronic stress decreased NR4A1 levels.
compared to controls (unpaired t-test, \( t_{(12)} = 4.72, p = 0.005 \)). We found the same comparing chronic CORT to control groups (unpaired t-test, \( t_{(12)} = 6.65, p < 0.001 \)). The difference between acute and chronic stress was significant (unpaired t-test, \( t_{(12)} = 6.72, p < 0.0001 \), Figure 3C), featuring a possible sensitization phenomenon. One issue raised by these data is the discrepancy between NR4A1 activity and NR4A1 levels in the context of chronic stress and CORT exposure.

Transcriptional activity of neuronal NR4A1 depended on its nuclear localization, phosphorylation and turnover.

We explored the possibility that activity of neuronal NR4A1 could depend on nuclear export besides its levels. For this, we utilized brain-derived neurotrophic factor (BDNF) as tool in an \textit{in vitro} system to force NR4A1 nuclear export in cortical neurons because TrkB is abundant in pyramidal cortical neurons (Jeanneteau et al., 2008), NR4A1 nuclear export was achieved in PC12 cells with a related neurotrophin (Katagiri et al., 2000), and BDNF expression is decreased by chronic stress and chronic CORT exposure (Duman, 2004; Arango-Lievano et al., 2015), which is relevant to the neuropathology of stress-related disorders (Autry and Monteggia, 2012). As anticipated, BDNF suppressed transcriptional activity of NR4A1 in primary cortical neurons (unpaired t-test, \( t_{(6)} = 2.57, p = 0.042 \), Figure 4A). Subcellular fractionation to separate nuclear proteins from the cytosol indicated that NR4A1 was mostly nuclear in untreated neurons and mostly cytosolic post-BDNF treatment (Figure 4B).

Furthermore, we characterized three antibodies to distinguish between the cytosolic and nuclear isoforms of NR4A1 (Figure 4B): (i) the antibody against the phosphorylated residue Ser350 (p-NR4A1), (ii) the antibody raised against the epitope containing residues 325-374
(cyto-NR4A1), and (iii) the antibody against both nuclear and cytosolic isoforms for total protein levels (pan-NR4A1).

To demonstrate the link between NR4A1 transcriptional activity, phosphorylation and nuclear export, we used NR4A1 mutants. Deletion of the phosphorylation site (S350A), the nuclear localization sequences (ΔNLS), the DNA-binding motif (R337A) or the transactivation domain (ΔAF1), all impaired NR4A1 nucleo-cytoplasmic ratio and transcriptional activity in primary cortical neurons (main effect of mutants by ANOVA $F_{(3,8)} = 28, p = 0.0001$ post-hoc Dunnett’s test for comparing ΔNLS and WT: $p = 0.0001$; R337A and WT: $p = 0.0002$; ΔAF1 and WT: $p = 0.0004$, Figure 4C,D). Additionally, NR4A1 ubiquitination was higher at the cytosolic isoform than the nuclear (Figure 4E), and NR4A1 downregulation was higher at the cytosolic isoform than the nuclear (main effect of BDNF by 2-way ANOVA $F_{(1,6)} = 38.24, p = 0.0008$; interaction of BDNF with time $F_{(4,24)} = 12.18, p < 0.0001$, post-hoc Sidak’s test for comparing BDNF and vehicle at 2h, 4h, 6h: $p < 0.0001$ and at 8h: $p = 0.0002$, Figure 4F).

Altogether, these data indicated that cytosolic NR4A1 was not transcriptionally active and most likely degraded.

Stress and CORT altered the nuclear localization, phosphorylation and levels of NR4A1.

To determine NR4A1 subcellular localization in vivo, we used the antibodies characterized in vitro. The pan-NR4A1 antibody detected total levels in both nucleus and cytosol of pyramidal neurons (labeled with $thyl$-YFP) in mouse PFC at rest (Figure 5A). The ratio nucleus-cytosol of pan-NR4A1 increased by acute stress (unpaired t-test, $t_{(18)} = 7.53, p < 0.0001$), chronic stress (unpaired t-test, $t_{(17)} = 12.2, p < 0.0001$) and chronic CORT (unpaired t-test, $t_{(15)} = 14.1, p < 0.0001$). We used Western blot to quantify p-NR4A1 and cyto-NR4A1 in PFC lysates, as the
antibodies did not work for histology (Figure 5B). Acute stress increased p-NR4A1 compared to controls (unpaired t-test, $t_{(22)} = 2.69$, $p = 0.013$, Figure 5C) and also increased cyto-NR4A1 compared to controls (unpaired t-test, $t_{(12)} = 2.55$, $p = 0.025$, Figure 5D). In contrast, we found the opposite comparing chronic stress to controls (effect on p-NR4A1: unpaired t-test, $t_{(16)} = 2.74$, $p = 0.014$, Figure 5C; effect on cyto-NR4A1 levels: unpaired t-test, $t_{(11)} = 4.48$, $p = 0.0009$, Figure 5D) and chronic CORT to controls (effect on p-NR4A1: unpaired t-test, $t_{(16)} = 2.95$, $p < 0.009$, Figure 5C; effect on cyto-NR4A1 levels: unpaired t-test, $t_{(12)} = 7.53$, $p < 0.0001$, Figure 5D). This was not a generalized effect on proteins sensitive to stress and CORT because FKBP51 was induced in these contexts (Figure 5B).

Again, different effects between acute and chronic stress on pan-NR4A1 nucleus-cytosol ratio (unpaired t-test, $t_{(17)} = 4.18$, $p = 0.0006$, Figure 5A), p-NR4A1 (unpaired t-test, $t_{(16)} = 2.71$, $p = 0.015$, Figure 5C) and cyto-NR4A1 (unpaired t-test, $t_{(11)} = 3.9$, $p = 0.0025$, Figure 5D), featured a putative sensitization phenomenon. Based on in vitro and in vivo data, we proposed a hypothetical model (Figure 5E) in which stress signals could influence transcriptional activity of neuronal NR4A1 by ways of phosphorylation, nuclear export and degradation by the ubiquitin-proteasome system.

**Genes regulated by neuronal NR4A1 are involved in mitochondrial uncoupling.**

NR4A1 activity regulates the expression of genes involved in cellular metabolism and cytoskeleton structure (Chao et al., 2007; Fassett et al., 2012; Chen et al., 2014). Here, we examined the expression of mitochondria-regulatory genes because both neuronal mitochondria and neuronal excitability were influenced by NR4A1 in a seizure model (Zhang et al., 2009). Compared to GFP, NR4A1 modified the expression of uncoupling proteins...
(UCP2: unpaired t-test $t_{(6)} = 5.1, p = 0.002$; and UCP4: unpaired t-test $t_{(6)} = 2.18, p = 0.035$; see Table 1), an effect blocked by BDNF as it caused NR4A1 nuclear export in cortical neurons. We used glutamate rather than CORT to simulate the effects of chronic stress in vitro because inhibitors of glutamate receptors blocked the effects of CORT and chronic stress on dendritic spine plasticity and neurotransmission (Duman, 2004; Popoli et al., 2012). We found that NR4A1 regulated more genes in the context of glutamate stimulation: UCP4 (unpaired t-test $t_{(6)} = 4.94, p = 0.0026$), MFN1 (unpaired t-test $t_{(6)} = 2.03, p = 0.044$), FIS1 (unpaired t-test $t_{(6)} = 3.48, p = 0.013$), and OPA1 (unpaired t-test $t_{(6)} = 2.3, p = 0.033$), unraveling a context-dependent effect. The consistent upregulation of UCP4 mRNA at rest and after glutamate stimulation suggested that NR4A1 could affect ATP stocks by uncoupling mitochondria proton transport from respiration (Liu et al., 2006; Klotzsch et al., 2015).

**NR4A1 transactivation domain was required to elicit mitochondrial proton leak.**

To monitor mitochondrial membrane potential that depends on the mitochondrial proton gradient, we used time-lapse imaging of the potentiometric mitochondrial dye tetramethylrhodamine (TMRM) in neurons (Figure 6A). Compared to GFP, NR4A1 reduced: (i) mitochondrial membrane depolarization to glutamate (unpaired t-test, $t_{(41)} = 2.73, p = 0.0091$), (ii) mitochondria membrane repolarization during washout (unpaired t-test, $t_{(41)} = 3.05, p = 0.0039$), and (iii) mitochondria energetic competence in the oligomycin test (unpaired t-test, $t_{(18)} = 2.27, p = 0.035$) because it hyperpolarized mitochondria in GFP neurons but depolarized them in NR4A1-transfected neurons (Ward et al., 2000). These observations (Figure 6B) indicated that NR4A1 unlike its mutant ΔAF1 caused mitochondrial uncoupling.
Stimulation with glutamate decreased whole cell ATP levels (main effect of glutamate: $-59 \pm 2.9\%$, 2-way ANOVA $F_{(1,10)} = 106.8, p < 0.0001$ post hoc Bonferroni’s test $p = 0.0007$).

This utilization of ATP stocks was exaggerated by NR4A1 compared to GFP (unpaired t-test, $t_{(16)} = 2.14, p = 0.024$, Figure 6B) but far from the ATP-depleting effect of oligomycin (unpaired t-test, $t_{(16)} = 3.17, p = 0.009$, Figure 6C). Consistent with a mild shortage of ATP, phosphorylation of the adenosine monophosphate-activated kinase (AMPK) increased modestly by NR4A1 compared to GFP ($+33.8 \pm 5.2\%$, unpaired t-test, $t_{(31)} = 5.55, p < 0.0001$, Figure 6D) and phosphorylation of its substrate acetyl-CoA carboxylase (ACC) increased modestly by NR4A1 compared to GFP ($+34 \pm 7.8\%$, unpaired t-test, $t_{(31)} = 4.54, p < 0.0001$, Figure 6D). In contrast, ΔAF1 lacking transcriptional activity had no effects on AMPK signaling.

NR4A1 used the AMPK catabolic pathway to reduce dendritic spine number.

To clarify a hypothetical link between AMPK signaling and morphological plasticity evoked by NR4A1, we used pharmacological modulators (Figure 6E). We obtained mild modulation of AMPK in primary cortical neurons by STO609 ($-7 \pm 4.1\%$ for p-AMPK and $-29 \pm 6.8\%$ for p-ACC, unpaired t-test, $t_{(10)} = 3.46, p = 0.006$) and A769662 ($+24.2 \pm 4.9\%$, unpaired t-test, $t_{(10)} = 4.79, p = 0.0007$ for p-ACC), respectively inhibitor of CAMKK activating kinase of AMPK (Mairet-Coello et al., 2013) and activator of AMPK (Scott et al., 2014). In transfected neurons, we found a functional interaction between NR4A1 and STO609 that reverted the low dendritic spine number above control levels (main effect of constructs by 2-way ANOVA $F_{(2, 296)} = 12.06, p < 0.0001$; main effect of STO609 $F_{(1, 296)} = 16.5, p < 0.0001$; interaction of constructs and
STO609 $F_{(2, 296)} = 21.28, p < 0.0001$, post hoc Tukey’s test for comparing STO609 and vehicle on NR4A1 cells $p < 0.0001$; on ΔAF1 cells $p > 0.99$; on GFP cells $p = 0.99$, Figure 6F and for cumulative distributions see Figure 6-1). Such an interaction was absent between ΔAF1 and STO609, suggesting redundancy between the NR4A1 and AMPK pathways.

We tested further this hypothetical framework with the AMPK activator A769662, as it is predicted to reduce dendritic spine number in neurons expressing ΔAF1 but not in neurons expressing NR4A1. We found no functional interaction between NR4A1 and A769662. On the contrary, A769662 reverted the effect of ΔAF1 on dendritic spine number (main effect of constructs by 2-way ANOVA $F_{(2, 300)} = 22.8, p < 0.0001$; main effect of A769662 $F_{(2, 300)} = 14.03, p = 0.0002$; interaction of constructs and A769662 $F_{(1, 300)} = 1.96, p = 0.14$, post hoc Tukey’s test for comparing A769662 and vehicle on ΔAF1 cells $p = 0.0039$; on NR4A1 cells $p = 0.6$; on GFP cells $p = 0.79$, Figure 6F and for cumulative distributions see Figure 6-1).

To confirm in vivo the functional link between AMPK and NR4A1 pathways, we used NR4A1 knockouts (Figure 7). An effect of genotype on p-AMPK (unpaired t-test, $t_{(15)} = 2.66, p = 0.017$) and p-ACC was observed in the PFC (unpaired t-test, $t_{(15)} = 6.8, p = 0.0074$). An effect of genotype was also observed on the levels of protein markers of excitatory synapses (P190A: unpaired t-test, $t_{(10)} = 4.42, p = 0.0013$ and Drebrin: unpaired t-test, $t_{(10)} = 5.88, p = 0.0002$, Figure 7D). This is consistent with the decrease of protein markers of excitatory synapses in primary cortical neurons expressing NR4A1 compared to ΔAF1 (Figure 7-1).

Altogether, these data indicated that AMPK signaling is an effector pathway of NR4A1 that alters dendritic spine number.

NR4A1 levels in human PFC of MDD patients.
Mechanistic data support a causative role of NR4A1 on synaptic loss in PFC neurons in the context of stress and disrupted CORT levels, as both increased its neuronal activity in animal models. In humans, synaptic loss in PFC neurons and disrupted CORT levels are established neuropathological features of MDD that are consistent with a putative impaired activity of NR4A1 (Duman et al., 2016). In a previous study (Duric et al., 2010), we showed that NR4A1 transcripts were up-regulated by 1.72 fold ($p = 0.029$, t-test corrected for false discovery rate) in a whole-genome microarray profiling of human hippocampus in MDD. In the same human cohort (for demographic information, see Figure 8-1), we analyzed NR4A1 mRNA levels in the medial PFC. Quantitative PCR analysis showed that NR4A1 transcripts were significantly higher in PFC of MDD subjects (fold increase 1.39, unpaired t-test, $t_{(47)} = 3.13$, $p = 0.018$) when compared to matched psychiatrically healthy controls (Figure 8). Factorial analysis revealed no effect of gender ($p = 0.372$) and no effect of antidepressant medication prescription ($p = 0.139$).

NR4A1 levels in human PFC of AD patients.

Similarities in AD and MDD with respect to volume reduction, neuronal atrophy and loss of connectivity in PFC could underlie common deficits in intracellular signaling network (van Veluw et al., 2012; Sampath et al., 2017). Synaptic loss in PFC neurons and disrupted CORT levels are neuropathological features of cognitive deficits across disorders with overlap in the expression of clinical state, established in MDD and AD (Uylings and de Brabander, 2002; Knobloch and Mansuy, 2008; Lupien et al., 2009). This prompted us to assess NR4A1 levels in PFC of human subjects clinically diagnosed with AD and age matched cognitive healthy controls (for demographic information see Figure 9-1). We used Western blots to discriminate...
between pan-NR4A1 and cyto-NR4A1 levels, as NR4A1 activity cannot be determined post-mortem (Figure 9A). Levels of pan-NR4A1 correlated with that of AMPK (Pearson $r = 0.53$, $p = 0.0003$, Figure 9B). Higher levels of AMPK (unpaired t-test, $t_{(39)} = 2.17$, $p = 0.035$, Figure 9C) and pan-NR4A1 (unpaired t-test, $t_{(39)} = 2.31$, $p = 0.025$, Figure 9D) were found in AD compared to controls. But lower levels of cyto-NR4A1 were found in AD compared to controls (unpaired t-test, $t_{(39)} = 2.34$, $p = 0.024$, Figure 9E). These data provided a molecular signature (Figure 9F) proportionate to cognitive scores in the mini-mental state examination (MMSE) test (see Figure 9-1B for cyto-NR4A1: Pearson $r = -0.37$, $p = 0.041$; pan-NR4A1: Pearson $r = 0.36$, $p = 0.019$; AMPK: Pearson $r = 0.48$, $p = 0.011$) and to the levels of synaptic markers (see Figure 9-1B for PSD95: Pearson $r = -0.38$, $p = 0.013$; Drebrin: Pearson $r = -0.44$, $p = 0.003$; PI90A: Pearson $r = -0.31$, $p = 0.049$). Factorial analysis revealed no effect of gender on any markers (see figure 9-1C for details).

DISCUSSION

NR4A1 is a transcription factor that we found deregulated in PFC of animal models of stress and excessive CORT levels, and in humans diagnosed with mental health diseases for which disrupted CORT levels and stress are established aggravating factors (de Quervain et al., 2004; Holsboer and Ising, 2010; Machado et al., 2014; Duman et al., 2016). Our study provides the first substantial support for an idea long speculated about. That is, dendritic spine excitatory synaptic reduction following chronic stress or disrupted CORT levels is a compensatory mechanism resulting from over-activity of cortical networks (Popoli et al., 2012). Although synaptic attrition and reorganization might compromise cognitive functions (Sampath et al.,

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Adaptive response to protect neurons from damages of over-excitation

As an activity-dependent immediate-early gene with high inducible pattern, high turnover rate and an option for deactivation by nuclear export, NR4A1 presents with the necessary attributes to temporarily adjust cellular fate to the triggering stimulus (Maxwell and Muscat, 2006; Helbling et al., 2014). Such cell-autonomous and time-locked expression of NR4A1 should help contain the neuronal damages from over-excitation by tempering the reception of excitatory signals, and/or the response to such excitation. We found evidence supporting both possibilities, as NR4A1 reduced the number of excitatory synapses and the mitochondrial response to glutamate. Decreasing synapse number in pyramidal neurons could favor neuronal adaptation and behavioral flexibility when levels of glutamate and CORT are excessive in PFC (Popoli et al., 2012; Chattarji et al., 2015). Stress-induced atrophy of dendritic territories in PFC corresponded with aberrant levels of mitochondria-related proteins in synaptosomes and with cognitive impairment (Lopes et al., 2016). This extends previous findings in a model of epilepsy in which NR4A1 expression protected hippocampal neurons from seizure-induced...
damage by rendering mitochondria more resistant to cellular stress and by reducing neuronal excitability (Zhang et al., 2009).

**From adaptation to maladaptation**

NR4A1 expression in cortex and hippocampus responded to gradual acute stress but showed tolerance to chronic stress (Umemoto et al., 1994). The contrary was observed in other brain regions, indicating circuit specificity (Campos-Melo et al., 2011). This could result from differential epigenetic modifications at the *Nr4a1* locus as a function of genetic background, development and stressors (Kember et al., 2012; Mizuno et al., 2012; Tognini et al., 2015). The subcellular distribution of NR4A1 is also affected differently by acute and chronic stress even though the last stressor is the same, which suggests a change of tune in the immediate response to the stressor. This could be due to BDNF expression, subjected to opposite changes on acute and chronic stress (Bath et al., 2013; Jeanneteau and Chao, 2013), as it deactivates NR4A1 by ways of nuclear export, phosphorylation, and downregulation by the ubiquitin-proteasome system. Nuclear retention of NR4A1 upon chronic stress is consistent with its prolonged activity that surpassed that induced by acute stress even though NR4A1 levels were higher in PFC neurons after acute than chronic stress. This is consistent with the low levels of cyto-NR4A1 despite the high levels of total NR4A1 found in PFC of human subjects diagnosed with AD. Levels of cyto-NR4A1 also correlated with measures of synaptic markers and cognitive function. This is mirrored by the decreased expression of BDNF in the human AD brain (Ginsberg et al., 2017). However, both high and low levels of NR4A1 have been linked to mental health issues as cortical levels of NR4A1 were reported decreased in autism and schizophrenia (Corley et al., 2016; Li et al., 2016), and NR4A1 knockouts exhibit learning
disabilities (McNulty et al., 2012) as well as transgenic mice with dominant negative NR4A1 that showed defects of synaptic plasticity (Hawk et al., 2012; Bridi and Abel, 2013).

A mechanistic framework to study signaling loops between mitochondria and synapses

An important question raised by our study is to which extent the reduction of excitatory synapse number resulted from mitochondrial uncoupling. Manipulations of AMPK signaling established a functional link between NR4A1 transcriptional activity, AMPK and excitatory synapses, which is corroborated by previous studies implicating AMPK in the regulation of synapse number, synaptic transmission, learning disabilities and amyloid toxicity (Potter et al., 2010; Vingtdeux et al., 2011; Mairet-Coello et al., 2013; Ma et al., 2014). Uncoupling of mitochondrial respiration from proton transport resulted in shortage of ATP stocks (Nicholls and Ward, 2000), activation of AMPK signaling (Weisova et al., 2012) and dendritic spine attrition (Dickey and Strack, 2011). We found that NR4A1 required its transcriptional activity to increase the proton leak in mitochondrial membranes and to increase ATP utilization in the context of glutamate stimulation. Previous studies showed that NR4A1 modulates mitochondria functions via genomic and non-genomic mechanisms (Close et al., 2013; Pawlak et al., 2015). Thus, NR4A1-target genes are likely important for regulating metabolic and morphological aspects of neuronal functions. NR4A1 modified the expression of several mitochondria-regulatory genes but only the UCPs (UCP2, UCP4 and UCP5) were counter-modified by BDNF as it deactivated NR4A1. UCP4 dissipates transmembrane proton gradient in mitochondria to moderate respiration and to reduce reactive oxygen species damage from oxidative phosphorylation (Liu et al., 2006; Oita et al., 2009; Zhao and Bruemmer, 2010; Klotzsch et al., 2015). Previous silencing of UCP4 rendered mitochondria less resistant to
glutamate excitotoxicity (Chan et al., 2006). Assuming that NR4A1-dependent induction of UCP4 could decrease dendritic spine number in the context of chronic stress and chronic CORT exposure, our study provides, a mechanistic framework for investigating whether mitochondrial uncoupling could serve as a rheostat to isolate mitochondrial respiration from excessive excitatory stimulation that would endanger neuronal survival.

Methodological limitations

NR4A1 activity was not measured directly in human cortical tissues because it was not possible in postmortem biopsies. Monitoring of p-NR4A1 could have strengthened the other readouts (total protein or RNA levels and cyto-NR4A1) that only reflect indirectly NR4A1 activity. But we did not test for p-NR4A1 in human PFC tissues, as it could be equivocal in postmortem analysis. Previous study of fresh fibrotic tissues indicated that p-NR4A1 was inversely correlated with total NR4A1 levels and activity (Palumbo-Zerr et al., 2015), which is consistent with our findings in human brain. The effect of medication on NR4A1 expression and activity is possible but prescription medications were not disclosed as per HIPAA policies for a pathological study. The drugs used to manipulate AMPK activity provided only mild modulatory effects. But there are no direct inhibitors of AMPK that would suppress its activity, nor there are currently other activators specific and more potent than A769662 (Steinberg and Kemp, 2009; Scott et al., 2014). The magnitude of stress and CORT-mediated attrition of dendritic spines depends on age (Bloss et al., 2011; McEwen and Morrison, 2013). Our study investigated the PFC of adolescent animals guided by previous reports in rodents of similar age subjected to similar procedures (Gourley et al., 2013; Swanson et al., 2013; Anderson et al., 2016). In particular, our studies were conducted on various animal species (KO mice and KO...
rats) and strains (C57BL6 and CD1) by distinct investigators. Despite the heterogeneity of the models; knockout, knockdown and dominant negative approaches, all showed a role of NR4A1 in activating AMPK and reducing excitatory synapses number in cortex. The in utero electroporation system offered the advantages of unilateral targeting of sparse cortical neurons during embryogenesis but impact on behavior was not informative. In contrast, constitutive knockouts exhibited behavioral phenotype and permitted cross-validation of biochemical and morphological findings. Yet, NR4A1 is expressed in many cell types, which could bias interpretations of KO data (Hawk and Abel, 2011; Safe et al., 2016). For instance, we found discrepancies in the effects of NR4A1 KO compared to its knockdown on dendritic spine number in PFC. Importantly, hypothalamic-pituitary-adrenal axis signaling was normal in NR4A1 deficient mice, indicating that CORT secretion and response are not biased in this model (Crawford et al., 1995).

Perspectives

There is no known endogenous ligand for NR4A1 but synthetic ligands with agonistic and antagonistic properties are being developed to regulate cellular fate in a variety of disease models (Safe et al., 2016). Drug-induced activation of NR4A1 to compensate for its low expression in a model of autism reduced the number of surplus synapses offering perspectives for therapeutic intervention (Li et al., 2016). There are also opportunities for clinical applications of NR4A1 ligands that would promote NR4A1 downregulation, nuclear export or transcriptional blockade.
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Figure 1. Chronic activation of the glucocorticoid stress pathway required NR4A1 for dendritic spines attrition in pyramidal neurons of mouse cortex.

(A) Experimental timeline: doxycycline-inducible NR4A1 construct (with GFP reporter) was electroporated in utero and doxycycline administered in drinking water for the indicated days between PND21 and PND31. During this period, mice were exposed either to daily unpredictable stress or CORT injections (15 mg/kg). See extended Figure 1-1.

(B) Spine density at apical tuft dendrites of cortical pyramidal L2/3 neurons of S1 and PFC electroporated with NR4A1 (Mean ±SEM of N = 5 mice/ group, ANOVA post-hoc Bonferroni’s test comparing 0d vs 3d: #p = 0.0168; *p < 0.0001; 0d vs 10d: *,#p < 0.0001). See extended Figure 1-2.

(C) Spine density at apical tuft dendrites of cortical pyramidal L2/3 neurons electroporated with NR4A1 constructs in S1. Mice were either reared in standard home cage conditions or exposed to chronic unpredictable stress. Mean ±SEM of N=5-7 mice/ group: ANOVA post-hoc Bonferroni’s test comparing NR4A1 and GFP *p = 0.0031; stress and controls #p = 0.0015; Interaction of stress and post-hoc Bonferroni’s test ΔAF1 #p = 0.0018).

(D) Knockdown of transfected NR4A1 in HEK cells with shRNA#1 and #2 compared to scramble (sc) sequence.

(E) Effect of NR4A1 knockdown on stress-induced spine loss at apical tuft dendrites of pyramidal L2/3 neurons in S1 (Mean ±SEM of N=6 mice/ group, ANOVA post-hoc Bonferroni’s test comparing stress and controls in scramble group *p = 0.0018). See extended Figure 1-3.
Effect of NR4A1 knockdown on CORT-induced spine loss at apical tuft dendrites of pyramidal L2/3 neurons in S1 (Mean ±SEM of N=6-7 mice/group, ANOVA post-hoc Bonferroni’s test comparing stress and controls in scramble group *p = 0.0017). See extended Figure 1-3.

Figure 2. Knockout of NR4A1 protected against adverse effects of chronic CORT injections.

(A) Apical tuft dendrites of Golgi-impregnated pyramidal L2/3 neurons in PFC from NR4A1 KO mice exposed to vehicle or CORT daily injections between PND21 and PND31.

(B) Distribution of dendritic spine territories at apical tuft of the layer 2/3 neurons in PFC (N dendrites = 72 +/-, 61 +/- for vehicle groups; N dendrites = 110 +/-, 107 +/- for CORT groups). Comparison with K-S test *, # p < 0.0001, ^p = 0.0016.

(C) Group effects on spine density (Mean ±SEM of N=5-6 mice/group, ANOVA post-hoc Bonferroni’s test comparing effect of genotype and CORT treatment *, # p < 0.0001, ^p = 0.0066).

(D) Immobility time in the TST of CORT-injected NR4A1 KO mice compared to wildtype littermates (Mean ±SEM of N=5-6 mice/group, ANOVA post-hoc Sidak’s t-test comparing total immobility time between genotypes *p < 0.05).

Figure 3. Chronic activation of the glucocorticoid stress pathway increased NR4A1 transcriptional activity in pyramidal L2/3 neurons of mouse PFC.

(A) Luciferase activity at NR4A1 reporter NurRE-luc compared to a minimal reporter construct introduced with a GFP reporter in cortical L2/3 neurons by electroporation in utero.
Luc activity monitored at PND31 ex vivo after microdissection of GFP-labeled PFC tissue. Data normalized to total mg protein of GFP dissected tissues are expressed as percentage of the NurRE-luc construct in control resting mice (mean ±SEM of N = 4 mice with minimal promoter / group, and 6 mice with NurRE/ group, unpaired t-test, \( ^* p = 0.0005, ^* p = 0.033, ^* p = 0.006, ^* p = 0.005 \) compared to controls, \( ^* p < 0.0001, ^* p < 0.0001 \) comparing indicated treatment to controls, \( ^* p < 0.0001 \) comparing acute and chronic stress).

(B) Western blots of PFC lysates from PND31 mice subjected to acute stress, chronic stress or CORT injections compared to controls.

(C) Protein levels of NR4A1 (mean ±SEM of N = 7 mice/ group) expressed as percentage of controls. Unpaired t-test, \( ^* p = 0.0016, ^* p < 0.0001, ^* p < 0.0001 \) comparing indicated treatment to controls, \( ^* p < 0.0001 \) comparing acute and chronic stress.

**Figure 4.** NR4A1 transcriptional activity in cortical neurons depends on its nuclear localization, phosphorylation and turnover.

(A) NR4A1 activity expressed as percentage of untreated controls impeded after stimulation with 25 ng/ml BDNF for 3 hrs (mean ±SEM of 4 independent experiments, unpaired t-test, \( ^* p = 0.042 \)). NurRE-luc reporter was co-transfected with recombinant NR4A1 in primary cortical neurons by electroporation and luciferase activity monitored in lysates.

(B) Antibodies to detect the nucleo-cytoplasmic isoforms (pan-NR4A1), cytosolic isoform (cyto-NR4A1) and phosphorylated isoforms (p-NR4A1 at Ser350) of recombinant NR4A1 electroporated in primary cortical neurons. Ratio of proteins in cytosol versus nuclear (C/N, means of 4 independent samples/ group) altered after stimulation with 25 ng/ml BDNF for 3 hrs. HSP90 and HDAC2 are loading controls.
(C) NurRE-luc reporter activity in primary cortical neurons transfected with the indicated constructs. Data normalized to GFP controls (mean ±SEM of 3 independent experiments, ANOVA post-hoc Dunnett’s test comparing WT and mutants *p < 0.0005).

(D) Deletion of Ser350 or NLS sequences altered NR4A1 C/N ratio in primary cortical neurons (means of 4 independent samples/ group normalized to WT+BDNF).

(E) Poly-ubiquitination of NR4A1 immunoprecipitated from lysates of primary cortical neurons stimulated with 25 ng/ml BDNF for 3 hrs.

(F) Withdraw of doxycycline for the indicated time destabilized NR4A1 recombinant protein levels in primary cortical neurons. Mean ±SEM of 4 independent experiments, ANOVA post-hoc Sidak’s test for interaction of BDNF and time *p < 0.0001.

Figure 5. Chronic activation of the glucocorticoid stress pathway decreased NR4A1 phosphorylation and cytosolic distribution in mouse PFC.

(A) Endogenous NR4A1 protein is both nuclear and cytosolic in pyramidal cortical neurons of PFC (thy1-YFP). Proportion of total 10,600 cells with NR4A1 localized equally in nucleus and cytosol (N=C), more in nucleus than cytosol (N>C) and less in nucleus than cytosol (N<C). Mean ±SEM of N = 10 controls, 10 acute stress, 7 CORT and 9 chronic stress mice, unpaired t-test *p = < 0.0001 comparing indicated group to controls and #p = 0.0006 comparing acute vs chronic stress.

(B) Western blots of PFC lysates from PND31 mice subjected to acute stress, chronic stress or CORT injections compared to controls.

(C) Phosphorylation of NR4A1 (S350-P) expressed as percentage of controls. Mean ±SEM of N = 12 control mice, 12 acute stress, 6 CORT and 6 chronic stress, unpaired t-test, *p = 0.013,
\(^p = 0.0094, \quad \&^p = 0.014\) comparing indicated treatment to controls, \(\&^p = 0.0153\) comparing acute and chronic stress.

(D) Cytosolic NR4A1 expressed as percentage of controls. Mean ±SEM of N = 7 control mice, 7 acute stress, 6 CORT and 6 chronic stress, unpaired t-test, \(*p = 0.025, \quad \&^p < 0.0001, \quad \&^p = 0.0009\) comparing indicated treatment to controls, \(\&^p = 0.0025\) comparing acute and chronic stress.

(E) Model of NR4A1 functional transport in cortical neurons. Induction of NR4A1 by acute stress increased nuclear export and phosphorylation. Chronic stress prolonged NR4A1 nuclear residency, which failed to contain its activity.

Figure 6. Activation of NR4A1-AMPK pathway reduced dendritic spine number in vitro.

(A) Time-lapse imaging of TMRM fluorescence in primary cortical neurons (DIV14 with 10 d doxycycline treatment). Yellow box is one example ROI.

(B) \(\Delta F (F_{F0}/F0 \times 100)\) traces are means ±SEM of N = 10 GFP, 10 NR4A1 and 7 \&AF1 neurons subjected to all treatments subsequently. Statistical comparison between GFP and NR4A1 groups at indicated epochs (last 3 min of each treatment) by unpaired t-test during 100 μM glutamate \(*p = 0.009\) (N = 16 GFP, 27 NR4A1 cells), during washout \(\&^p = 0.0039\) (N = 16 GFP, 27 NR4A1 cells) and during 1 μM oligomycin \(\&^p = 0.035\) (N = 10 GFP, 10 NR4A1 cells).

(C) Dissipation of ATP upon stimulation of primary cortical neurons (DIV14 with 10 d doxycycline treatment) with 100 μM glutamate compared to 1 μM oligomycin for 1 hr. Mean ±SEM of N = 8 GFP, 10 NR4A1 and 5 \&AF1 independent samples, unpaired t-test comparing GFP and NR4A1 \(*p = 0.024\).
(D) Phosphorylation of AMPK and its substrate ACC in primary cortical neurons (DIV14 with 10d doxycycline) after stimulation with 25 μM glutamate for 3 hrs (N = 18 GFP, 15 NR4A1, 5 ΔAF1 samples/group). Pearson correlation between levels of p-ACC and p-AMPK normalized to GFP as controls. Group comparison by unpaired t-test for p-ACC: NR4A1 vs GFP p < 0.0001 and ΔAF1 vs GFP p = 0.009; for p-AMPK: NR4A1 vs GFP p < 0.0001 and ΔAF1 vs GFP p = 0.004.

(E) Modulation of AMPK by 10 μM STO609 and 1 μM A769662 for 24 hrs in primary cortical neurons (DIV14). Pearson correlation between levels of p-ACC and p-AMPK normalized to vehicle controls (N = 6 samples/group). Comparison by unpaired t-test for p-ACC: STO609 vs vehicle p = 0.0061 and A769662 vs vehicle p = 0.0007; for p-AMPK: STO609 vs vehicle p = 0.2 and A769662 vs vehicle p = 0.0013.

(F) Effect of STO609 (S) and A769662 (A) compared to vehicle (V) on dendritic spine density in primary cortical neurons expressing GFP, NR4A1 or ΔAF1 (DIV21 with 10d doxycycline). Mean ±SEM of N= 99 GFP, 192 NR4A1 and 160 ΔAF1 dendrites, ANOVA post-hoc Tukey’s test for comparing NR4A1 and ΔAF1 **p < 0.0001; GFP and NR4A1 $p = 0.0019; GFP and ΔAF1 $p = 0.002; STO609 and vehicle on NR4A1 cells $p < 0.0001, A769662 and vehicle on ΔAF1 cells $p = 0.023; interaction of NR4A1 and A769662 *p < 0.045. See extended Figure 6-1.

Figure 7. AMPK signaling and levels of synaptic marker in PFC of NR4A1 knockouts.

(A) AMPK signaling in PFC of NR4A1 KO rats.

(B) Means ±SEM of 11 -/- compared 6 +/+. Data expressed as percentage of controls (unpaired t-test comparing genotypes *p = 0.017 and $p = 0.007).
(C) Levels of synaptic markers in PFC of NR4A1 KO rats

(D) Means ±SEM of 6 -/- compared 6 +/+. Data expressed as percentage of controls (unpaired

t-test comparing genotypes *p = 0.0013 and #p = 0.0002). See extended Figure 7-1.

Figure 8. Abnormal NR4A1 transcript levels in human MDD brains.

Upregulated levels of NR4A1 mRNA in PFC of MDD subjects. Means ±SEM normalized to

Tubulin (N = 25 CTR, 24 MDD unpaired t-test, t(47) = 3.13, *p = 0.018). See Figure 8-1 for
demographic information. See extended Figure 8-1.

Figure 9. Abnormal levels of AMPK and NR4A1 in human AD brains.

(A) Representative image of AMPK protein and NR4A1 nucleo-cytoplasmic isoforms in

human cortex. See extended Figure 9-1.

(B) Pearson correlation (p = 0.0003) between AMPK and pan-NR4A1 levels in human PFC

from AD (N = 24) and CTR (N = 17). Data are Western blots optical densities normalized to

GAPDH.

(C) Upregulation of AMPK protein levels in human PFC (Mean ±SEM of N=24 AD and 17

CTR normalized to GAPDH levels, unpaired t-test, *p = 0.035).

(D) Downregulation of NR4A1 cytoplasmic isoform in human PFC (Mean ±SEM of N = 24

AD and 17 CTR normalized to GAPDH levels, unpaired t-test, *p = 0.024).

(E) Upregulation of NR4A1 total isoforms in human PFC (Mean ±SEM of N=24 AD and 17

CTR normalized to GAPDH levels, unpaired t-test, *p = 0.0258).

(F) Pearson correlations between MMSE scores and levels of AMPK (p = 0.011), cyto-

NR4A1 (*p = 0.041), pan-NR4A1 ("p = 0.019) or synaptic markers PSD95 ("p = 0.013),

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Drebrin \((p = 0.003)\), P190A \((\#p = 0.049)\). Data are normalized to CTR (Mean ±SEM of \(N = 17\) subjects with score of 30-25, \(N = 12\) with score of 18-10 and \(N = 12\) with score of 9-0). See extended Figure 9-1 for details.

**TABLES**

**Table 1. Effect of neuronal NR4A1 on mitochondria-regulatory genes expression.**

Messenger RNA levels determined by q-PCR on total RNA extracts of primary cortical neurons (DIV14 with 10 d doxycycline treatment). Data expressed as percentage of GFP controls (Mean ±SE of \(N = 4\) independent experiments). Unpaired t-test for comparing effect of NR4A1 with GFP in untreated control cells \(*p < 0.036\), for comparing effect of NR4A1 with GFP in BDNF-treated cells (25 ng/ml for 3 hrs), and for comparing effect of NR4A1 with GFP in glutamate-treated cells (100 μM for 3 hrs) \(*p < 0.045\).
Extended data

Figure 1-1: Developmental expression of NR4A1 in PFC
Western blot with pan-NR4A1 antibody and GAPDH as loading control. Mean ±SE of N = 3 mice at each time point. Unpaired t-test for comparing the indicated time point with E18 *p < 0.035. Refers to Figure 1A.

Figure 1-2: Synaptic markers of excitatory synapses in layer 2/3 pyramidal neurons of PFC.
Confocal images of dendritic segments of pyramidal L2/3 neurons in PFC at PDN31 labelled with GFP plasmid electroporated in utero at E15.5, and co-stained with PDS95 and synaptophysin antibodies. Markers are where expected: PDS95 occurred in dendritic spines as puncta and dendritic spines were juxtaposed or near to synaptophysin puncta. Refers to Figure 1B.

Figure 1-3: Cumulative distribution of spines density at apical tuft dendrites of L2/3 pyramidal neurons in vivo.
(A) Comparisons with the Kolmogorov-Smirnov test between sc and #1 *p < 0.0001; sc and sc+stress *p < 0.0001; #1 and #1+stress p = 0.56; sc+stress and #1+stress §p < 0.0001 (Number of dendrites = 36 shRNA sc, 30 shRNA#1, 30 shRNA sc+stress and 23 shRNA#1+stress). Refers to Figure 1E.
(B) Comparisons with the Kolmogorov-Smirnov test between sc and #1 *p = 0.0008; sc and sc+CORT *p < 0.0001; #1 and #1+CORT p = 0.1; sc+CORT and #1+CORT §p < 0.0001
Figure 6-1: Cumulative distribution of dendritic spines density in cultured cortical neurons.

Comparisons with the Kolmogorov-Smirnov test between GFP and NR4A1 \( *p = 0.005 \); GFP and ΔAF1 \( *p = 0.001 \); NR4A1 and ΔAF1 \( *p < 0.0001 \); for the effect of STO609 between GFP and NR4A1 \( \#p < 0.0001 \); GFP and ΔAF1 \( \#p = 0.036 \); NR4A1 and ΔAF1 \( \#p = 0.0016 \); for the effect of A769662 between GFP and NR4A1 \( \#p = 0.001 \); NR4A1 and ΔAF1 \( \#p = 0.031 \)

(Number of dendrites = 99 GFP (V: 42; S: 23; A: 34), 192 NR4A1 (V: 85; S: 47; A: 60) and 160 ΔAF1 (V: 57; S: 47; A: 56). Refers to Figure 6F.

Figure 7-1: NR4A1 acts as transcription factor to decrease synaptic proteins content.

Doxycycline-induced expression of NR4A1 decreased levels of excitatory synapse proteins in primary cortical neurons (DIV14, doxycycline treatment for last 10 days). This effect required NR4A1 transcriptional activity given that doxycycline-induced expression of ΔAF1 had no effect. Mean ±SEM of N = 4 independent experiments. Group comparisons between doxycycline and no doxycycline at the indicated markers for the effect of NR4A1 by unpaired t-test \( *p < 0.001 \), \( **p < 0.0005 \), for the effect of ΔAF1 \( \#p < 0.014 \), and for comparing NR4A1 and ΔAF1 at the indicated markers \( \uparrow p = 0.03 \), \( \uparrow\uparrow p = 0.002 \), \( \uparrow\uparrow\uparrow p < 0.004 \). Refers to Figure 7D.

Figure 8-1: Demographic information of MDD subjects and controls.
Figure 9-1. NR4A1 protein levels in AD correlated with measures of synaptic markers and cognitive function.

(A) Demographic information. F, female; M, male; FC, frontal cortex (medial anterior: Brodmann area 9-10); PMI, postmortem interval (hours); MMSE, mini mental state examination test; Medications of persons during life were not accessible (NA) as per HIPAA guidelines for a pathological study. Refers to Figure 9A.

(B) Protein levels of NR4A1, AMPK and synaptic markers in PFC of AD and CTR. Data are means ± SEM of the optical density (OD) ratio between markers and GAPDH, and normalized to the CTR group. Pearson correlations between MMSE scores and protein levels in N = 17 CTR and 24 AD. Refers to Figure 9F.

(C) There is no significant effect of gender on the expression of markers within CTR and AD groups. Data are means ± SEM of the OD ratio between markers and GAPDH. Refers to Figure 9F.
| TREATMENTS                      | DRP1 | MFN1 | MFN2 | FIS1 | OPA1 | UCP2 | UCP4 | UCP5 |
|--------------------------------|------|------|------|------|------|------|------|------|
| ratio (NR4A1 / GFP):           | % SE | % SE | % SE | % SE | % SE | % SE | % SE | % SE |
| Untreated controls             | 127.6 ± 1.5 | 128.7 ± 2.8 | 113.5 ± 1.3 | 106.6 ± 1.6 | 111.0 ± 0.8 | 22.3 ± 0.4 | 157.5 ± 26.3 | 112.8 ± 17.8 |
| 25 ng/ml BDNF                  | 119.5 ± 1.6 | 122.3 ± 2.2 | 104.8 ± 1.1 | 97.9 ± 0.8 | 75.7 ± 0.3 | 87.4 ± 0.6 | 126.4 ± 16.2 | 77.8 ± 11.8 |
| 25 mM glutamate                | 95.9 ± 0.7 | 127.9 ± 1.0 | 96.7 ± 2.0 | 129.6 ± 1.2 | 107.8 ± 1.1 | 85.6 ± 1.7 | 157.9 ± 30.5 | 88.9 ± 14.5 |
| 100 mM glutamate               | 121.3 ± 1.6 | 70.1 ± 3.5 | 90.1 ± 4.5 | 159.8 ± 20.7 | 155.5 ± 24.7 | 61.5 ± 28.6 | 176.9 ± 14.9 | 98.2 ± 9.2 |