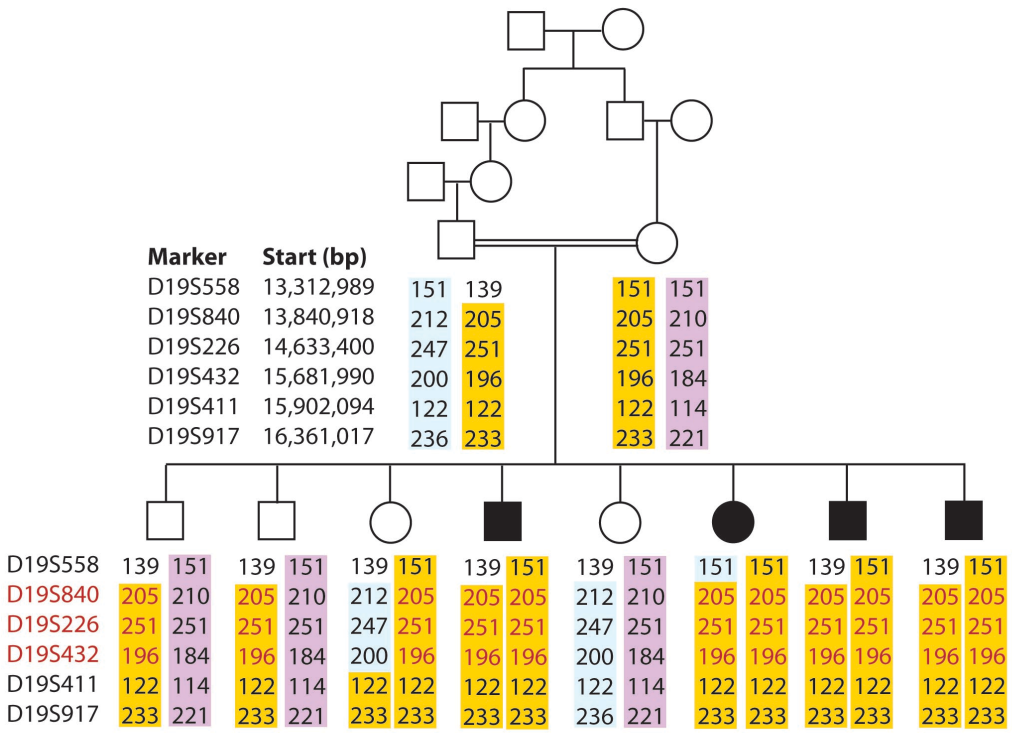
Manzini et al. Supplemental Information

Supplemental figures:



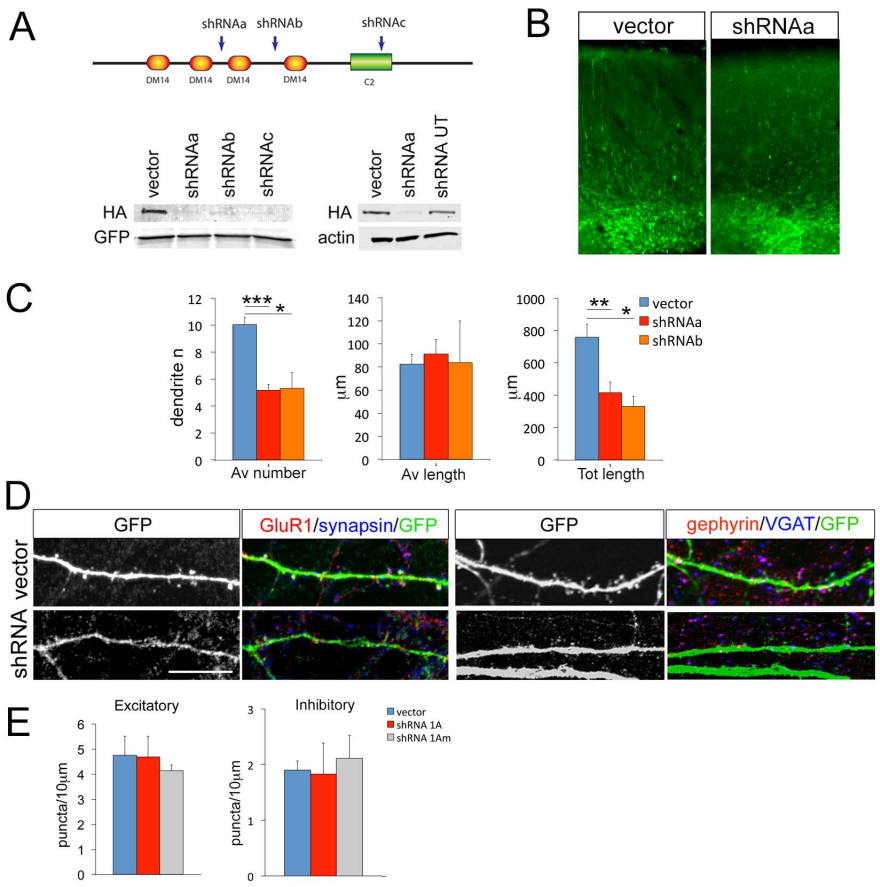
Minimum microsatellite haplotype

Marker	Start (bp)	Hom allele
D19S558	13,312,989	het
D19S840	13,840,918	205
CC2D1A	14,016,956-14,031,194	
D19S226	14,633,400	251
D19S385	14,702,584	149
D19S514	15,025,465	252
D19S929	15,160,440	252
D19S432	15,681,990	196
D19S252	15,728,156	108
D19S411	15,902,094	122

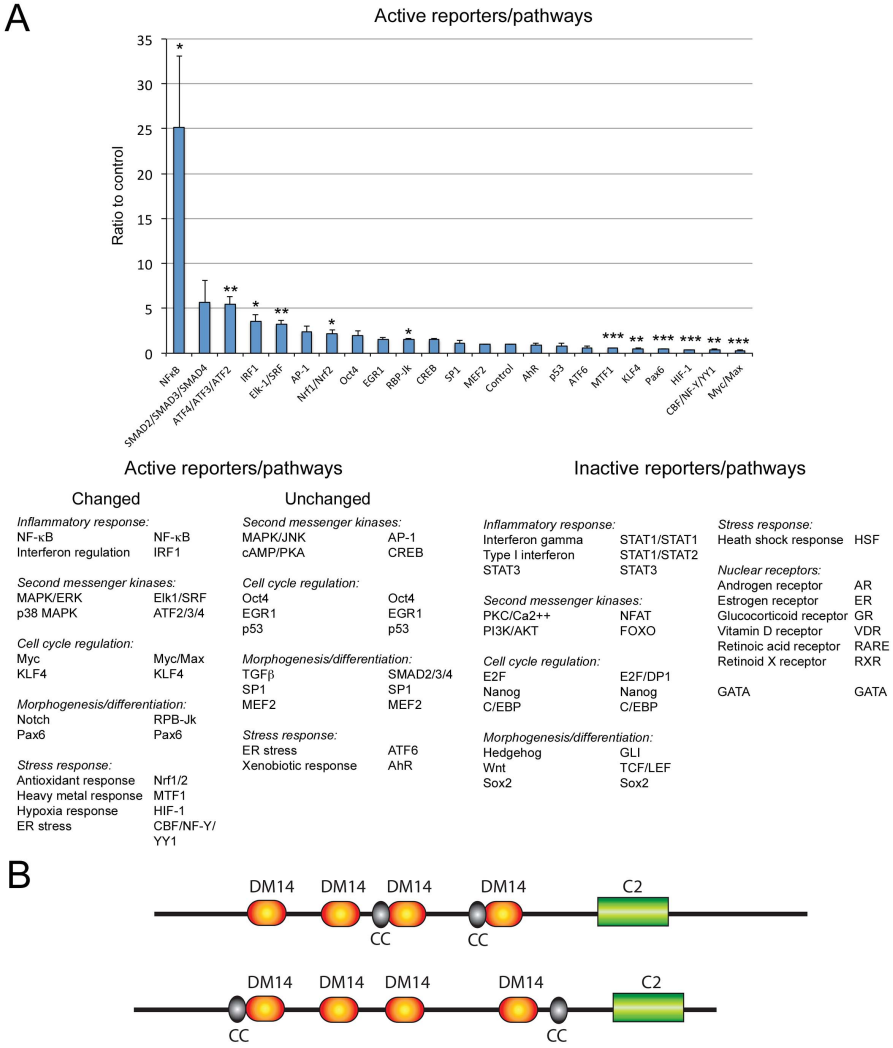
SNP haplotype around CC2D1A

Marker	Start (bp)	Hom allele
RS10419600	13,701,840	G
RS10409538	13,712,064	C
RS1422837	13,754,417	A
RS12462927	13,772,375	G
RS16979306	13,802,510	G
RS10422746	13,938,653	T
CC2D1A	14,016,956-14,031,194	
RS10419533	14,032,512	A
RS10419451	14,163,973	C
RS11667662	14,384,770	T
RS12460783	14,416,401	A
RS12982444	14,418,721	C
RS11670064	14,447,350	A

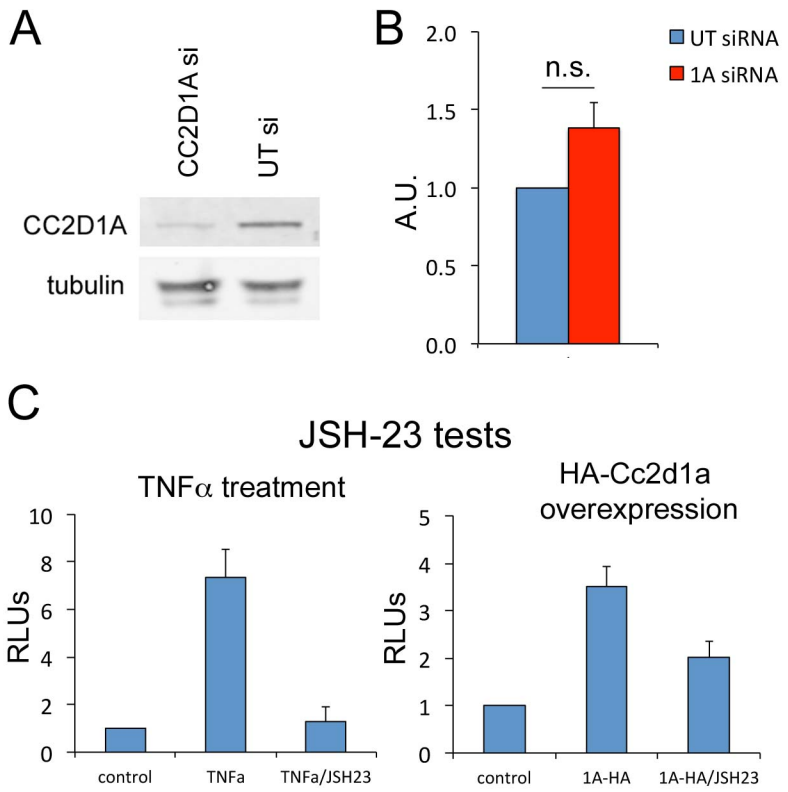
Supplemental Figure 1. Founder haplotypes shared among families 1, 2 and 3. Related to Figure 1. Microsatellite haplotype for family 3 showing the minimum region shared among all affected individuals in families 1,2 and 3. Extended minimum microsatellite and SNP haplotype are shown to the right.



Supplemental Figure 2. Additional data on the effects of Cc2d1a knockdown in neurons. Related to Figure 2. A. Multiple shRNAs were designed targeting different regions of the *Cc2d1a* mRNA. shRNA location is indicated over the protein schematic. Western blot analysis shows that three different shRNAs were highly efficient in removing expression of Cc2d1a. An additional control shRNA (shRNA UT) was designed by introducing 5 missense changes in shRNAa and this control shRNA does not reduce Cc2d1a expression. **B.** *In utero* electroporation at E15.5 did not affect neuronal migration at E18. **C.** Two independent shRNAs (a and b) yielded identical results on dendritic arborization in hippocampal neurons. **D-E.** Density of excitatory synapses identified by colocalization of the AMPA receptor subunit GluA1 and synapsin on a GFP positive dendrite and inhibitory synapses identified by colocalization of the postsynaptic scaffold gephyrin and the presynaptic GABA transporter VGAT was not changed. Scale bar: 10µm



Supplemental Figure 3. Additional data on the effects of Cc2d1a overexpression. Related to Figure 3. A. Summary of luciferase assays performed using the Cignal Finder 45-Pathway Reporter Arrays. Reporters were considered inactive when their luciferase levels were identical to the negative controls in both experimental and control conditions, while active reporters showed a response above negative control levels. Active reporters were considered unchaged if they showed no change in luciferase activity levels between experimental and control conditions. **B.** CC2D1A and CC2D1B proteins show great structural similarity (DM14:Drosophila melanogaster 14 domain; CC: coiled coil domain).



Supplemental figure 4. Additional data on Cc2d1a regulation of NF-κB. Related to Figure 4. A. Efficent knock-down of human CC2D1A is obtained using a pool of siRNAs in HEK-293 cells, compared to untargetable (UT) siRNA oligonucleotides. **B.** No significant increase in NF-κB transcriptional activity is observed following CC2D1A knock-down in HEK-293 cells. **C.** Effectiveness of JSH-23 in inhibiting NF-κB trascriptional activity. 20μM of JSH-23 greatly reduced NF-κB activation following treatment with either 50ng/ml of TNFα or HA-Cc2d1a overexpression.

Supplemental Table. Clinical data. Related to clinical description in first results section
Families 1 and 2

Pedigree No. (Gender) **Age when last evaluated**	**1:2 (F)** **15 y**	**1:3 (F)** **13 y**	**1:4 (M)** **6y**	**2:1 (M)** **11 y**	**2:3 (F)** **7 y**
Perinatal history	Full-term NSVD	Full-term NSVD	Full-term NSVD	Full-term NSVD	Full-term NSVD
Early motor development	Delayed	Delayed	Delayed	Reportedly normal	Delayed
Initial symptoms	Delayed compared to other children. No unusual or excessive interests.	Speech delay. Social with peers. Socially delayed but appropriate. Imaginative play.	Lack of response to name. By 3-4 years, behind peers in socialization. Later: walking around in circles. Collected rubber balls of different sizes and color and lined them up. No imaginative play. Hand clapping. Used to take mother by the hand to make her do things for him. Little pointing at objects.	Seizures; difficulty in interacting with his peers; preoccupation with toy-cars.	Speech delay. Poor social interactions. Spinning. Preoccupation with gadgets (phone charger).
Seizures	Not present	Not present	Not present	Present	Suspected.
Aggressive behavior	Not present	Not present	Not present	Occasional: when demands are not met.	Not present
Regression of skills	Not reported	Not reported	Not reported	Not reported	Not reported
Dysmorphic features	None	None	None	None	None
Mental status exam	Fixed smile. Poor eye contact. Few words. Clinical impression: Intellectual disability (ID). No clear evidence of ASD.	Fixed smile. Poor eye contact. Few words. Clinical impression: Intellectual disability. No clear evidence of ASD.	Oblivious of his surroundings. No rapport with the examiner. Poor eye contact. Trying to spin the chair. Flat expression. Hand flapping present. Mumbling with no clear speech. CARS Score: 38. Clinical Impression: ASD and ID	Passive. Mumbling tone. Poor eye contact. Short phrases but no reciprocal conversation. Rocking movements. SCQ score: 14; CARS score: 38. No IQ score. Clinical Impression: ASD and ID	Hand flapping. Finger flicking. Aimless wandering in circles. Overactive. Visual scanning of objects but poor eye contact with the examiner. Occasional grunting noises. SCQ: 26; CARS: 50. No IQ score. Clinical Impression: ASD and ID

NSVD: Normal spontaneous vaginal delivery, CARS: Childhood Autism Rating Scale, SCQ: Social and Communication Questionnaire
Note: Individual 1:1, male, aged 17 years, is reportedly institutionalized because of cognitive and speech delay with severe aggression.

Family 3

Pedigree No. (Gender) **Age when last evaluated** **(Duration of follow-up)**	**3:1 (M)** **32y** **(≥11y)**	**3:2 (F)** **22y** **(≥11y)**	**3:3 (M)** **20y** **(≥11y)**	**3:4 (M)** **14y** **(≥11y)**
Perinatal history	Full-term NSVD	Full-term NSVD	Full-term NSVD	Full-term NSVD
Early motor development	Delayed: walked after 2y	Normal	Delayed: walked about 2y	Normal
Initial symptoms	Abnormal behavior (2½ y): Playing with dirt and swallowing foreign objects (e.g. coins).	Delayed speech: "Mama and "Baba" at 3y, two words sentences at 6y.	Delayed speech: Single words at 3y.	Delayed speech: Single words by 3y and two word sentences at 5y.
Seizures	Not present	Not present	Not present	One episode of febrile convulsion at 2y
Aggressive behavior	Occasional: when demands are not met.	Not present	Occasional: during the period of adolescence.	Not present
Regression of skills	Not present	Not present	Not present	Not present
Dysmorphic features	None	None	None	None
Mental status exam	Leiter International Performance Scale-Revised [Leiter-R] (24y): Full IQ Score of 30. Clinical impression: ID. No evidence of ASD	Test of Non-verbal Intelligence- III [TONI-III] (16y): Standard score of 70 Clinical impression: ID. No evidence of ASD	Standford Binet Intelligence Scale (14y): IQ <32, with a corresponding mental age of 2y 11mo Clinical impression: ID. No evidence of ASD	Bayley Scales of Infant Development (8y): Mental Development Index Score <50, with a corresponding age equivalent of 26 mo Clinical impression: ID. No evidence of ASD

Family 4

Pedigree No. (Gender) **Age when last evaluated**	**4:1 (M)** **36y**	**4:2 (M)** **34y**	**4:3 (M)** **29y**	**4:4 (M)** **19y**	**4:5 (M)** **15y**
Perinatal history	Full-term NSVD	Full-term NSVD	Full-term NSVD	Full-term NSVD	Full-term NSVD
Early motor development	Normal	Delayed: walked at 2y	Normal	Normal	Normal
Initial symptoms	Normal speech onset, but limited communication, echolalia.	Normal speech onset, but limited communication, not able to learn.	Delayed speech, which remained poorly developed and not understandable.	Normal speech onset, but limited communication.	Normal speech onset, but limited communication, echolalia.
Seizures	Not present	Not present	Not present	Not present	Not present
Aggressive behavior	Not reported, but "easily angered"	Not present	Not present	Not present	Not present
Regression of skills	Not present	Not present	Not present	Not present	Not present
Dysmorphic features	None	Slightly microcephalic, protruding ears	Slightly protruding ears	None	None
Mental status exam	Understands verbal instruction, but paucity of spontaneous communication. Good eye contact. Attended school for cognitively impaired children, but was not able to learn. Clinical impression: ID. No clear evidence of ASD.	Understands verbal instruction and communicates in small sentences. Was not sent to school because he is more severely affected than 4:1. Compulsive about routine. Scratches himself when stressed. Clinical impression: ID. No clear evidence of ASD.	Understands simple verbal commands, but speech has never been comprehensible. Does not indicate hunger and does not show interest in television. Uncanny facial recognition of people only met once, though cannot pronounce names. Clinical impression: ID/ borderline ASD.	Described as a "permissive automaton": extremely passive with no affect, but perfectly follows commands and routines. Diminished spontaneous communication, but good eye contact and socially appropriate behavior. Clinical impression: ID. No evidence of ASD.	Understands simple verbal commands, but has trouble imitating. Inattentive and self-absorbed, poor eye contact. Constantly self-talking. Clinical impression: ASD/ID

Supplemental Experimental Procedures

Linkage analysis and DNA sequencing

Genomic DNA was purified from lymphocytes separated from peripheral blood using commercial kits (Qiagen). Genomic DNA from all available family members was used for genome-wide SNP analysis on Axiom Chip platform (Affymetrix). For microsatellite analysis, highly polymorphic microsatellite markers were chosen from the Marshfield database in the University of California Santa Cruz Genome Browser. Fluorescently labeled polymerase chair reaction (PCR) primers (Applied Biosystems) were used to amplify DNA samples using standard conditions and PCR products were resolved on an Applied Biosystem 3031xl Genetic analyzer. Exome sequencing for Family 4 was completed using the Agilent Sure Select v4 capture kit followed by paired-end sequencing on an Illumina HiSeq machine. Sanger sequencing of *CC2D1A* (NM_017721) coding region was performed on PCR products after amplification of genomic DNA. PCR primers were designed for each exon including at least 50bp of flanking intronic sequences. The effect of the mutation on mRNA splicing was further evaluated by two-step RT-PCR. Primer sequences are available upon request.

Patient cell lines and mutated protein detection

Transformed lymphoblastoid cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum, glutamine (2mM) and penicillin/streptomycin (100U/100mg) (all from Invitrogen). Protein lysates were obtained by boiling a cell pellet in Laemmli sample buffer (Bio-Rad Laboratories). Western blotting was performed using standard protocols on Invitrogen equipment. Presence of CC2D1A was assessed using a mouse polyclonal antibody raised against the full-length human CC2D1A protein (AbCam), using rabbit anti-alpha tubulin antibody (AbCam) to control for loading and appropriate IRDye secondary antibodies (LI-COR Biosciences) for detection on an Odyssey Imager (LI-COR Biosciences).

Quantitative PCR primers

Cc2d1a-For 5'-GCTGGCAGAGCTAAATGAGGT-3' and Cc2d1a-Rev 5'-GTGCAGACTGGTAGAGGGTTA-3'.

ShRNA constructs

For short hairpin shRNA construction, RNAi sequences were as follows: shRNAa – 5'AATTGTCAAGCAATACCAA3'; shRNAb – 5'AGGAGACTCCTAAGAAGCA3'; shRNAc – 5'AGCAGTTCAAACTCTGCAT3'. Oligonucleotides containing the selected sequenced in a short hairpin configuration were cloned into a version of pSilencer 1.0 (Ambion) modified to also express GFP (kind gift of Azad Bonni, Harvard Medical School). Mutated shRNA sequences were designed by inserting 5 single nucleotide changes in effective shRNAs. ShRNA efficiency was tested by measuring knock-down of HA-tagged murine Cc2d1a upon transfection into HEK293 cells on Western blot using both β actin (rabbit anti-β actin; AbCam) and GFP (chicken anti-GFP; AbCam) as controls. All secondary antibodies were from Li-Cor Biosciences for detection on an Odyssey Imager (LI-COR Biosciences).

Cell culture and morphological analysis

Hippocampal cultures were prepared from E17-E18 Swiss Webster mouse pups and plated on 8-well Lab-Tek chamber slides (Nunc) coated with poly-L-ornithine (Sigma-Aldrich) at a density of $2.5X10^5$ cells/cm^2. Cultures were maintained in Neurobasal medium with B27 supplement (all Invitrogen). In all cultures transfections were performed 1 day after plating using Lipofectamine 2000 (Invitrogen) as described by Dean et al., 2003. Cells were fixed with 4% paraformaldehyde at the time-points indicated and transfected neurons were immunostained for GFP to reveal dendrite morphology. Images for at least 10 independent cells per condition per experiment were collected via an unbiased method using a Zeiss M2 Imager microscope. Dendrite length and number were measured using the NeuronJ plugin for ImageJ software (Meijering et al., 2004). For spine analysis, neurons were fixed at 14 div and a portion of the dendrite proximal to the cell body was imaged at high magnification (63X) for at least 10 independent cells per condition using a Zeiss LSM Meta510 confocal microscope. Spines were counted and scored manually in the visible field and the length of the dendrite where spines were counted was measured using NeuronJ to determine spine density.
For synaptic puncta analysis, hippocampal neurons were fixed at 14 div with 4% paraformaldehyde and immunostained for GluA1 (rabbit anti-GluA1, AbCam) and synapsin I (mouse anti-synapsin I, Synaptic Systems) for excitatory synapses and for gephyrin (rabbit anti-gephyrin, Synaptic Systems) and VGAT (mouse anti-VGAT, Synaptic System) for inhibitory synapses. All cultures were also counterstained for GFP to identify transfected cells for analysis and imaged on a Zeiss LSM510 or LSM700 confocal microscope. Puncta colocalization on GFP positive dendrites was counted using Imaris software (Bitplane) and puncta decorated by all three antibodies were considered synaptic contacts. Density was measured by dividing the total number of puncta by the length of the dendrites in the field of view.

HEK293 and neuronal luciferase assays

Experiments testing NF-κB activity in HEK293 cells were performed using a firefly luciferase reporter construct containing four NF-κB binding sites in the promoter region and pRL-TK were *Renilla* luciferase is driven by the thymidine kinase promoter (kind gift of Douglas McDonald, Boston Children's Hospital). NF-κB reporter and pRL-TK were cotransfected in HEK293 cells with Cc2d1a or Cc2d1b overexpressing constructs. HA-Cc2d1b (NM_177045) was synthesized by Genewiz and cloned into pcDNA3. For TNFα treatments cells were treated with 100ng/mL for 2 hours.
For luciferase assays hippocampal neurons were prepared at E17-18 and $2X10^6$ cells were seeded in each well of a 6-well plate. Cells were transfected at 1 div with a ratio of 1:0.8:0.8 of shRNA or overexpression construct:NF-kB reporter:pRL-TK as described above. Luciferase assays were performed at 2 or 7 div using the Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer, and luciferase values were calculated as a ratio between firefly and *Renilla* luciferase in each experimental condition.

References

Dean, C., Scholl, F. G., Choih, J., DeMaria, S., Berger, J., Isacoff, E., & Scheiffele, P. (2003). Neurexin mediates the assembly of presynaptic terminals. *Nature Neuroscience*, *6*(7), 708–716. doi:10.1038/nn1074

Meijering, E., Jacob, M., Sarria, J.-C. F., Steiner, P., Hirling, H., & Unser, M. (2004). Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry. Part a : the Journal of the International Society for Analytical Cytology*, *58*(2), 167–176. doi:10.1002/cyto.a.20022