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A circadian clock in hippocampus is regulated by interaction between oligophrenin-1 and Rev-erba

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Running title

Circadian clock in hippocampus regulated by oligophrenin-1-Rev-erba interaction

Oligophrenin-1 regulates dendritic spine morphology in brain. Mutations in the oligophrenin-1 gene cause intellectual disability. We report a new partner of oligophrenin-1, Rev-erb α : a nuclear receptor that represses the transcription of circadian oscillators. We show in mouse brain that oligophrenin-1 interacts with Rev-erb α , causing it to locate to dendrites, reducing its repressor activity, and protecting it from degradation. More importantly we demonstrate a circadian oscillator in hippocampus, involving the clock gene *bmal1*, modulated by Rev-erb α and requiring oligophrenin-1 for normal oscillation. We also show that synaptic activity induces Rev-erb α localization to dendrites and spines, a process mediated by AMPA receptor activation and requiring oligophrenin-1. Our data reveal new interactions between synaptic activity and circadian oscillators, and delineate a new means of communication between nucleus and synapse that may provide insight into normal plasticity and the etiology of intellectual disability.

Defective formation or function of synapses in the central nervous system during development results in disorders of learning and memory, including autism and intellectual disability¹⁻³. X-linked intellectual disability (XLID) is a heterogeneous condition caused by single gene mutations on chromosome X¹⁻³. Over half the genes found mutated in XLID code for synaptic proteins involved in actin cytoskeleton rearrangement, synaptic plasticity, synapse formation or neurotransmission; although the precise roles of most of these genes remain unknown⁴.

One of the genes responsible for XLID is OPHN1, which encodes a synaptic RhoGTPase-activating protein (oligophrenin-1) that, among other things, regulates dendritic spine shape in the brain⁵⁻⁷. OPHN1 involvement in XLID was established by the identification of XLID-associated OPHN1 mutations (balanced translocation t(X; 12) or frameshift deletion) in XLID patients⁸. Subsequently OPHN1 mutations were identified in families with intellectual disability associated with cerebellar hypoplasia or epilepsy⁹⁻¹¹.

The oligophrenin-1 protein comprises an amino terminal BAR (*Bin-Amphiphysin-Rvs*) domain which binds curved membranes; a pleckstrin homology domain thought to confer membrane binding specificity through interaction with phosphoinositides; a central RhoGAP domain; and three carboxyl-terminal proline-rich sequences, which are putative SH3-binding sites for endocytic adaptor proteins^{7,8,12}. Oligophrenin-1 is broadly expressed in brain with enrichment in the hippocampus, cortex, and cerebellum. It is associated with F-actin in growth cones and dendritic spines, suggesting participation in synapse formation or function^{5,6}. Oligophrenin-1 is also associated with Homer⁶, an adaptor molecule that influences dendritic spine morphology and synaptic transmission through interaction with Shank and with glutamate receptors¹³⁻¹⁵. In hippocampal slices and neuronal cultures, inactivation of oligophrenin-1 function affects the maturation and reduces the density of dendritic spines^{6,7,12}. The oligophrenin-1 (*ophn1*) knockout mouse has impaired spatial learning, altered social behavior, novelty-driven hyperactivity, and enlarged cerebral ventricles⁷. Loss of oligophrenin-1 also reduces synaptic vesicle endocytosis in

the pre-synaptic compartment^{12,16} and affects the internalization and stability of AMPA receptors post-synaptically^{12,16}.

In the present study we show that oligophrenin-1 interacts with Rev-erb α . Rev-erb α is a nuclear receptor that suppresses gene transcription and is involved in circadian clock regulation¹⁷. The heme group is a physiological ligand of Rev-erb α that enhances its thermal stability and results in the recruitment of the co-repressor N-CoR¹⁸. Rev-erb α knockout mice show several defects during the postnatal development of the cerebellar cortex, with delayed proliferation and migration of granule cells from the external granule cell layer, together with increased apoptosis of neurons in the internal granule cell layer¹⁹.

We show that oligophrenin-1 interacts with Rev-erb α to protect it from degradation, cause it to localize in dendrites, and suppress its repressor activity. More importantly we show the presence of a circadian oscillator in hippocampus that is modulated by Rev-erb α and requires the presence of oligophrenin-1 for normal oscillation.

Results

Oligophrenin-1 interacts with Rev-erb α in neurons and COS7 cells and induces Rev-erb α localization to the cytoplasm of COS7 cells overexpressing both proteins

Using the carboxy-terminal of oligophrenin-1 as bait in the yeast two-hybrid system, we screened 1.5×10^6 clones of a human fetal brain cDNA library. Four interacting cDNAs were isolated (clones 11, 19, 23, 40; Fig. 1a) all of which encoded Rev-erb α .

We next used the two-hybrid system to assess the interaction of each of two fragments (550-633 aa and 634-802 aa) of the C-terminus of oligophrenin-1 as bait, with a minimal Rev-erb α cDNA clone (which does not contain the DNA binding domain) as prey (Fig. 1b). Activation of all three reporter genes (HIS3, LacZ and URA3) (Fig. 1b) indicated that the proximal fragment had greater affinity for Rev-erb α than the distal fragment, suggesting that the oligophrenin-1/Rev-erb α interaction is complex and involves different regions of the carboxy terminal of oligophrenin-1.

To confirm the interaction between oligophrenin-1 and Rev-erb α , we examined their co-immunoprecipitation when overexpressed in COS7 cells. Full-length myc-tagged Rev-erb α was co-transfected either with wild type (WT) hemagglutinin-tagged oligophrenin-1 (HA-oligophrenin-1) or with HA-oligophrenin-1 Δ C. Oligophrenin-1 Δ C has a deleted carboxy-terminal end and mimics one of the forms present in intellectual disability ⁸. Rev-erb α coimmunoprecipitated with WT oligophrenin-1, but not with oligophrenin-1 Δ C (Fig. 1d).

Next, we studied the localization of these proteins in transfected COS7 cells. When expressed alone, Rev-erb α localized to the nucleus, visualized by colocalization with DAPI (nuclear marker) (Fig. 1c). When oligophrenin-1 was expressed alone it was present in the cytoplasm in regions enriched in F-actin (sub-cortical actin and stress fibers) with no detectable localization in the nucleus (Fig. 1c) as previously described ⁵. On co-expression with oligophrenin-1, Rev-erb α was present in both nucleus and cytoplasm (Fig. 1c) where it co-localized with oligophrenin-1.

Importantly, the localization of Rev-erb α to the cytoplasm was dependent on interaction with the oligophrenin-1 C terminus, since Rev-erb α remained in the nucleus when co-expressed with oligophrenin-1 Δ C, the mutant lacking the C-terminal tail and unable to bind Rev-erb α (Fig. 1c).

To obtain evidence for oligophrenin-1-Rev-erb α interaction in vivo, we performed GST pull-down experiments on rat brain extracts and cultured hippocampal neuron lysates. The first 83 (550-633aa) and last 126 residues of the oligophrenin-1 C terminus fused to GST (but not GST alone) pulled down Rev-erb α from rat brain and hippocampal neuron lysates (Fig. 1e). As in the two-hybrid system (Fig. 1b), the proximal part of the oligophrenin-1 C terminus (aa 550-633), containing two proline-rich regions, interacted more strongly with Rev-erb α than the distal part (Fig. 1e).

Co-immunoprecipitation experiments from rat brain lysate (Fig. 1f) also showed that oligophrenin-1 and Rev-erb α interact. These data establish that oligophrenin-1 binds Rev-erb α via its C-terminal in neurons and COS7 cells, and causes Rev-erb α to localize in the cytoplasm of COS7 cells.

Oligophrenin-1 reduces Rev-erb α repression of transcription and protects it from degradation

To assess the functional significance of the oligophrenin-1/Rev-erb α interaction we investigated oligophrenin-1's effect on transcription repression by Rev-erb α using a reporter assay in heterologous cells (HEK293T). Previous studies have shown that Rev-erb α 's ability to repress transcription is strongly dependent on Rev-erb α protein binding to its response element (RE),^{20,21} and can be followed by monitoring the activity of the luciferase reporter gene under control of the SV40 promoter coupled to the RE (pSV40-RE). In HEK cells, we overexpressed the reporter construct alone (pSV40-RE), and used other constructs to overexpress GFP (control), myc-tagged Rev-erb α , myc-Rev-erb α plus HA-tagged oligophrenin-1, or myc-Rev-erb α plus HA-oligophrenin-1 Δ C.

We first showed that myc-Rev-erb α overexpression caused four-fold repression of reporter gene (luciferase) transcription in HEK cells compared to cells overexpressing pSV40-RE alone (Fig. 2a). Neither HA-oligophrenin-1 alone (data not shown) nor GFP alone (Fig. 2a) had any effect on reporter gene repression. However, luciferase expression was significantly higher (1.5 fold) in cells expressing myc-Rev-erb α and HA-oligophrenin-1 (transfection with 600 ng of each construct) compared to cells expressing myc-Rev-erb α alone (Fig. 2a). We also found that the repression activity of Rev-erb α increased significantly (decreasing luciferase activity) as the amount of myc-Rev-erb α used in the transfection process (150, 300, 600 ng) increased (constant quantity of HA-oligophrenin-1 plasmid) (Supplementary Fig. 1a). Furthermore, in cells expressing myc-Rev-erb α and HA-oligophrenin-1 Δ C, luciferase expression was reduced to levels similar to those of cells overexpressing myc-Rev-erb α alone (Fig. 2a). These data show that oligophrenin-1 reduces the repressor activity of Rev-erb α .

To investigate whether oligophrenin-1 reduces Rev-erb α repressor activity by causing Rev-erb α to locate outside the nucleus, we measured luciferase activity and myc-Rev-erb α expression in the nucleus and cytoplasm of HEK cells co-expressing myc-Rev-erb α with different amounts (150, 300

and 600 ng) of the plasmid encoding HA-tagged oligophrenin-1. We found that luciferase activity increased (repression activity of Rev-erb α decreased) with the amount of HA-oligophrenin-1 transfected (Supplementary Fig. 1b). Subcellular fractionation showed that HA-oligophrenin-1 only accumulated in cytoplasm (Fig. 2b), while myc-Rev-erb α accumulation in cytoplasm increased with the amount of HA-oligophrenin-1 used in the transfection procedure (0.25 ± 0.005 with 150 ng vs. 0.33 ± 0.005 with 300ng, $p \leq 0.001$; 0.25 ± 0.005 with 150 ng vs. 0.39 ± 0.006 with 600ng, $p \leq 0.0001$; 0.33 ± 0.005 with 300ng vs. 0.39 ± 0.006 with 600ng, $p \leq 0.01$) (Fig. 2b). Furthermore, the ratio of Rev-erb α in cytoplasm to total quantity present increased with increasing luciferase activity (Supplementary Fig. 1c) indicating that the repression activity of Rev-erb α correlated with its nuclear localization.

In the next set of experiments we investigated whether oligophrenin-1 influenced Rev-erb α stability. Glycogen synthase kinase 3 β (GSK3 β) phosphorylates Rev-erb α causing it to stabilize²², while kinase inhibition by lithium or small interfering RNA leads to near complete loss of endogenous Rev-erb α protein.²² We transfected HEK cells (that endogenously express GSK3 β), with GFP (as control of transfection efficiency) and myc-tagged Rev-erb α , alone or in combination with the dominant-negative (DN) form of HA-tagged GSK3 β ²², or with oligophrenin-1, and also with both HA-GSK3 β DN and oligophrenin-1. Forty-eight hours after transfection we assessed myc-Rev-erb α (Figs. 2c and 2d). Myc-Rev-erb α was destabilized in cells co-expressing HA-GSK3 β DN compared to cells not expressing HA-GSK3 β DN (0.02 ± 0.001 vs. 2.5 ± 0.15 , $p \leq 0.0001$) (Fig. 2d). But levels of myc-Rev-erb α were higher in cells overexpressing oligophrenin-1 together with myc-Rev-erb α and HA-GSK3 β DN than those overexpressing myc-Rev-erb α with HA-GSK3 β DN but no oligophrenin-1 (3.2 ± 0.15 vs. 0.02 ± 0.001 , $p \leq 0.0001$) (Figs. 2c and 2d). These findings show that, in heterologous cells, oligophrenin-1 stabilizes Rev-erb α in the absence of active endogenous GSK3 β .

To investigate how oligophrenin-1 protects Rev-erb α from degradation, HEK cells overexpressing myc-Rev-erb α alone or with HA-oligophrenin-1 (600 ng of plasmid transfected) were incubated for

4h at 37°C, with or without the proteasome inhibitor MG132 (10μM), and the distribution of myc-Rev-erba in nucleus and cytoplasm assessed. In HEK cells not treated with MG132 and expressing myc-Rev-erba, myc-Rev-erba accumulated in the nucleus, as shown by Western blot (Fig. 2e). In cells also expressing HA-oligophrenin-1, myc-Rev-erba was present both in nucleus and cytoplasm (Fig. 2e). The ratio of cytoplasmic to total (nucleus plus cytoplasm) myc-Rev-erba was 0.16 ± 0.007 in cells expressing myc-Rev-erba alone, and 0.44 ± 0.006 in cells expressing myc-Rev-erba plus HA-oligophrenin-1, $p \leq 0.0001$ (Fig. 2e). When proteasomes were blocked by MG132, the accumulation of myc-Rev-erba in nucleus and cytoplasm was similar in cells expressing myc-Rev-erba alone and in those also expressing HA-oligophrenin-1 (Fig. 2e); the respective cytoplasmic to total ratios of myc-Rev-erba were 0.578 ± 0.0022 and 0.560 ± 0.004 (Fig. 2e). These findings indicate that, in heterologous cells, oligophrenin-1 protects myc-Rev-erba from proteasome degradation.

We next performed subcellular fractionation to assess Rev-erba protein levels by Western blot in the nuclear fraction (N) and post-nuclear supernatant (S) of hippocampus from oligophrenin-1 WT and KO mice⁷. In WT hippocampus, Rev-erba was present in N and S, while in KO hippocampus, Rev-erba was only present in N (at a similar level to WT) (Fig. 2f), and overall levels of Rev-erba protein (N+S) were higher in WT than KO (1.62 ± 0.06 vs. 0.95 ± 0.03 , $p \leq 0.001$) (Fig. 2f, right). Furthermore, the S to N ratio of Rev-erba was higher in WT than KO (0.26 ± 0.01 vs. 0.48 ± 0.01 , $p \leq 0.001$) (Fig. 2f).

These findings were confirmed by our immunofluorescence study of Rev-erba localization, which showed that in WT neurons, Rev-erba was prominent in nuclei and was also present in dendrites; while in KO neurons, Rev-erba immunostaining was still clearly evident in nuclei, but appeared less evident in dendrites (Supplementary Fig. 2). As control we also examined the localization of the transcription factor Sp1 in KO and WT mouse neurons (Supplementary Fig. 2), finding no differences in Sp1 immunostaining between oligophrenin-1 KO and WT mice (Supplementary Fig. 2).

To test if oligophrenin-1 absence caused an increase in endogenous Rev-erb α degradation in dendrites, hippocampal neurons overexpressing oligophrenin-1 siRNA or scrambled siRNA^{6,12} were incubated for 4h at 37°C, with or without (untreated) proteasome blocker MG132 (10 μ M), and the distribution of endogenous Rev-erb α in soma and dendrites assessed. In untreated neurons overexpressing scrambled siRNA, Western blot showed that Rev-erb α accumulated in soma and dendrites; while in neurons overexpressing oligophrenin-1 siRNA, Rev-erb α was present only in soma (Fig. 2g). On quantification, Rev-erb α levels were higher in untreated neurons overexpressing scrambled siRNA than those overexpressing oligophrenin-1 siRNA (1.85 \pm 0.016 vs. 1.11 \pm 0.028, $p\leq$ 0.0001) (Fig. 2g). When proteasomes were blockaded by MG132, Rev-erb α protein was present in both soma and dendrites (Fig. 2g) and levels were similar irrespective of whether scrambled siRNA or oligophrenin-1 siRNA was overexpressed (Fig. 2g). In addition, Rev-erb α protein levels were higher in MG132-treated than untreated neurons. These findings show that, in hippocampal neurons, oligophrenin-1 stabilizes endogenous Rev-erb α in dendrites by protecting it from proteasome degradation.

Altered circadian oscillations in hippocampus of Ophn-1^{-/-} mice

Since Rev-erb α is known to repress its own promoter, we assessed the consequences of oligophrenin-1 loss on Rev-erb α transcription²¹. We used RT-PCR to determine the expression of Rev-erb α mRNA at circadian times (CT) 18, 0, 6, and 12 hours in dark/dark conditions (DD), in the hippocampus, cortex and cerebellum of oligophrenin-1 WT and Ophn-1^{-/-} mice. In the hippocampus of both WT and Ophn-1^{-/-} animals, Rev-erb α mRNA expression oscillated over 24 hours. In WT, the observed peak was between CT 3-9 hours, while in Ophn-1^{-/-} mice the observed peak was between CT9-15 hours (Fig. 3a) showing shifted oscillation of Rev-erb α expression in hippocampus of Ophn-1^{-/-} mice ($F(3;24)=3.96$; $P<0.02$) (Fig. 3a). The differences between genotypes were most significant ($p<0.05$) at CT12 (0.81 \pm 0.077 in Ophn-1^{-/-}, 0.656 \pm 0.035 in WT) and CT18 (0.587 \pm 0.032 in Ophn-1^{-/-}; 0.438 \pm 0.013 in WT). In cortex, Rev-erb α mRNA oscillations

over 24 hours were similar between genotypes, with the difference that Rev-erb α mRNA levels were higher overall in oligophrenin-1 Ophn-1^{-/-} animals ($F(1,23)=9.81$; $p=0.005$) (Fig. 3d). In cerebellum, Rev-erb α mRNA also oscillated over 24 hours (Supplementary Fig. 3a) and expression levels were similar in WT and Ophn-1^{-/-}. These findings are consistent with greater expression of Rev-erb α mRNA in the cortex and hippocampus of oligophrenin-1 Ophn-1^{-/-} animals and a modest shift in the oscillation of Rev-erb α mRNA in the hippocampus of Ophn-1^{-/-} compared to WT animals.

We next assessed the expression of Rev-erb α protein in WT and Ophn-1^{-/-} animals over 24 hours in DD conditions. Protein levels oscillated over 24 hours in both WT and Ophn-1^{-/-} hippocampus, and were similar at CT0 and CT6, but at CT12 and CT18 were higher in WT than Ophn-1^{-/-} (1.64 ± 0.018 vs. 1.38 ± 0.014 , $p<0.05$ at CT12; 2.03 ± 0.004 vs. 1.74 ± 0.007 , $p<0.05$ at CT18) (Fig. 3b). In cortex, Rev-erb α protein also oscillated, with levels similar at all CTs in Ophn-1^{-/-} and WT (Fig. 3e). These findings indicate that loss of oligophrenin-1 interaction increases Rev-erb α protein degradation in the hippocampus at times around CT12-CT18, suggesting that oligophrenin-1 regulates Rev-erb α stability during circadian rhythms.

The reduced Rev-erb α protein at CT12 and 18 probably explains the increased Rev-erb α mRNA at the same time points, since the protein is no longer present at sufficient levels to repress its own transcription. These in vivo findings confirm that oligophrenin-1 stabilizes Rev-erb α in hippocampus.

We next assessed the expression of Bmal1 mRNA at CT 18, 0, 6 and 12 h, in hippocampus, cortex and cerebellum of oligophrenin-1 Ophn-1^{-/-} and WT mice. Bmal1 is a clock gene known to be a target of Rev-erb α repression¹⁷. Bmal1 transcript levels oscillated over 24 hours in both Ophn-1^{-/-} and WT hippocampus, but were significantly higher in Ophn-1^{-/-} at CT18 than WT (0.632 ± 0.024 vs. 0.474 ± 0.016 , $p<0.05$) (Fig. 3c). In cortex the oscillation of Bmal1 mRNA expression over 24 hours was similar to that in hippocampus. ANOVA showed that the WT and Ophn-1^{-/-} oscillations of Bmal1 transcripts differed ($P=0.037$) but pair-wise Bonferroni tests were not significant (Fig.

3f). Bmal1 mRNA expression also oscillated in cerebellum with oscillations similar in *Ophn-1*^{-/-} and WT (Supplementary Fig. 3b). These results suggest that oligophrenin-1 regulates Rev-erb α stability during its circadian oscillations to thereby affect expression of the master clock gene, Bmal1, in specific brain areas, particularly hippocampus.

Oligophrenin-1 causes Rev-erb α to localize to dendrites

To investigate the molecular basis of oligophrenin-1's ability to regulate the transcriptional activity of Rev-erb α , we manipulated oligophrenin-1 and Rev-erb α expression in cultured neurons. First, we investigated the localization of GFP- and myc-tagged Rev-erb α overexpressed in cultured hippocampal neurons, in combination with WT HA-oligophrenin-1, and in combination with HA-oligophrenin-1 Δ C. We found that exogenous Rev-erb α protein expression, as revealed by myc immunostaining, appeared concentrated in the nucleus but was also clearly present in dendrites (Fig. 4a; left panel). When oligophrenin-1 was co-expressed, Rev-erb α still accumulated in the nucleus but also co-localized with oligophrenin-1 in dendrites and dendritic spines.

The staining intensity of myc-Rev-erb α in dendrites was greater with oligophrenin-1 than with GFP control (98.76 ± 15.96 vs. 12.19 ± 2.86 , $p < 0.001$) (Fig. 4; left and middle panels and b). Rev-erb α in dendrites was markedly reduced when oligophrenin-1 Δ C was co-expressed with Rev-erb α (5.48 ± 2.18 vs. 12.19 ± 2.86 , $p = 0.046$) and abolished with oligophrenin-1 siRNA (0.51 ± 0.03 vs. 12.19 ± 2.86 , $p = 0.005$) (Fig. 4, right and bottom panels and b). These findings indicate that oligophrenin-1 and Rev-erb α associate in hippocampal neurons where they co-localize in dendrites and spines (as also shown in supplementary Fig. 2).

Chemically-induced synaptic activity induces Rev-erb α localization to dendrites

Synaptic activity induces a variety of changes in neurons, ranging from transient posttranslational modifications to modulation of gene expression programs²³. The signals generated in synapses must therefore be transported to the nucleus, and it has been shown, in sensory neurons of mollusk *Aplysia* and hippocampal neurons of mice, that the classical nuclear import pathway plays a critical

role in transporting synaptically generated signals into the nucleus during learning-related forms of plasticity²⁴. Furthermore, a recent study showed that synaptic activity induced by NMDA activation drives oligophrenin-1 into dendritic spines¹². We therefore investigated whether increasing excitatory synaptic activity – by blocking inhibitory GABAergic transmission using the GABA receptor antagonist bicuculline and blocking synaptic activity with the Na-channel blocker tetrodotoxin (TTX) – had reciprocal effects on the localization of exogenous and endogenous Rev-erb α in the neuron. Hippocampal neurons were co-transfected with GFP and myc-Rev-erb α at DIV11, and treated with either 2 μ M TTX or 40 μ M bicuculline for 1.5 h at DIV20. Bicuculline resulted in major localization of myc-Rev-erb α in dendrites, whereas TTX reduced myc-Rev-erb α localization in dendrites (bicuculline: 115.18 \pm 18.29 vs. untreated 12.19 \pm 2.86, p <0.001; TTX: 0.56 \pm 0.09. vs. untreated 12.19 \pm 2.86, p <0.01) (Figs. 5a and 5b).

These findings indicate that Rev-erb α localization is modified by changes in synaptic activity (as induced by bicuculline and TTX). As control, we also tested whether synaptic activity influenced the localization of the overexpressed transcription factor HA-Sp1. Neither bicuculline nor TTX had any effect on HA-Sp1 localization in hippocampal neurons (Supplementary Fig. 4a).

In additional experiments, we tested whether glutamate receptors were required for Rev-erb α localization to dendrites and spines. Dendritic localization of Rev-erb α in bicuculline-treated neurons was prevented by addition of the AMPA receptor antagonist NBQX (100 μ M) (10.65 \pm 2.5 vs. 115.18 \pm 18.29, p <0.001 Figs. 5a and 5b) but not by addition of the NMDA receptor inhibitor APV (100 μ M) (106.8 \pm 11.55 vs. 115.18 \pm 18.29, p =0.3, Figs. 5a and 5b), indicating that AMPA receptor activation is required for the localization of Rev-erb α to dendrites. Application of bicuculline to neurons overexpressing oligophrenin-1 siRNA did not induce Rev-erb α localization to dendrites.

We next examined effects of oligophrenin-1 overexpression and chemically-induced changes in synaptic activity on endogenous Rev-erb α localization. We found that bicuculline-treated, but not TTX-treated, neurons expressed more endogenous Rev-erb α in dendrites than untreated cells (1.6

times more, $p < 0.05$). Neither bicuculline nor TTX had any effect on the localization of endogenous Sp1 in hippocampal neurons (Supplementary Fig. 4b). We also found that cells overexpressing oligophrenin-1 expressed significantly more Rev-erb α in dendrites than cells not overexpressing oligophrenin-1 (1.4 times more; $p < 0.05$) (Figs. 6a and 6b). In neurons transfected with oligophrenin-1 siRNA and treated with bicuculline we found a marked reduction (significant) in staining for Rev-erb α in dendrites compared to untreated neurons (2.9 times lower, $p < 0.01$ Figs. 6a and 6b).

These results suggest that oligophrenin-1 is required for the synaptic activity-induced localization of Rev-erb α to dendritic spines. To corroborate this, we fractionated cell bodies (soma fraction) from pre- and postsynaptic components (dendrite fraction) in untreated and bicuculline-treated cultured rat hippocampal neurons²⁵. As expected, bicuculline induced Rev-erb α accumulation in the dendrite fraction and reduced the quantity of protein in the soma (ratio of density in dendrite to soma: 2.62 ± 0.09 treated; 1.3 ± 0.09 untreated; $p < 0.01$) (Fig. 6c).

We next set-up an in vivo experiments, administering intraperitoneal kainate to a wild-type mouse model^{26,27} to stimulate neuronal discharge in brain, and then examining the distribution of Rev-erb α in hippocampal slices. In untreated hippocampus, Rev-erb α was mainly in nucleus, while after kainate stimulation, Rev-erb α appeared distributed throughout the neuropil and often co-localized with synaptic marker PSD95 (Supplementary Fig. 5).

Taken together these results show that oligophrenin-1 regulates endogenous Rev-erb α localization to dendrites through oligophrenin-1-mediated control of AMPA receptor stability at the synapse.

Oligophrenin-1 interaction regulates endogenous Rev-erb α transcription in hippocampal neurons

We next examined the effect of chemically-induced synaptic activity on the production of Rev-erb α transcripts in hippocampal neurons in the presence and absence of endogenous oligophrenin-1.

First, we assessed Rev-erb α mRNA (RT-PCR) in cultured hippocampal neurons from oligophrenin-

1 WT and KO mice. Rev-erb α mRNA levels were higher in Ophn-1^{-/-} neurons (0.37 ± 0.01 in KO vs. 0.25 ± 0.005 in WT, $p < 0.05$) (Fig. 7a). Bicuculline treatment (24h) caused a significant increase in Rev-erb α mRNA expression in WT neurons compared to untreated (Fig. 7a) (0.48 ± 0.04 vs. 0.25 ± 0.005 $p < 0.01$). In Ophn-1^{-/-} neurons, Rev-erb α mRNA expression unchanged between treated than untreated neurons. Thus bicuculline-induced synaptic activity increased Rev-erb α transcript expression in the presence of oligophrenin-1 but not in its absence (Fig. 7a).

We next transfected hippocampal neurons with various types of oligophrenin-1 siRNA and/or oligophrenin-1 constructs, and treated them or not with bicuculline (40 μ M, 24h). In untreated neurons, Rev-erb α mRNA levels were higher when oligophrenin-1 siRNA was overexpressed compared to overexpressed scrambled si RNA (0.36 ± 0.004 vs. 0.28 ± 0.007 ; $p < 0.05$) (Fig. 7b). Bicuculline treatment increased Rev-erb α mRNA levels in neurons overexpressing scrambled siRNA compared to untreated (0.37 ± 0.007 vs. 0.28 ± 0.007 ; $p < 0.01$) but had no effect on oligophrenin-1 knockdown neurons (0.36 ± 0.004 untreated vs. 0.31 ± 0.003 treated) (Fig 7b). These findings indicate that, in hippocampal neurons, bicuculline-induced synaptic activity increases the transcription of Rev-erb α mRNA but only in the presence of oligophrenin-1.

Finally, to gain insight into whether the oligophrenin-1/Rev-erb α interaction mediates Rev-erb α repressor activity, we investigated the effects of oligophrenin-1 overexpression and suppression on Rev-erb α transcripts. Hippocampal neurons were transfected with oligophrenin-1 siRNA together with an oligophrenin-1 variant resistant to this siRNA (siRNA rescue) or with oligophrenin-1 siRNA together with a variant of oligophrenin-1 Δ C resistant to siRNA (siRNA rescue Δ C). In untreated neurons overexpressing siRNA and the rescue construct, Rev-erb α mRNA levels were similar to those in untreated neurons overexpressing scrambled siRNA (0.29 ± 0.014 vs. 0.28 ± 0.007) (Fig. 7b). Bicuculline application caused Rev-erb α mRNA to increase (compared to untreated) in neurons expressing oligophrenin siRNA plus the oligophrenin-1 variant resistant to siRNA (siRNA-rescue) (0.29 ± 0.014 untreated vs. 0.39 ± 0.012 treated, $p < 0.01$), but not in neurons transfected with oligophrenin-1 siRNA plus the oligophrenin-1 Δ C variant resistant to siRNA

(siRNA rescue ΔC) (Fig. 7b). These results show that oligophrenin-1 increases synaptic activity-stimulated Rev-erba transcription in neurons which presumably has an important effect on Rev-erba's suppressor activity.

Discussion

We have delineated a surprising interaction between the synaptic protein oligophrenin-1 and the transcription repressor Rev-erba. We found that oligophrenin-1 reduced the repressor activity of Rev-erba, by causing it to locate outside the nucleus and protecting it from degradation induced by GSK3 β inactivation (which normally stabilizes Rev-erba by phosphorylation). Furthermore, in the absence of oligophrenin-1, Rev-erba levels decreased in the soma and its gene repressor activity reduced.

We have also delineated a circadian oscillator in hippocampus, which is modulated, as expected, by Rev-erba but requires the presence of oligophrenin-1, since in its absence (Ophn-1^{-/-}), our data suggest that the rhythmic expression of Rev-erba in dark-dark conditions is about 6 hours later than the WT peak. This same oscillator was present in cortex and cerebellum. In cortex, oligophrenin-1 absence had a significant effect (ANOVA) on Rev-erba oscillations but levels did not differ significantly at any time point. This finding suggests that oligophrenin-1's effect is not confined to hippocampus but is also present in other parts of the brain. In cerebellum Rev-erba rhythms were unaffected by oligophrenin-1 absence in dark/dark conditions. The different results between brain regions may be related to *ophn1* gene expression pattern, which shows enrichment in hippocampus compared to cortex and cerebellum. The expression of the clock gene *Bmal1*, regulated by Rev-erba,¹⁷ was only weakly altered in the hippocampus of oligophrenin-1 Ophn-1^{-/-} mice.

How can the oligophrenin-1/Rev-erba interaction be interpreted? The literature provides no indication that Rev-erba has a functional role in dendrites/spines, although such a role cannot be excluded *a priori*. For example, it has been shown that Rev-erba's nuclear repressor activity depends on the oxidation state of its heme moiety and the ability of nitric oxide (NO) to bind heme

in reducing conditions. Since NO is a neurotransmitter involved in long term potentiation²⁸ we speculate that a similar mechanism might occur in synapses: by binding to Rev-erb α heme, NO might regulate the transcriptional repressor activity of Rev-erb α in target neurons to influence the expression of protein complexes in the dendritic spines and long-term synaptic plasticity.

Oligophrenin-1's previously unsuspected role in circadian rhythms is equally intriguing. Our data suggest the interpretation that the repression activity Rev-erb α is regulated in a cyclic fashion by its degradation, and that oligophrenin-1, together with GSK3 β , control its localization and stability. In humans Rev-erb α repressor activity is known to have circadian variation²⁹. Oligophrenin-1 would serve to stabilize Rev-erb α within the cytoplasm protecting it from degradation; only a minor fraction of Rev-erb α appears to be transported with oligophrenin-1 to dendritic spines. In KO mice, oligophrenin-1 absence results in increased degradation of Rev-erb α and slower buildup in the nucleus, leading to higher Rev-erb α transcript levels in the later stages of the 24 h cycle (Fig 3a), since Rev-erb α protein represses Rev-erb α transcription.²¹ The functional consequences of oligophrenin-1 loss on the *bmal1* clock remain to be explored. It is thought that Rev-erb α oscillations act as an additional stabilizing loop helping to maintain the precision of circadian rhythms¹⁷.

Our other finding, that bicuculline-induced synaptic activity increases Rev-erb α transcript levels but only in the presence of oligophrenin-1, reinforces the idea that oligophrenin-1 is involved in the control of circadian rhythms in hippocampus. It was initially believed that only the suprachiasmatic nucleus was involved in generating circadian rhythms. However in mammals genes are expressed in circadian rhythms in hippocampus³⁰⁻³², olfactory bulb³³, cerebellum³⁴ and other regions³⁵. Increased synaptic activity can be a marker of the initiation of activity within the sleep-wake cycle of animals, and our data suggest that oligophrenin-1-dependent increase in Rev-erb α transcription as a result of synaptic activity may be a means of entraining hippocampal circadian oscillations to activity signals from other parts of the brain. In *Drosophila*, levels of key

synaptic proteins α -DLG, synapsin, and syntaxin have been related to behavioral states correlated with the sleep-wake cycle.³⁶

Rev-erb α transcription is rhythmic because it is enhanced by Clock-Bmal1, and repressed by Per-Cry^{37,38} and Rev-erb α protein²¹. Rev-erb α also represses Bmal1 transcription¹⁷.

We also found that that synaptic activity in vivo (WT animal) increased the localization of Rev-erb α protein to cytoplasm and dendrites. Our other experiments demonstrated that synaptic activity-induced Rev-erb α localization to dendrites is mediated by AMPA receptors results. Again, this may not have functional significance, but could be an epiphenomenon arising from interaction between ‘synaptic’ oligophrenin-1 and ‘nuclear’ Rev-erb α . It is known that the oligophrenin-1 localization to dendritic spines is driven by synaptic activity where it complexes with, stabilizes, and selectively activates AMPA receptors³⁹.

To conclude, our data clearly reveal some interactions between synaptic activity and circadian oscillators and implicate oligophrenin-1 in its regulation, delineating a new means of communication between synapse and nucleus^{24,36,40}. These findings are further indication^{36,41} that cognitive ability is influenced by circadian rhythms, and promise to provide insight into normal plasticity and the etiology of intellectual disability. Although the effect of oligophrenin-1 loss on circadian synaptic activity was modest, we speculate that it may have more profound effects during development and may be a major factor leading to mental compromise in persons with OPHN1 mutations.

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Figure legends

Figure 1. Oligophrenin-1 interacts with Rev-erb α in yeast two-hybrid system, COS7 cells and in neurons

(a) Schematic representation of oligophrenin-1 and the C-terminus domain of oligophrenin-1, used as bait for the yeast two-hybrid screening, and the results of the yeast two-hybrid screening with schematic representation of the four positive clones of the Rev-erb α protein. Two-hybrid interaction was quantified based the activation of the three reporter genes (HIS3, LacZ, URA 3) (3+, activation of all three reporter genes; 2+, activation of HIS3 and LacZ reporters; 1+, activation of HIS3). Small numbers refer to amino acid residues.

(b) Mapping of oligophrenin-1/Rev-erb α interaction by two-hybrid test. We used two fragments of the C-terminus of oligophrenin-1 (550-633 and 634-802 amino acids) as bait and a Rev-erb α fragment (263-614 amino acids) as prey. Interactions quantified as in (a).

(c) Interaction between oligophrenin-1 and Rev-erb α was confirmed by co-immunoprecipitation experiments in COS7 cells. HA-tagged wild-type oligophrenin-1 co-immunoprecipitated with myc-Rev-erb α , while the mutated form of oligophrenin-1 (HA-oligophrenin Δ C did not.

(c) Distribution of Rev-erb α and oligophrenin-1 in COS7 cells. Myc-tagged Rev-erb α localizes in the nucleus. HA-oligophrenin-1 distributes in the cytoplasm (upper panel). Ectopic expression of WT HA-oligophrenin-1 together with myc-Rev-erb α shows co-localization of both proteins in the cytoplasm. When Rev-erb α is expressed alone (visualized by DAPI staining, top panel right), and co-expressed with the mutant form of HA-oligophrenin-1, it is confined to the nucleus (bottom panel). Scale bar 10 μ m.

(d) Interaction between oligophrenin-1 and Rev-erb α was confirmed by co-immunoprecipitation experiments in COS7 cells. HA-tagged wild-type oligophrenin-1 co-immunoprecipitated with myc-Rev-erb α , while the mutated form of oligophrenin-1 (HA-oligophrenin Δ C did not.

(e) GST pull down experiments. Diagram with the two fragments of the C-terminal tail of oligophrenin-1 used in the GST pull-down assay (top). Purified GST-550-633 aa binds Rev-erba more strongly than GST-676-802 aa in lysates from rat brain and hippocampal neurons. (f) Co-immunoprecipitation of oligophrenin-1 and Rev-erba in rat brain lysate.

Figure 2. Oligophrenin-1 reduces transcriptional repression by Rev-erba

(a) Oligophrenin-1 reduces transcriptional repression by Rev-erba. HEK cells were transfected with pSV40-RE vector alone, with pSV40-RE plus GFP, with pSV40-RE plus Rev-erba, with pSV40-RE plus Rev-erba plus oligophrenin-1, and also with Rev-erba plus oligophrenin-1ΔC. The graph shows that Rev-erba protein markedly represses transcription of the pSV40-RE construct (measured by luciferase activity) compared to Rev-erba absence. When oligophrenin-1 is also expressed with Rev-erba it suppresses Rev-erba repression so that luciferase activity increases. When oligophrenin-1ΔC is expressed with Rev-erba, luciferase activity is reduced to levels to similar to those in cells with no oligophrenin-1 overexpression. Values are means ± SEM. The difference between pSV40-RE plus Rev-erba and pSV40-RE plus Rev-erba plus oligophrenin-1 is significant (student *t*-test, * $p < 0.05$).

(b) Western blots show localization of myc-Rev-erba in cytoplasm and nucleus in HEK cells transfected with various concentrations of HA-oligophrenin-1. Total protein extract from HEK cells was separated by SDS-PAGE and probed with myc antibody, then stripped and re-probed with HA and tubulin antibody. HA-oligophrenin-1 is only present in cytoplasm. The histograms show that the amount of myc-Rev-erba in cytoplasm increases with the amount of HA-oligophrenin-1 added to the transfection procedure. Data are means ± SEM (at least three experiments) of blot intensities of myc-Rev-erba as ratio of density in cytoplasm to total, normalized against tubulin; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ (ANOVA, Bonferroni post test).

(c) Oligophrenin-1 protects Rev-erba from degradation. Same constructs used as in experiments reported in (a). Expression of the dominant-negative form of GSK3β (HA-GSK3βDN) in HEK

cells results in near total absence of myc-Rev-erb α (lane 1). Presence of oligophrenin-1 inhibits degradation of myc-Rev-erb α protein (lane 3) in the presence of HA-GSK3 β DN.

(d) Histograms quantify myc-Rev-erb α expression normalized to tubulin. Means \pm SEM of at least three experiments; **** $p < 0.0001$ (ANOVA, Bonferroni post test).

(e) Western blots show the distribution of myc-Rev-erb α in cytoplasm (C) and nucleus (N) of HEK cells overexpressing myc-Rev-erb α alone or with HA-oligophrenin-1, after incubation with or without the MG132. Histograms show quantification (as ratio of density in cytoplasm to total) of myc-Rev-erb α . In the absence of proteasome inhibitor MG132, Rev-erb α is degraded unless oligophrenin-1 is present (left lanes); in the presence of MG132 Rev-erb α is not degraded. Means \pm SEM; *** $p < 0.001$ (ANOVA, Bonferroni post test).

(f) Western blots show localization of Rev-erb α in the nuclear fraction (N) and post-nuclear supernatant (S) of hippocampus from oligophrenin-1 WT and KO mice. PSD-95 is a synaptic marker; nuclear protein 4eBP1 was used as nuclear marker. Quantification shows total (S+N) levels of Rev-erb α protein are higher in WT than KO hippocampus (1.62 ± 0.06 vs. 0.95 ± 0.03 , *** $p \leq 0.001$, Student t -test) and that the ratio of S Rev-erb α to N Rev-erb α is higher in hippocampus of WT and KO mice (*** $p < 0.001$, Student t -test). Values are means \pm SEM.

(g) Western blots show Rev-erb α in dendritic and soma fractions of hippocampal neurons overexpressing oligophrenin-1 knockdown siRNA or scrambled siRNA, treated or untreated with MG132. In untreated neurons with knockdown oligophrenin-1, Rev-erb α appears largely absent from dendrites and the total quantity is less than in neurons overexpressing scrambled siRNA. When proteasome degradation is blocked by MG132 Rev-erb α levels increase and the protein is present in both dendrites and soma. Histograms show the quantification of total (soma + dendritic) Rev-erb α in untreated and MG132-treated hippocampal neurons overexpressing oligophrenin-1 siRNA or scrambled siRNA. Values are means \pm SEM; ** $p < 0.01$ **** $p < 0.0001$ (ANOVA, Bonferroni post test).

Figure 3. Altered circadian expression of Rev-erb α and clock gene mRNAs in Ophn-1^{-/-} mice.

(a and d) Histograms showing relative expression of Rev-erb α mRNA in hippocampus and cortex of oligophrenin-1 WT and Ophn-1^{-/-} mice under DD conditions at circadian times (CT) 18, 0, 6, and 12 h. Samples were taken after 30 h (CT18), 36 h (CT0), 42h (CT6) and 48 h (CT12) of continuous darkness which started at 7 pm (so CT0 was at 7 am). Values are means \pm SEM of at least three experiments. ANOVA, Bonferroni post hoc test: * $p < 0.05$.

(b and e) Western blots showing Rev-erb α protein in hippocampus and cortex of Ophn-1^{-/-} and WT mice at CT18, CT0, CT6, and CT12. Total protein extract from WT or Ophn-1^{-/-} of hippocampus and cortex was separated by SDS-PAGE and probed with Rev-erb α antibody, then stripped and re-probed with oligophrenin1 and tubulin antibody. Histograms show mean blot intensities (\pm SEM, at least three experiments) normalized against tubulin. ANOVA, Bonferroni post hoc test: * $p < 0.05$. At CT12 and CT18, Rev-erb α protein expression is significantly lower in oligophrenin Ophn-1^{-/-} than WT mice hippocampus homogenates.

(c and f) Histograms showing relative expression of Bmal1 mRNA in hippocampus and cortex of oligophrenin-1 WT and Ophn-1^{-/-} mice under DD conditions at circadian times (CT) 18, 0, 6, and 12 h. Values are normalized to cyclophilin A mRNA. ANOVA, Bonferroni post hoc test: * $p < 0.05$.

Figure 4. Accumulation of Rev-erb α in dendrites and spines induced by overexpression of oligophrenin-1 in neurons

(a) In mature neurons (DIV20), myc-Rev-erb α localizes particularly in nucleus, but it is also present in cell body and dendrites (left panel). Overexpression of WT HA-oligophrenin-1 results in accumulation of myc-Rev-erb α in dendrites and spines (middle panel). When mutant oligophrenin-1 is overexpressed there is no myc-Rev-erb α accumulation in dendrites and spines (right panel). Oligophrenin-1 knockdown with siRNA abolishes myc-Rev-erb α staining outside the nucleus (bottom panels). Scale bar, 10 μ m.

(b) Histograms show the intensity of GFP-and myc-tagged Rev-erb α staining in dendrites and spines of cultured hippocampal neurons in transfected as in (a). Values are means \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA, Tukey test).

Figure 5. Synaptic activity induces localization of Rev-erb α at synapses

(a) AMPA receptor and oligophrenin-1 mediate Rev-erb α localization in dendrites and dendritic spines. Hippocampal neurons were cotransfected with GFP and myc-Rev-erb α on DIV11 and at DIV20 were treated for 1.5 h with the indicated drugs. Bicuculline (40 μ M) but not TTX (2 μ M) induces localization of myc-Rev-erb α in dendrites and spines. Dendritic localization of myc-Rev-erb α in bicuculline-treated neurons is prevented by addition of the AMPA receptor antagonist NBQX (100 μ M), but not by addition of the NMDA receptor inhibitor APV (100 μ M). Application of bicuculline to neurons transfected with oligophrenin-1 siRNA does not result in localization of myc-Rev-erb α to dendrites or spines. Scale bar 10 μ m.

(b) Histograms show intensity of myc-Rev-erb α staining in dendrites and spines in neurons transfected as in (a). Bicuculline but not TTX results in major localization of myc-Rev-erb α in dendrites that is prevented by addition of NBQX but not by addition of APV. Oligophrenin-1 knockdown with oligophrenin-1 siRNA abolishes Rev-erb α localization in dendrites even in the presence of bicuculline. Values are means \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA, Tukey test).

Figure 6. Oligophrenin-1 is required for the synaptic activity-induced localization of endogenous Rev-erb α in dendrites

(a) Localization of endogenous Rev-erb α in mature hippocampal neurons (DIV20). Endogenous staining of Rev-erb α in (left to right) untreated (UT), bicuculline-treated (40 μ M 1.5 h), TTX-treated (2 μ M 1.5 h), bicuculline-treated (40 μ M 1.5 h) neurons overexpressing oligophrenin-1

siRNA, and untreated neurons overexpressing GFP-oligophrenin-1. The lower right panel shows staining for oligophrenin-1 in oligophrenin-1-overexpressing neurons. Scale bar 10 μ m.

(b) Quantification of endogenous staining for Rev-erb α in dendrites of neurons treated as indicated in (a). Bicuculline treatment results in major localization of endogenous Rev-erb α in dendrites.

TTX treatment and oligophrenin-1 knockdown with oligophrenin-1 siRNA reduces Rev-erb α localization in dendrites even in the presence of bicuculline. Values are means \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA, Tukey test).

(c) Western blots quantifying (as ratio of density in dendrite to cell body) localization of Rev-erb α in dendrite fraction and soma fraction after subcellular fractionation of untreated and bicuculline-treated mature cultured hippocampal neurons (DIV20). Values are means \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (student t -test). PSD-95 is a synaptic marker; nuclear protein 4eBP1 was used as soma marker.

Figure 7. Oligophrenin-1 interaction is required for Rev-erb α mRNA expression in hippocampal neurons

(a) Histograms show relative expression of Rev-erb α mRNA in bicuculline-treated (Bic) and untreated (NT) hippocampal neurons from WT and Ophn-1^{-/-} mice. Transcript levels are significantly higher in treated WT and untreated Ophn-1^{-/-} than untreated WT. Means \pm SEM; $n = 3$ * $p < 0.05$, ** $p < 0.01$ (ANOVA, Bonferroni post test).

(b) Histograms show the relative expression of Rev-erb α mRNA in bicuculline-treated (Bic) and untreated (NT) hippocampal neurons overexpressing either oligophrenin siRNA, scrambled siRNA, oligophrenin-1 siRNA together with an oligophrenin-1 variant resistant to this siRNA (siRNA-rescue), or oligophrenin-1 siRNA together with oligophrenin-1 Δ C resistant to this siRNA (siRNA-rescue Δ C). Bicuculline-induced synaptic activity increases Rev-erb α transcript levels only in the presence of WT oligophrenin-1 (scrambled siRNA, siRNA-rescue). Values are means \pm SEM; $n = 3$ * $p < 0.05$, ** $p < 0.01$ (ANOVA, Bonferroni post test).

Methods

Yeast two-hybrid screening and cDNA constructs

For two-hybrid experiments, a fragment corresponding to the C-terminus of oligophrenin-1 (aa 550-802) was cloned into the pDBLeu vector, in frame with the GAL4 binding domain, and used as bait to screen a human fetal brain cDNA library (ProQuest Pre-made cDNA Libraries) that had been cloned into the pPC86 vector. Positive colonies grew on plates containing 10 mM 3-AT without tryptophan, leucine, or histamine and expressed all three reporter genes: HIS3, LacZ and URA3. cDNA plasmids from positive clones were recovered via DH5 α *E. coli*, plated on Amp and sequenced.

For further two-hybrid experiments, two DNA fragments corresponding to the oligophrenin-1 C-terminus aa 550-633 and aa 634-802 -were subcloned into pDBLeu and used as bait; the DNA fragment corresponding to aa 263-614 of Rev-erb α was subcloned into pPC86 plasmid and used as prey. Oligophrenin-1 with the hemagglutinin (HA) tag and Rev-erb α with the myc tag (tags inserted at N-terminus) were each subcloned into the GW1-CMV expression vector (British Biotechnology, United Kingdom). The truncated HA-oligophrenin-1 Δ C (aa 1-550) was made by PCR amplification with appropriate oligonucleotides and also subcloned into the GW1-CMV expression vector together with HA-tag at the N-terminus.

The GST-oligophrenin-1 fragments corresponding to aa 550-633 and aa 676-802 were cloned into the pGEX4T-3 vector. The oligophrenin-1 siRNA target sequence was 5' gaacctatctaccacactc3'; the corresponding scrambled siRNA was gctcacccttctactctc were cloned into lentiviral PII 1.3 vector; both were described and characterized by Kobayashi et al 2009. For rescue experiments, the constructs were made as described by Kobayashi et al 2009.

Oligophrenin-1 was a gift from Prof. Van Aelst (Cold Spring Harbor Laboratory New York, USA). GSK3 β DN was a gift from Dr. J. Jaworski (International Institute of Molecular and Cell Biology, Warsaw); pGL2RE-SV40 was a gift from Dr. V. Laudet²¹.

Luciferase assay

Luciferase was assayed using the Dual Luciferase Report Assay System (Promega, Milan, Italy)^{42,43}.

Coimmunoprecipitation and GST-pull down assays

Transfected COS7 cells were lysed with 50mM TRIS-HCl, 150mM NaCl, 1mM EDTA, 1% NP40, 0.5% deoxycholate, 0.05% SDS, and protease inhibitors (lysis buffer). Lysate samples (100 µg protein) were incubated overnight at 4°C with rabbit anti-HA-tag and mouse anti-myc-tag (Santa Cruz Biotechnology, California), both at 5µg/ml, in buffer A (200 mM NaCl, 10 mM EDTA, 10 mM Na₂HPO₄, 0.5% NP-40, 0.1% SDS, 10 mM NaF, plus Ser/Thr- and Tyr-phosphatase inhibitor cocktails).

Protein A agarose beads (Santa Cruz Biotechnology, California) washed in buffer A were added and incubation continued for 2 h. The beads were pelleted by centrifugation, washed five times with buffer A, resuspended in sample buffer for SDS-PAGE, and boiled for 5 min. The beads were again pelleted by centrifugation, and the supernatants applied to 7.5% SDS-PAGE. Protein bands were transferred to nitrocellulose membranes (Amersham) at 80V for 120 minutes at 4°C. Primary antibodies were applied overnight in blocking buffer (20 mM Tris , pH7.4, 150mM NaCl, 0.1% Tween20, and 3% dried non-fat milk). Secondary antibodies (HRP-conjugated anti anti-mouse, or anti rabbit) (Amersham) were used at 1:2000 dilution. The signal was detected using an ECL detection system.

For glutathione S-transferase (GST) pull-down experiments, hippocampal neuron lysates and rat brain extracts were incubated with 30 µg GST fusion protein immobilized on GST 4B beads (GE Healthcare) for 3 h at 4°C, washed five times in lysis buffer, and resuspended in 25 µl SDS sample buffer. GST alone served as control. Samples were separated by SDS-PAGE, followed by Western blotting with appropriate antibodies.

GST fusion proteins were prepared in BL21 *E. coli* and purified by standard procedures.

The following antibodies and dilutions were used: mouse anti-HA-tag 1: 200(Roche Applied Science); rabbit anti Myc-tag 1:200 (Upstate Cell Signaling Solutions); and rabbit anti-Rev-erba 1:500 (gift from V. Laudet, Laboratoire de Biologie Moléculaire de la Cellule, Lyon).

Primary embryonic rat hippocampal and cortical neurons: culture, transfection, staining and quantification

Primary rat hippocampal neurons were prepared from embryonic 18-19 day rat brains⁴⁴ and plated on poly-D-lysine (30µg/ml) coated coverslips at densities of 75,000/well for immunochemistry, and at 300,000/well for biochemistry experiments. After 13-14 days in vitro, cultures were transfected using the calcium phosphate method⁴⁵

COS7 and human embryonic kidney 293T (HEK) cells at 50-70% confluence (24 hr after plating in 6-well plates or plating on glass coverslips in 12-well plates) were transfected with cDNA expression constructs (1-2µg DNA per well) using a JetPEI transfection kit (Invitrogen) for 4-5 h at 5% CO₂, 37°C. The transfected cells were washed twice with PBS and grown for 24-48 h in Dulbecco's modified Eagle medium plus 10% fetal bovine serum and 1% penicillin/streptomycin, before fixation for immunocytochemistry or lysis for coimmunoprecipitation.

Hippocampal neurons and COS7 cells were fixed in 4% paraformaldehyde-4% sucrose for 10 min. Primary and secondary antibodies were applied in gelatin detergent buffer (30mM phosphate buffer at pH 7.4 containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl). The following antibodies and dilutions were used: rabbit anti-Rev-erba, 1:100 (Abcam, UK); rabbit anti-oligophrenin-1, 1:100; and rabbit anti-Tuj1 1: 200 (all gifts from Dr. P. Billuart, Institut Cochin, Paris); rabbit anti-HA-tag, 1:200 (Santa Cruz Biotechnology Inc., California) and 4'-6-diamidino-2-phenylindole, 0.1µg/ml (gift from Dr. B. Chini, CNR Institute of Neuroscience, Milan); Cy3- or FITC-conjugated secondary antibodies (Jackson Immnunoresearch, West Grove, PA).

Fluorescent images were acquired with a Biorad MRC1024 confocal microscope, using a Nikon 40x and 60x objectives with sequential acquisition setting at 1280 x 1024 pixel resolution. Image

data were a Z series projection of about 5-10 images, each averaged 2-4 times and taken at 0.4–0.7 μm depth intervals. Morphometric measurements were made with Metamorph image analysis software (Universal Imaging Corporation). Fluorescent intensity of staining of endogenous synaptic proteins was measured as described⁴⁶. Image acquisition and morphometric quantification were performed by investigators blind to experimental conditions. Measurements are means \pm standard error of the mean (SEM).

The following additional antibodies and dilutions were used: rabbit anti-SNK, 1:50 (gift from Dr. D. Pak, Georgetown University, Washington); rabbit anti-PSD-95 1:500 (Neuromab), rabbit anti 4eBP1 1:250 (Cell Signaling); mouse anti α -tubulin 1:1000 (Sigma), rabbit anti-Sp1 1:100 (Upstate).

Fractionation experiments from cultured neurons

Three week-old high-density cultures of rat hippocampal neurons were treated with papain (0.1 mg/ml) for 10 minutes at room temperature, rinsed 3 times with PSB and covered with 3 ml/dish buffer (10mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.35 M sucrose) and passed through a wide bore pipette to break down the network. Fractions were pooled and mixed 1:1 with sucrose buffer (0.8 M sucrose, 10mM Tris-HCl (pH 7.4), 0.5 mM EGTA) and centrifuged at 1,500 g for 10 min. The pellet (P1) contained cell bodies (soma). The supernatant was recovered and centrifuged at 28,000 g for 10 min to produce P2 which was free of somatic contamination and enriched in pre- and post-synaptic structures. P1 and P2 were separately resuspended in buffer for SDS-PAGE. For quantification of immunoblot signals total band intensity was measured with ImageQuant software (Bio-Rad), and values normalized to the α -tubulin signal.

Statistical analyses

Data are expressed as means with standard errors of the mean (\pm SEM). The significance of differences was tested by unpaired Student t test (two means) or ANOVA with appropriate post tests (more than two means).

Drug treatments

Tetrodotoxin (TTX), bicuculline, (2R)-amino-5-phosphonopentanoate (APV), 6-nitro-7-sulphamoylbenzoquinpxaline (NBQX) were from Tocris Cookson. TTX and bicuculline were dissolved in water; APV and NBQX in dimethyl sulphoxide. Hippocampal neurons cultured 20 days were treated with drugs for 1.5 h at 37°C, then washed and fixed for immunocytochemistry. Kainic acid (Sigma-Aldrich, Milan Italy) was administered intraperitoneally (15m/kg) and the animals were sacrificed 2 hr later.

MG132 (Calbiochem, Darmstadt, Germany) was added to the incubation medium of neurons (3 μ m for 4 h) and COS7 cells (10 μ m for 4h).

Quantitative RT-PCR

Total mRNA was extracted using RNeasy Plus kit (Qiagen, Valencia, CA. cDNA was synthesized from from Dnase I-treated RNA using the QuantiTect reverse transcription kit (Qiagen,) according to the manufacturer's instructions. mRNA transcripts were quantified by Taqman Q-PCR 3 (Applied Biosystems, Foster city, CA, USA) on a Prism 7900 thermal cycler and sequence detector (Applied Biosystems). All primers and probes were from Applied Biosystems. Reactions were performed in triplicate. Average delta-Ct values normalized to GAPDH or cyclophilin A (housekeeping gene) were used to calculate gene fold induction, relative to control set to 1. Relative RNA levels were expressed as percentage of maximum value obtained for each experiment. The primers used were:

Mouse NRD1 (ID #Mm00520708_m1), mouse Bmal1 (ID #Mm00500226_m1) and Gapdh (ID #Mm99999915_g1) (from Applied Biosystems). Cyclophilin A forward: 5'-gtgactttacagccataatg; reverse: 5'-acaagatgccaggacctgtat, gene ID cyclophilin A (or Ppia) (uc007hyn.1).

Animal care

Experimental procedures were performed in accordance with the European Communities Council Directive (86/809/EEC) on the care and use of animals, and were approved by the Ethics Committee of our Institute.

All animals (wild type and oligophrenin-1-mutant mice) were housed singly in cages in a room at $23 \pm 1^\circ\text{C}$ under a 12/12 light-dark cycle (LD bright light 200 lx). For the DD experiments (n=4, animals per time per genotype) the DD period started at 7 pm. The CT18 samples were taken after 30h of continuous dark; the other CTs followed at 6h intervals: CT0, CT6 and CT12 after 36, 42 and 48 h of continuous darkness, respectively.

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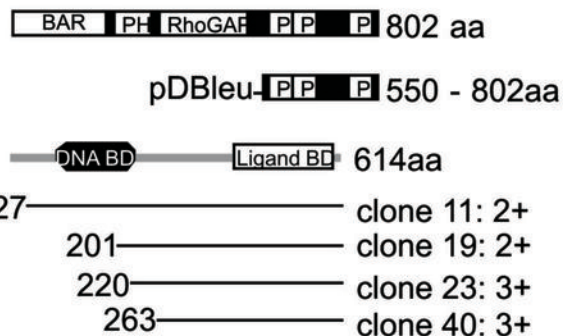
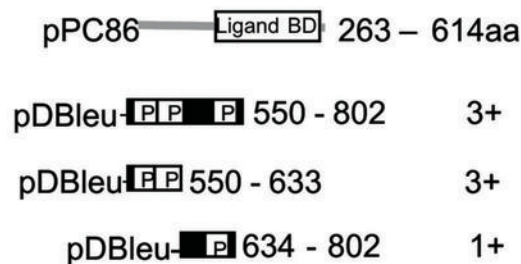
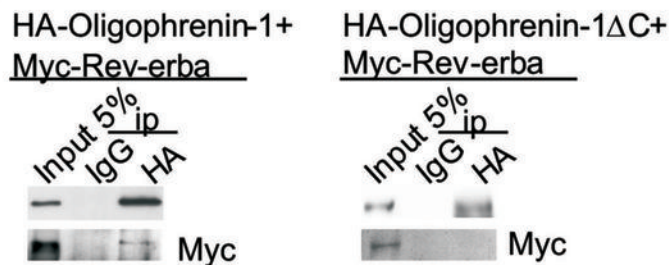
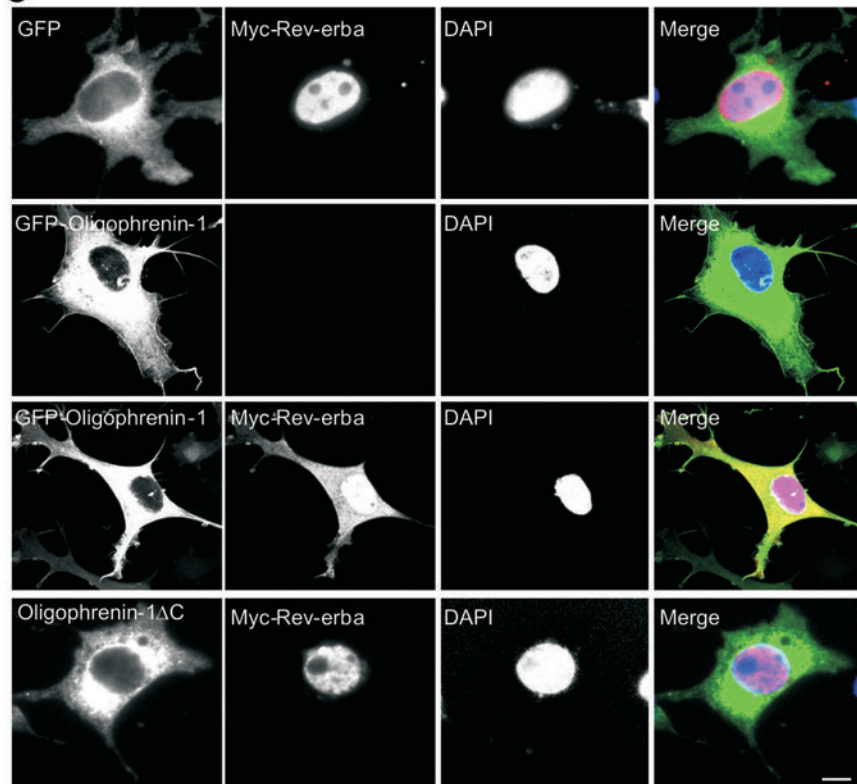
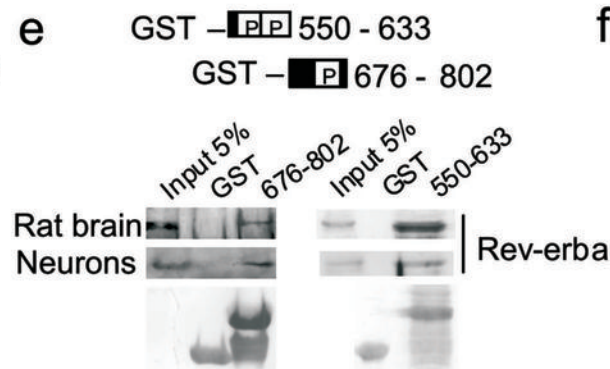
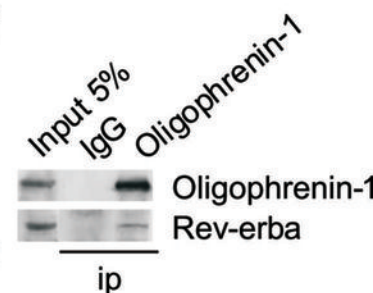
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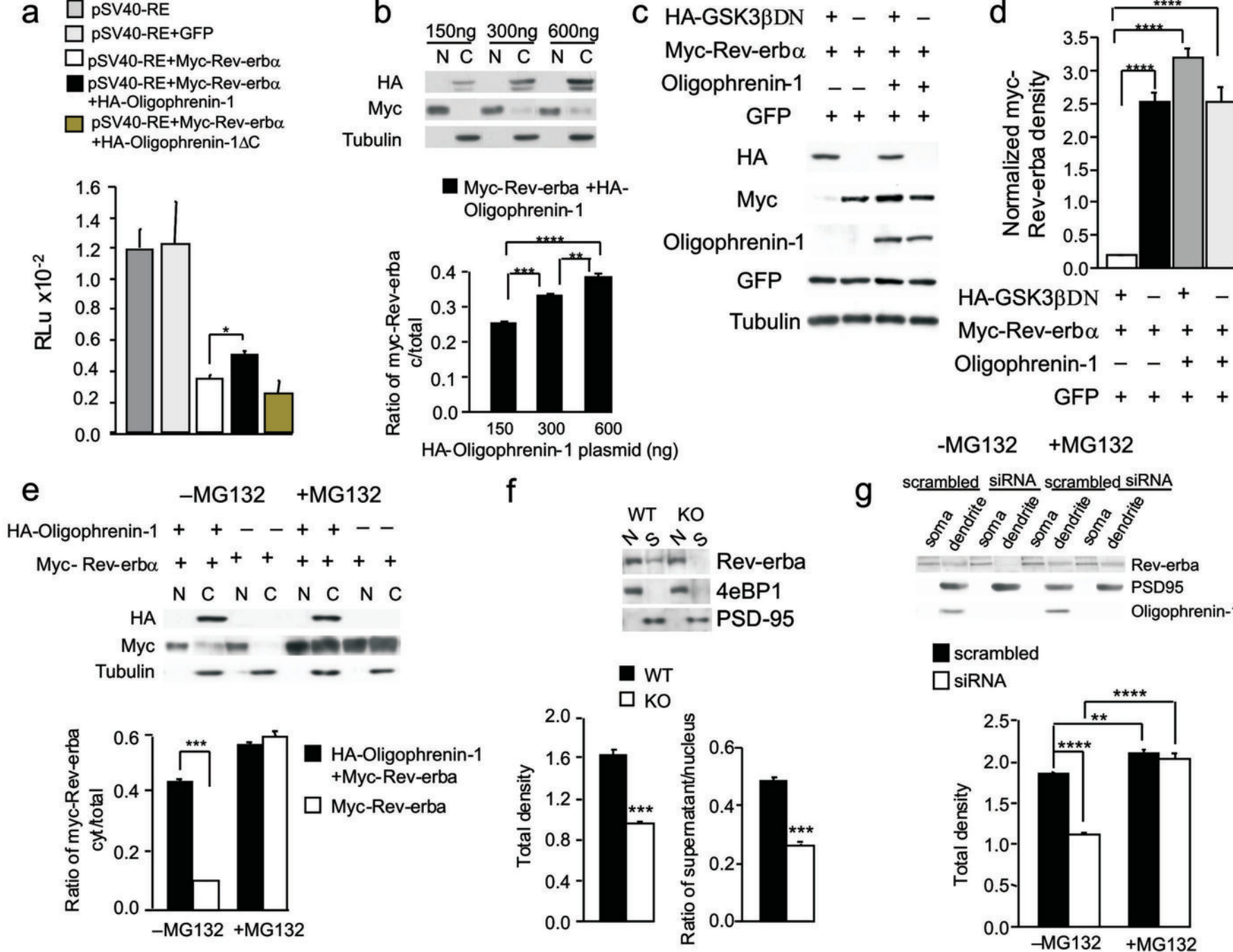
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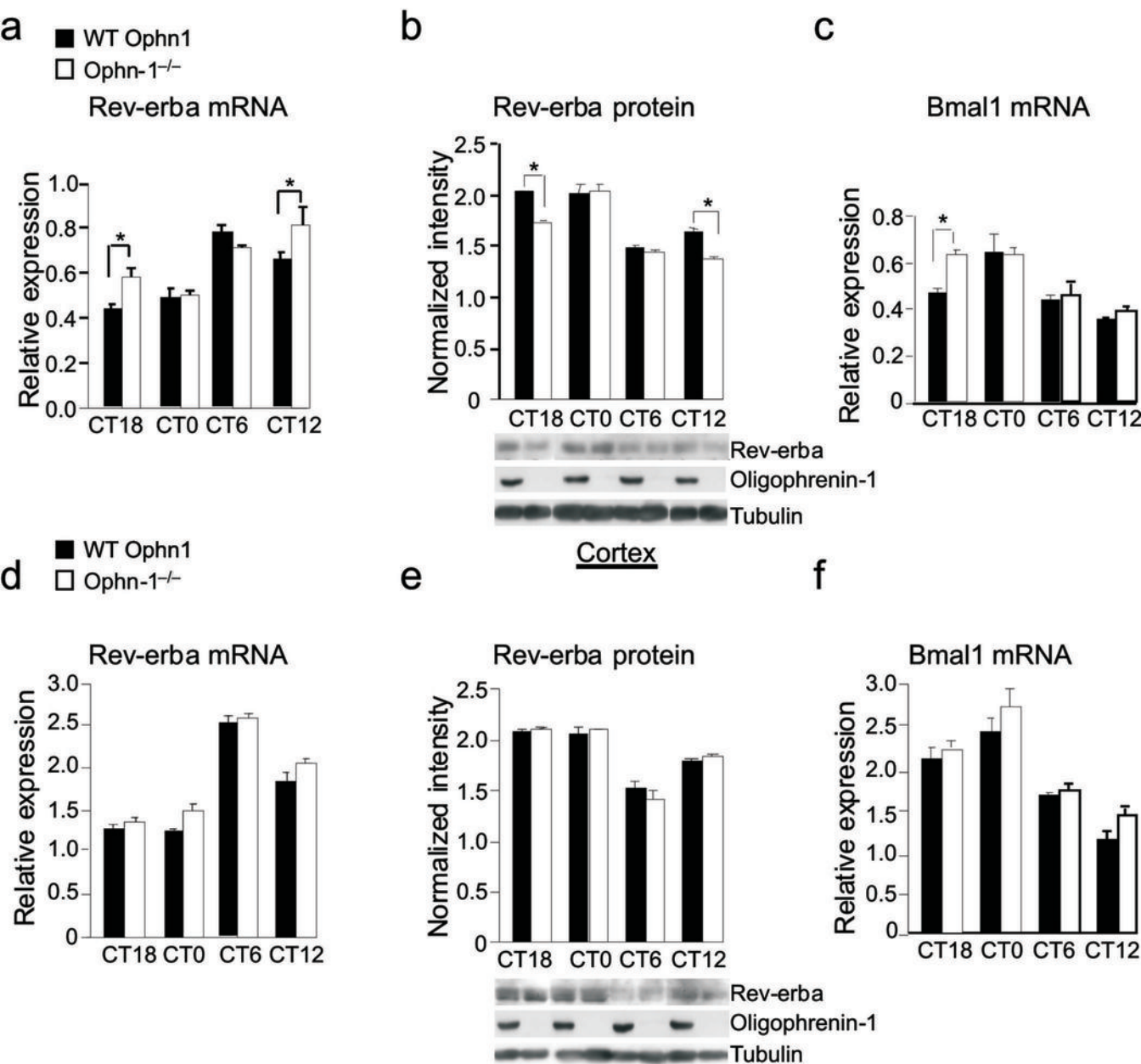
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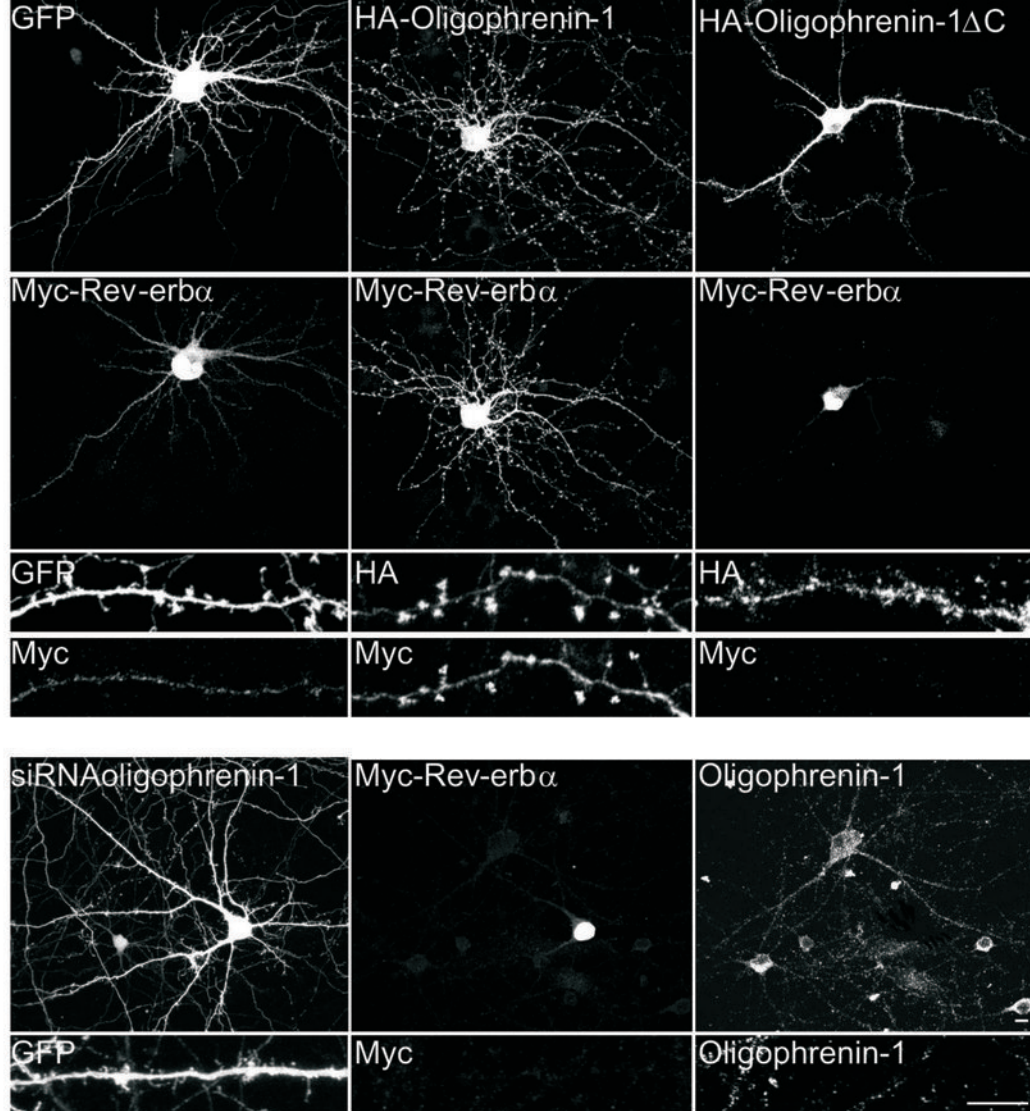
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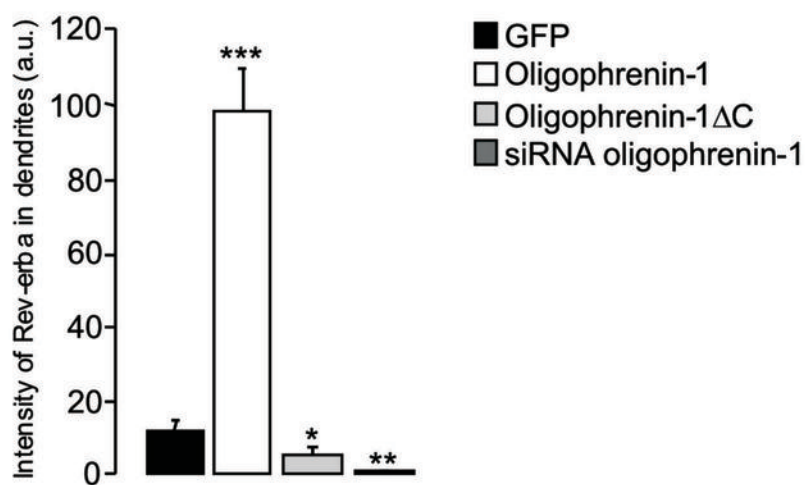


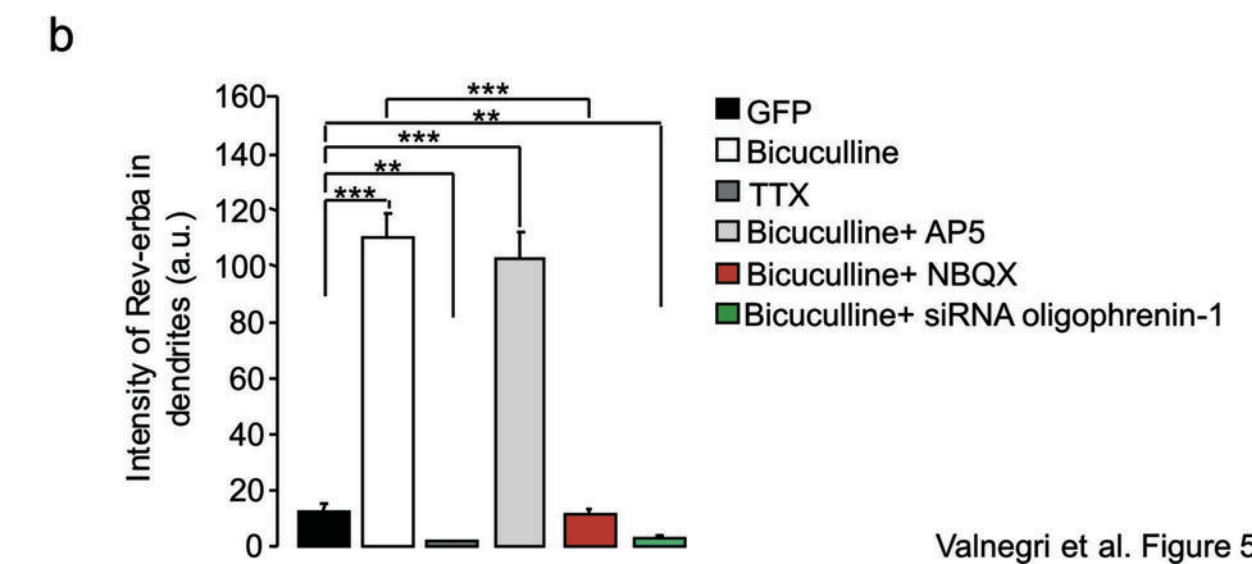
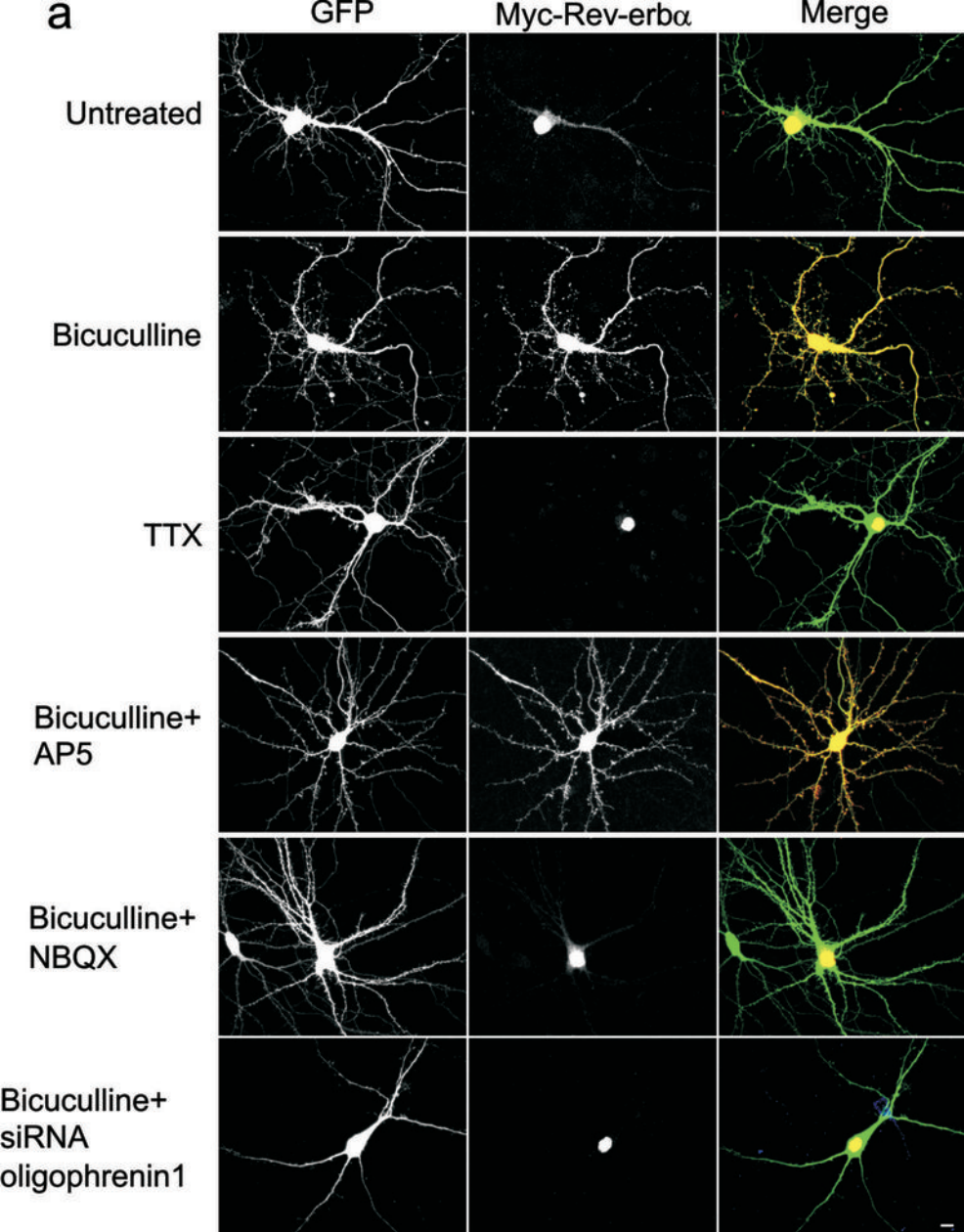


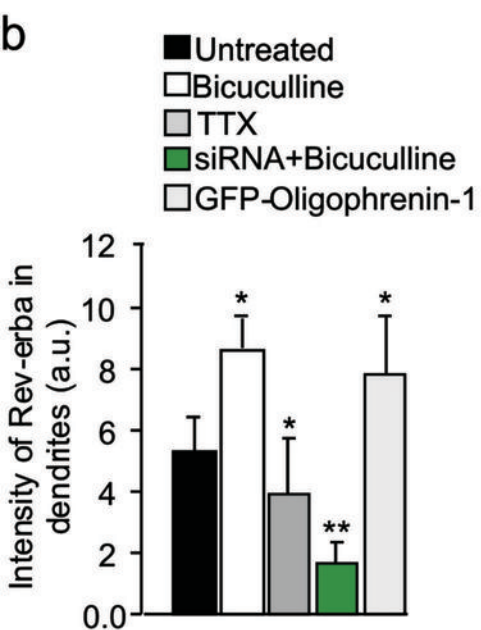
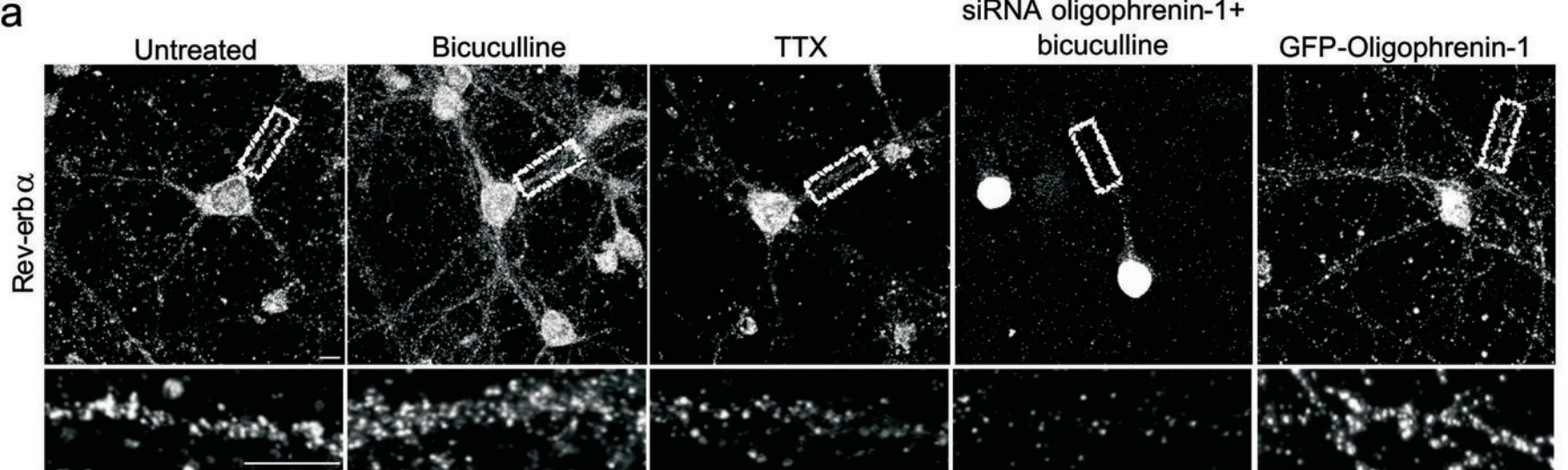
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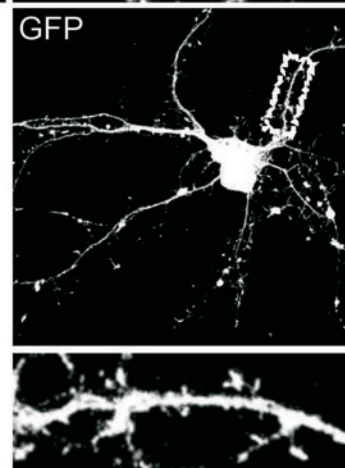
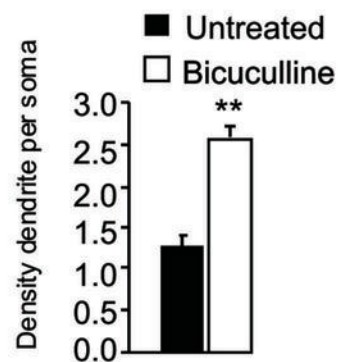
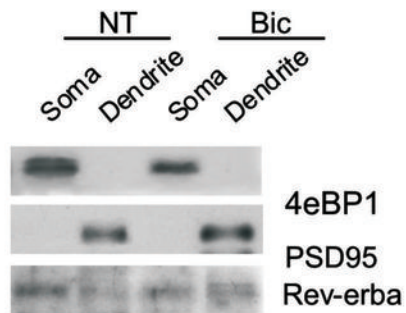
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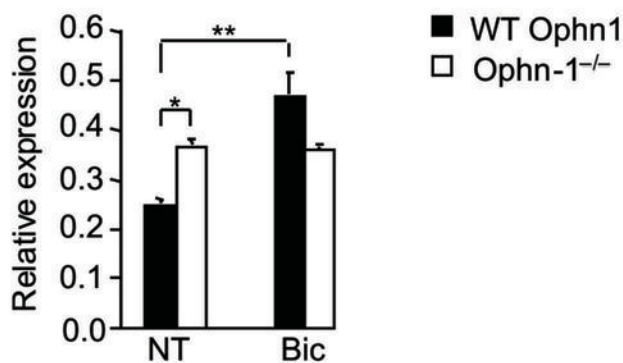


c



a

Rev-erba mRNA

**b**

Rev-erba mRNA

