**Supplementary Figure 1. Ultrastructural characterization of P2 fraction.** EM analysis of P2 fraction obtained from cultures processed at DIV6 (A) and DIV16 (B) showed sub-cellular structure resembling synaptosome. Arrows indicate post-synaptic density, arrowhead presynaptic bouton filled with synaptic vesicles. Scale bar=100nm.

**Supplementary Figure 2. Biochemical investigation of DIV6 cultures.** We monitored biochemical fractions obtained from DIV6 neurons by western-blotting with antibodies raised against synaptic (A), cytoskeletal (B) and cytosolic markers (C). NR2A=NMDA-R subunit 2A, s-physin=synaptophsin, RpS6= ribosomal protein S6. For each protein blotted on the left the histograms on the right report on y-axis the optical density (O.D.) found in each fraction normalized versus the input amount. Noteworthy, we found that NR2A is present at low level in the P2 fraction, i.e. crude synaptosome, of immature neurons as reported in literature (1).

**Supplementary Figure 3. Subcellular localization of STREP-PD from P2 hits.** (A) To test the performance of our protocol, we assayed by western-blotting the distribution of selected hits from STREP-PD from P2 fraction. (B) Neurons were fixed at DIV16 and stained with antibodies raised against the indicated hits (red) plus antibodies specific for the pre-synaptic marker SV2A (green). The selected hits show high overlap with synaptic structures (merge). Scale bar=10 µm.

**Supplementary Figure 4. Neuronal arborization develops in vitro.** (A) Cortical neurons were infected at DIV1 with a GFP-expressing virus (miRNA control). Cells were fixed at the indicated DIVs and imaged by mean of confocal laser microscopy. Scale bar= 20µm. (B) Distribution of neurites in each branching order. Data are expressed as mean±SEM; * p<0.05 vs DIV4, one-way ANOVA test; n=20 neurons for each experimental case.
Supplementary figure 5. Synapses mature morphologically along \textit{in vitro} culture. Cortical neurons were cultured until the indicated DIV and then fixed and prepared for electron microscopy. (A) Representative images showing synaptic buttons and sub-cellular structure, namely postsynaptic density (PSD, arrow), active zone (AZ, arrowhead), synaptic vesicle (SV, hollow circle) and docked synaptic vesicle (docked SV, filled circle). (B) Morphological analysis of synaptic structures in term of PSD length, PSD width, AZ length, SV total pool, number of docked SV and mean SV diameter for each synapse revealed that pre and postsynaptic structures increase their complexity during development \textit{in vitro}. Images were acquired at 40000X. Scale bar= 500nm. Data are expressed as mean±SEM; * $p<0.01$ vs DIV4, ANOVA test, n= 4, 10 synaptic contacts for each experimental case were measured.

Supplementary Figure 6. Development of tools to modulate Negr1 expression. (A) Western blotting analysis of RAB3A expression in lysate obtained from cortexes of mice sacrificed at postnatal day 0 (P0), 2 (P2) and 7 (P7). (B) Quantification of RAB3A amount in postnatal cortexes showed that its did not change significantly during \textit{in vivo} maturation. Data were normalized versus P0 amount.(C) Four cassettes containing Negr1 specific silencing sequences were cloned in the LVTH backbone that co-expresses GFP via IRES sequence. In order to asses the efficiency of our four silencing constructs, we transfected FLAG-Negr1 and silencing constructs or empty vector with a ratio of 1:3 in N2A cells. Three independent silencing miRNAs (A2, D2, C7) efficiently down-regulated Negr1 expression in N2A cells, whereas one (C8) and the empty vector (CTRL) did not. (D) Next, we produced viral particles bearing silencing miRNA A2 and control miRNA C8. We infected cortical neurons at DIV1 and evaluated Negr1 protein level at DIV16. Western-blotting analysis showed that miRNA A2 strongly reduced endogenous Negr1 expression in neurons. (E) The panel reports protein optical density (O.D.) normalized versus control infection. (F) To confirm the specificity of our silencing approach, we introduced in FLAG-Negr1 two silent mutations in the
region targeted by miRNA A2 construct by site-direct mutagenesis. Such substitutions confer to the construct the resistance to miRNA Negr1 mediated silencing (Negr1-R). When we evaluated Negr1 protein level in N2A cells co-transfected with FLAG-Negr1 or FLAG-Negr1-R plus miRNA C8 or A2 (1:3 ratio), we confirmed that Negr1-R expression was not impaired by miRNA A2. (G) The panel reports protein optical density normalized versus the condition FLAG-Negr1+ miRNA C8 in the different experimental cases. Samples containing equal amount of proteins were resolved by SDS-PAGE. Data are based on protein optical density (O.D.) normalized as described and expressed as mean±SEM; Student T-test, * p<0.05 versus FLAG-Negr1 miRNA C8, ° p<0.05 versus FLAG-NEGR1 miRNA A2; n = 4 independent experiments.

**Supplementary Figure 7. Negr1 silencing affects dendritic development.** Cortical neurons were infected at DIV1 with miRNA control and miRNA Negr1. At DIV16, cells were fixed, stained with anti-MAP2 antibodies and imaged by means of confocal-laser microscopy. We considered MAP2 positive processes as dendrites and MAP2 negative processes lacking spines as axons. GFP (green), MAP2 (red), merge and tracing images are reported. Negr1 silencing reduced dendrites number while axon (arrowhead) was present in both miRNA control and miRNA Negr1 infected cells. Scale bar=20 µm

**Supplementary Figure 8. Negr1 silencing affects neurite development.** Cortical neurons were infected at DIV1 with miRNA control and miRNA Negr1-2 (bearing Negr1 silence sequence D1). At DIV16, cells were fixed and imaged by means of confocal-laser microscopy. (A-B) Morphology of neurons infected with miRNA control and miRNA Negr1-2 and relative tracing. (C-D) Quantification of neurite total length and number for each neuron. (E) Sholl analysis shows that the neurite arborization is severely affected by miRNA Negr1-2 infection. (F) miRNA Negr1-2 infection alters the distribution of neurites in each branching order. Scale bar= 20µm. Data are
expressed as mean±SEM; Student T-test, * p<0.01, n=3, 7 neurons for each experimental case were measured.

**Supplementary Figure 9. Negr1 modulates neuronal maturation in vivo.** Mice embryos were electroporated *in utero* at E15.5 with miRNA control or miRNA Negr1 and sacrificed at P7. (A) Confocal images of GFP fluorescence in coronal sections of mouse somatosensory cortices. Scale bar= 25 µm. (B) Sholl Analysis shows that the neurite arborization is severely affected by miRNA Negr1 infection. Student T-test, * p<0.05, n= 10 electroporated cells/experimental case.

**Supplementary Table 1. Protein identification.** Table reports on sheet “Protein list” gene symbol, protein name, accession number, molecular weight, the number of unique peptide found in the different experiments, GO annotation cellular component, predicted number of membrane-passing domain as computed by TMHMM 2.0 (TMHMM), GO annotation biological process. The sheet “protein export” reports MS/MS export view of the analysed samples.

**Supplementary Table 2. Peptide identification.** Table lists analytic reports for all peptides identified.

**Supplementary Table 3. Relative protein quantification.** Table on “significant hits” sheet reports information for differentially expressed proteins characterized by p-value < 0.05 as computed by T-test analysis. protein accession number, GO annotation cellular component, predicted number of membrane-passing domain as computed by TMHMM 2.0 (TM), prediction of secretion (SP), GO annotation biological process, number of peptides assigned, number of unique peptides used for the quantification, cumulative MASCOT confidence score, ANOVA p-value, T-test p-value, fold change between DIV16 and DIV6 (DIV16/DIV6), gene symbol, protein description and normalised
as well as raw abundances of cumulated peptides intensities recorded in each experimental
condition and spectral count. Sheet “membrane hits” list differentially expressed proteins localised
at the membrane, as indicated by GO analysis. Sheet “plot” includes a graph plotting DIV16/DIV6
ratio (expressed as Log10) for all differentially expressed protein identified. Table lists T-test p-
value, ratio DIV16/DIV6, Log10 of ratio DIV16/DIV6, gene symbol and protein description.

**Supplementary Table 4. Peptide report for supplementary table 3.**

Analytical details for all peptides used for relative quantification of proteins reported in
supplementary table 3 are listed, including m/z values, retention times, charge state, fold change
between DIV6 and DIV16 For each peptide, individual Mascot score, mass errors, peptide
sequence, peptide modification including position, normalised peptide abundances as well as
spectral counts observed in every sample.

**Supplementary references**

   NMDA receptor surface trafficking and synaptic subunit composition are developmentally