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Engrailed Homeoprotein Protects Mesencephalic Dopaminergic Neurons from Oxidative Stress

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Summary

Engrailed homeoproteins are expressed in adult dopaminergic neurons of the substantia nigra. In *Engrailed1* heterozygous mice, these neurons start dying at 6 weeks, are more sensitive to oxidative stress, and progressively develop traits similar to those observed following an acute and strong oxidative stress inflected to wild-type neurons. These changes include DNA strand breaks and the modification (intensity and distribution) of several nuclear and nucleolar heterochromatin marks. Engrailed1 and Engrailed2 are biochemically equivalent transducing proteins previously used to antagonize dopaminergic neuron death in *Engrailed1* heterozygous mice and in mouse models of Parkinson disease. Accordingly, we show that, following an acute oxidative stress, a single Engrailed2 injection restores all nuclear and nucleolar heterochromatin marks, decreases the number of DNA strand breaks, and protects dopaminergic neurons against apoptosis.

Introduction

Engrailed1 (En1) and Engrailed2 (En2), collectively Engrailed or En1/2 homeoproteins (HPs), play equivalent roles in the survival of adult mesencephalic dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) (Albéri et al., 2004; Sgadò et al., 2006). In the *En1*−/+; *En2*+/+ (*En1*+/−) mouse, mDA neurons degenerate progressively starting at 6 weeks (Sonnier et al., 2007). As in Parkinson disease (PD), death is higher in the SNpc than in the VTA and *En1*+/− mice display motor and non-motor behaviors (Sonnier et al., 2007).

This and a possible association between *EN* polymorphisms and the risk to develop PD (Fuchs et al., 2009; Haubenberger et al., 2011; Rissling et al., 2009) suggest that *Engrailed* might be in the PD pathway. Thanks to HP internalization properties (Joliot and Prochiantz,

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Author Contributions

H.R. and F.-X.B.d.T. contributed with O.M.-B. to the conception and realization of experiments. J.F. and R.L.J. were involved in the experimental work, the supervision of the junior investigators, and the conception of the study. A.P. coordinated the work, participated in the conception of the study, and wrote the paper with the help of R.L.J. primarily.

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2004), it was shown that Engrailed transduction saves mDA neurons in the *En1*+/− mouse (Sonnier et al., 2007) and in wild-type (WT) mice exposed to striatal 6-hydroxydopamine (6-OHDA), systemic 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridin (MPTP), or the toxic A30P variant of alpha-synuclein (Alvarez-Fischer et al., 2011). Engrailed survival activity involves its ability to stimulate the translation of mitochondrial complex I subunits Ndufs1 and Ndufs3, resulting in enhanced ATP synthesis (Alvarez-Fischer et al., 2011; Stettler et al., 2012). Indeed, Engrailed is a multifunctional protein regulating the translation of capped mRNAs (Brunet et al., 2005).

We now find that the loss of one *En1* allele leads to changes in the expression of many genes in the DNA damage response (DDR) and chromatin structure pathways, often observed upon oxidative stress (Canugovi et al., 2013), suggesting that Engrailed protects against oxidative stress. Indeed, mDA neurons from *En1*+/− mice present signs of DNA damage and chromatin alterations and are more sensitive to an acute oxidative stress. Moreover, Engrailed transduction protects against oxidative stress-induced phenotypes.

Results

mDA Neuron Gene Expression in the SNpc of *En1*+/− Mice

RNA sequencing (RNA-seq) analysis on microdissected SNpc was performed in WT and *En1*+/− mice. Comparable reads were obtained in WT and *En1*+/− mice with 989 differentially expressed genes ($p < 0.05$) (Figure S1A; Table S1). Analysis was performed on 6-week-old animals when all neurons are still present in the in *En1*+/− mice (Sonnier et al., 2007). Pathway Studio Ontology (gene set enrichment analysis, Pathway Studio software) indicates that the three most represented and significant groups are DNA repair ($p = 0.002$), chromatin remodeling ($p = 0.004$), and transcription factors ($p = 0.007$); Cell Process Pathways analysis also revealed differential apoptosis regulation genes ($p = 0.01$) (Figure S1B). Genes within these ontologies and pathways were ranked by increasing p values; Figure 1A highlights those with significant differences in read numbers.

Transcription factor genes represent the most abundant group (Figure S1B); Figure S1D ranks by p values those with modified expression in *En1*+/− mice. En2 infusion in the SNpc of WT mice also modifies transcription factor genes, but much less so for DDR and chromatin-modifying ones (Figure S1C) that were thus further investigated in the context of this study. The qRT-PCR (Figure S1E) confirmed that 6-week-old *En1*+/− mice display altered expression of several genes related to DNA damage, chromatin remodeling, and apoptosis (Asagoshi et al., 2010; Burikhanov et al., 2014; Choi et al., 2004; Hong et al., 2008; Ma and D'Mello, 2011; Melis et al., 2013; Yang et al., 2013; Zhang et al., 2013).

DNA Strand Breaks and Modified Chromatin Marks in *En1*+/− mDA Neurons

The mDA neurons in the SNpc of *En1*+/− mice (between 6 and 8 weeks of age) were examined for signs of DNA damage by following the DNA strand break marker γ-H2AX (Löbrich et al., 2010). This revealed the presence of multiple γ-H2AX foci in about 16% of tyrosine hydroxylase-positive (TH+) neurons in the SNpc (Figures 1B and 1C). Of note, DNA strand breaks do not necessarily lead to rapid cell death, as shown by the absence of

significant death in the *En1*+/− mutant between 24 and 48 weeks (Sonnier et al., 2007), even though 16% of the cells have multiple γ-H2AX foci at 24 weeks (Figure S1F). In WT mice, about 98% of mDA neurons display a single ring of γ-H2AX around the nucleolus (Figure 1B), also present in neurons throughout the brain. In line with the lesser sensitivity of VTA mDA neurons to the loss of one *En1* allele (Sonnier et al., 2007), γ-H2AX staining was similar in the VTA of WT and *En1*+/− mice (Figure 1C).

En1+/− mice also have signs of chromatin alteration. As shown in Figures 1D–1F, the pattern of perinucleolar and perinuclear H3K27me3 (K27 trimethylated histone H3) staining was changed in a significant fraction of *En1*+/− TH+ neurons. The size distribution of the DAPI-dense regions of heterochromatin (Guenatri et al., 2004) also shows a reduction in the percentage of large spots in the *En1*+/− mouse (Figure 1G). Heterochromatin changes often are associated with changes in the expression of long interdispersed nuclear elements (LINEs) (Beisel and Paro, 2011; Guetg and Santoro, 2012); accordingly, LINE expression was increased in the SNpc of *En1*+/− mutants (Figure S1G). The induction of apoptosis genes (Figure 1A) was confirmed by the TH/activated caspase-3 co-staining in the SNpc of *En1*+/− mice, never seen in WT littermates (Figure S1H).

Enhanced Sensitivity of *En1*+/− mDA Neurons to Acute Oxidative Stress

An acute oxidative stress was applied to mDA neurons by injecting 6-OHDA (a superoxide-producing drug specifically captured by mDA neurons) directly into the SNpc. This induced, within 6 hr, the loss of 35% of the TH+ neurons and the formation of multiple abnormal γ-H2AX foci in about 26% of the remaining ones (Figures 2A and 2B), only in the ipsilateral SNpc. Neuronal loss was paralleled by a reduction in TH protein and mRNA (*En1* mRNA also) (Figures S2A and S2B) and the presence of numerous activated caspase-3-positive mDA neurons (Figure S2C). Injecting 6-OHDA into *En1*+/− mice led to a higher percentage of TH+ neurons with γ-H2AX foci in the 6-OHDA-injected side (51%) and to a parallel increase in the loss of TH+ neurons (60%) (Figures 2A and 2B).

This demonstrates that endogenous En1 has a protective effect against oxidative stress. *En1* is primarily a repressor gene (Tolkunova et al., 1998). Therefore, an activator form of Engrailed, composed of the En1/2 homeodomain (for target recognition) fused to four copies of the VP16 transcriptional activator domain of the herpes virus (EnHD-VP64) should have an anti-En1 activity. EnHD-VP64 infusion indeed leads to mDA cell death (Figures S3A and S3B) and to an increase in the number of γ-H2AX foci (Figure S3C). This confirms that Engrailed protects against oxidative stress, in part through transcriptional repression.

Oxidative Stress Induces Changes in Heterochromatin Marks

Heterochromatin marks also were modified 6 hr after 6-OHDA injection in WT mice. The dense perinucleolar and perinuclear H3K27me3 staining in TH+ neurons of sham-injected mice was changed into diffuse nucleoplasmic staining in 6-OHDA-injected mice (Figure 2C). This change was quantified at the level of the nucleolus by measuring perinucleolar fluorescence intensity along one diameter (Figure 2D, left). Similarly, the ratio of H3K27me3 fluorescence intensities between the peripheral nuclear lamina and the nuclear stroma dropped from 1.4 to 1.0 (Figure 2D, right). Acute 6-OHDA also disrupted H3K9me3

(K9 trimethylated histone H3) and MeCP2 staining (Figures S2D and S2E), with the loss of neat lamin B2 staining (Figure S2F) and a change in the size distribution of DAPI-dense spots (Figure S2G). MeCP2 binds methylated CpGs and changes in its staining might reflect guanine oxidation (Skene et al., 2010).

Nucleolar stress, suggested by perinucleolar γ-H2AX loss upon 6-OHDA (Figure 2A), was verified by the drop from 70% (sham) to 30% (6-OHDA) of mDA cells with dense nucleolin staining (Figures 2E and 2F). This change was accompanied by a strong upregulation of ribosomal pre-45S RNA (Figure 2G), signaling nucleolar damage (Guetg and Santoro, 2012; Larson et al., 2012). In comparison, nucleolin staining, normal in *En1*+/− TH+ neurons at 6 weeks, showed signs of disruption at 1 year (Figure 2H). However, the expression of genes involved in nucleolus organization (RNA-seq) and qRT-PCR analysis of pre-45S rRNA suggested a change in nucleolar physiology in 6- to 8-week-old *En1*+/− mice (Figures S3D and S3E). Nucleolar disruption links to the p53 pathways for senescence and apoptosis (Teng et al., 2013), including in mDA neurons (Rieker et al., 2011). The qRT-PCR on RNA from SNpc of sham- and 6-OHDA-injected mice showed that the level of *p53* transcripts was increased by 50% upon 6-OHDA injection in parallel with that of *p21*, a p53 target (Sperka et al., 2012; Figure S2H). Finally, in line with the regulation of retrotransposition through MeCP2 (Muotri et al., 2010), LINE-1 mRNA levels were increased upon 6-OHDA injection (Figure S2I).

Engrailed Protects mDA Neurons from Oxidative Stress

To verify if Engrailed protects against oxidative stress, WT mice were unilaterally injected with 6-OHDA, and re-injected 30 min later with vehicle (sham) or En2. Analyses were done at 6 hr, 24 hr, and 7 days post-injections. The dramatic loss at 24 hr of TH+ cells in the SNpc of 6-OHDA/sham-injected mice was highly reduced in 6-OHDA/En2-injected mice (Figure 3A). Protection was still visible at 7 days with 40% and 20% of surviving neurons in En2-injected and sham mice, respectively (Figure 3B). En2 injection 24 hr (instead of 30 min) after 6-OHDA and analyzed 6 hr later showed no recovery, demonstrating that TH staining corresponds to true survival and not to TH re-expression (Figure S3F).

As in other models (Casafont et al., 2011; Li et al., 2013), 6-OHDA-induced apoptosis was paralleled by the expression of cell cycle markers (Figures S4A–S4D). Accordingly, cyclin A expression detected in En2-treated TH+ cells 6 hr post-injections had almost disappeared at 24 hr (Figure 3C). Neuronal rescue by En2 was associated with the reappearance of normal staining patterns for H3K27me3, nucleolin, and γ-H2AX (Figure 3D). The percentage of TH+ cells with WT perinucleolar H3K27me3 staining increased from 20% to 37% at 6 hr and reached 60% and 80% at 24 hr and 7 days, respectively. WT nucleolin pattern took longer to reappear as recovery was observed only at 24 hr with little changes between 24 hr and 1 week. Finally, the decrease in the number of γ-H2AX foci was slower with full recovery only at 7 days.

Rescue correlated with a significant increase in TH mRNA expression and a decrease in that of pre-45S rRNA already 6 hr after En2 injection (Figure 3E). The expression of selected genes related to cell cycle and apoptosis (Lim and Kaldis, 2013; Smith et al., 2003; Wang et al., 2009), upregulated in the *En1*+/− mouse or upon 6-OHDA injection, was repressed in

the SNpc of 6-OHDA/En2 mice (Figure 3E). Analyses were done at 6 hr when sham- and En2-injected animals still have similar numbers of mDA neurons (Figure 3B).

VTA neurons are less sensitive to the loss of one *En1* allele. This might reflect the expression of Otx2, another transducing HP (Prochiantz and Di Nardo, 2015), specifically in the VTA (Di Salvio et al., 2010b) and its enhanced expression in the *En1* mutant (Figure S1D). A protective effect of Otx2 was confirmed in the 6-OHDA model (Figure 3F). Conversely, Engrailed could act as a general midbrain survival factor. To address this point, embryonic day (E)14.5 midbrain neurons expressing Engrailed, but of which only 1%–2% were dopaminergic, were exposed to H_2O_2 with or without En2. Figure S4E illustrates that En2 decreased the number of DNA strand breaks induced by H_2O_2 in parallel with a decrease in the formation of comet tails that signal DNA damage.

Engrailed Activates Short- and Long-Term Survival Pathways

Protection by En2 requires its injection within 24 hr after 6-OHDA (Figure S3F). To identify genes in this early survival pathway, SNpc RNA from 6-OHDA/sham or 6-OHDA/En2 mice (6 hr) was sequenced and genes in the chromatin remodeling, DNA damage, apoptosis, and cell cycle pathways were analyzed according to Pathways Studio. Figures 4A and 4B rank by order of significance the genes with highest differences in read numbers, and Figure 4C provides qRT-PCR confirmation of enhanced expression for top representative genes in the four analyzed pathways. Genes in the apoptosis pathway were more represented than in the *En1*+/− mouse (Figure 1). This was not due to En2 injection per se (Figure S4F), suggesting that the rapid upregulation of anti-apoptotic pathways by En2 takes place specifically following the acute oxidative stress.

Paralleling *Gadd45b* decreased expression in 8-week-old *En1*+/− mice, the addition of cycloheximide in the rescue experiments demonstrated that *Gadd45b*, as opposed to *Pml* for control, did not require the translation of an intervening protein and is, thus, a direct target of Engrailed (Figure 4D). The high induction of *Gadd45b*/*g* and NF-κB suggested a role for c-Jun N-terminal kinase (JNK) signaling, a pathway implied in several neuro-degenerative diseases including PD (Coffey, 2014). Figures 4E and 4F confirm that the strong increase in p-JNK staining 6 hr after 6-OHDA was antagonized by En2 (Figure 4F).

Discussion

High levels of reactive oxygen species (ROS) are toxic, in particular at the DNA level (Vijg and Suh, 2013) where they directly induce DDR (O'Sullivan and Karlseder, 2012). In consequence, neurons with high metabolic activity, such as SNpc mDA neurons, producing high amounts of ATP and ROS, are at risk for degeneration (Canugovi et al., 2013). Chromatin remodeling and DDR pathways are interconnected as DNA damage induces chromatin changes, themselves necessary to give access to the DNA repair machinery (Madabhushi et al., 2014; Soria et al., 2012). This study places *Engrailed* genes as key regulators of DNA damage and chromatin changes that accompany chronic and acute forms of oxidative stress in mDA neurons.

The *En*+/− chronic model of oxidative stress in which mDA neurons show progressive but faster death rate than in WT mice demonstrates that, similar to *Otx2* dosage in the adult retina (Bernard et al., 2014), *Engrailed* dosage is important in the adult SNpc. Indeed, given that En1 and En2 are biochemically equivalent (Hanks et al., 1995), the loss of only one allele out of four is enough to accelerate cell death. In the context of aging and neurological diseases, this suggests that mDA neurons in the *En1*+/− mouse age faster and are more sensitive to 6-OHDA, a toxin used in animal models of PD.

PD, even in its familial forms, declares itself rather late in life, underscoring a risk associated with age. Even if *Engrailed* is not a PD gene, its anti-aging properties might explain the association of *EN1* polymorphisms and the risk to develop PD (Fuchs et al., 2009; Haubenberger et al., 2011; Rissling et al., 2009). In this context, it is noteworthy that several phenotypes observed either in the *En1*+/− mice or in the acute 6-OHDA model are reminiscent of observations made on PD patients or models. For example, the loss of MeCP2 in SNpc mDA neurons compromises the nigrostriatal dopaminergic pathway (Gantz et al., 2011); JNK and cyclin pathways are implied in PD (Coffey, 2014; Smith et al., 2003) and nucleolin diffusion was reported in PD patients (Rieker et al., 2011).

The low expression of the DA transporter explains the poor sensitivity of VTA mDA neurons to 6-OHDA, but not their relative resistance to degeneration in the *En1*+/− mutant since they also express *Engrailed*. A possibility is that Otx2, an HP expressed in the VTA and not in the SNpc, protects VTA neurons from death (Di Salvio et al., 2010a), thus dampening the loss of one *En1* allele and slowing down mDA neuron degeneration that eventually takes place (Sonnier et al., 2007). Indeed, Otx2 prevents the degeneration of retinal ganglion cells in a mouse model of glaucoma (Torero Ibad et al., 2011) and confers a protection similar to that of En2 in the 6-OHDA model (this study).

In a previous study, it was shown that Engrailed could protect mDA neurons in three mouse models of PD (Alvarez-Fischer et al., 2011). The study was very different in the sense that 6-OHDA was injected in the striatum and not injected directly into the SNpc, an acute and harsh procedure preventing long-term secondary effects. More importantly, Engrailed was infused in the SNpc 3 weeks before the insult and not injected 30 min after 6-OHDA injection. Finally, this study, contrary to the previous ones, establishes a role of Engrailed as a transcriptional and epigenetic regulator and not only at the level of protein translation, even if the two modes of action may concur to save the cells. This transcriptional and epigenetic level is very important as it suggests that Engrailed may have a long-lasting effect on mDA neuron survival.

One can thus propose a mechanism based on short-term and long-term effects. Short-term effects include the translation of mRNAs encoding mitochondrial proteins (Alvarez-Fischer et al., 2011) and, in particular but not only, the transcription of anti-apoptotic genes (this study) and the repression of apoptosis as already proposed (Albéri et al., 2004; Beltran et al., 2014), with a predominance, seemingly, of GADD45b/g, NF-κB, and JNK pathways. We propose that long-lasting effects relate to transcriptional and epigenetic mechanisms allowing both chromatin restructuration and DNA repair, two highly interconnected

pathways based on the regulation of the genes highlighted in our initial RNA-seq study comparing the transcriptome of WT and *En1*+/− SNpc.

In conclusion, the protective effects of Engrailed in PD models (Alvarez-Fischer et al., 2011), together with the present data, give credit to the idea of using Engrailed as a therapeutic protein acting at both the levels of mRNA translation and direct or indirect gene transcription.

Experimental Procedures

Animals

Mice were treated as per the guidelines for the care and use of laboratory animals (NIH) and the European Directive 86/609 (EEC Council for Animal Protection in Experimental Research and Other Scientific Utilization). Swiss OF1 WT (Janvier) and *En1*+/− mice (Hanks et al., 1995) were maintained in a conventional animal facility. Experimental groups consisted of 6- to 9-week-old mice.

In Vivo Treatments

For 6-OHDA injections, mice were placed in a stereotaxic instrument and a burr hole was drilled into the skull 3.3 mm caudal and 1 mm lateral to the bregma. The needle was lowered 4 mm from the surface of the skull and 6-OHDA (2 µl; 0.8 µg/µl, Sigma) or sham (NaCl 0.9%) injection was performed over 4 min. For Engrailed rescue experiments, a solution (2 µl) of bacterial recombinant En2 (300 ng; 4 µM) and colominic acid (3 µg) (Sonnier et al., 2007) or vehicle (NaCl 0.9%) was injected 30 min after 6-OHDA injection using the same coordinates. When indicated, cycloheximide (0.1 µg/µl, Sigma) was added. For Otx2 protein injection, a 2-µl solution containing 300 ng protein was used. Mice were killed at the indicated times for analysis. SNpc tissues for qRT-PCR and western blot analysis were obtained by performing 1-mm punches from 2-mm-thick frozen coronal slices.

For EnHD-VP64, mice were infused for 7 days with an osmotic mini pump (Alzet 1002, Charles River Laboratories) connected to a 4-mm-long cannula placed at the same stereotaxic coordinates as above. The pump was filled with 100 µl containing En-VP64 (400 nM, 0.9% NaCl or the equivalent volume of an empty-plasmid-containing bacterial extract) and colominic acid (1.5 µg/µl).

Image Quantification

Images were analyzed with ImageJ. For immunofluorescence, all quantifications were performed using 603 magnification and 0.7-µm-thick successive focal planes. For H3K27me3 nucleolar pattern analysis, a graph of the intensities of pixels along a line positioned through the nucleolus was created. Perinuclear/nuclear ratio of H3K27me3 fluorescence intensity was determined by measuring pixel density at the periphery of the nucleus and in the nucleoplasm. DAPI-dense regions in sham- and 6-OHDA-injected mice were quantified by measuring individual DAPI surface areas in each TH+ cell and plotting them as relative frequency distribution histograms.

Statistical Analysis

Statistical significance was determined using appropriate tests as indicated. Data are expressed as means ± SEM (*p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001 in all experiments).

Also see the Supplemental Experimental Procedures for RNA-seq, qRT-PCR, and immunostaining experiments.

Supplemental Information

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Engrailed regulates DNA damage response and chromatin remodeling
- Dopaminergic neurons are protected by Engrailed from oxidative stress
- Engrailed acts both at genetic and epigenetic levels to confer neuroprotection
- Engrailed homeoprotein transduction has therapeutic potential

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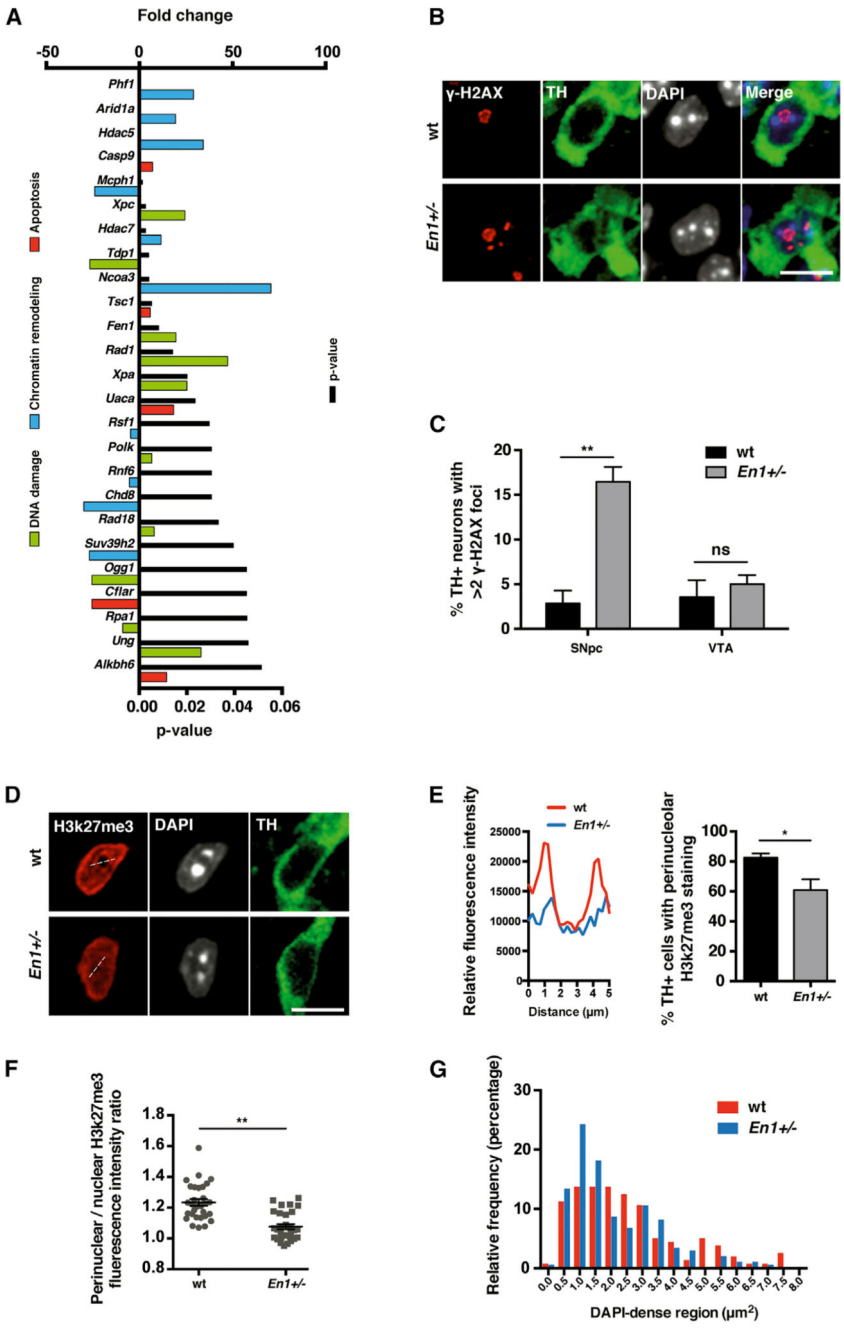

Figure 1. Altered Expression of DNA Damage and Heterochromatin Marks in*En1*+/− Mice

(A) The differentially expressed genes in *En1*+/− SNpc TH+ neurons belonging to DNA damage, chromatin remodeling, and apoptosis groups are ranked by p values.

(B) TH+ neurons in the SNpc of WT mice display a single ring of γ-H2AX staining; *En1*+/− TH+ neurons show additional γ-H2AX foci scattered in the nucleus. Scale bar, 10 μm.

(C) The percentage of TH+ neurons with more than two γ-H2AX foci in the SNpc increases from 2% in WT to 16% in 8-week-old *En1*+/− mice (n = 3; Student's t test). γ-H2AX

staining in TH+ neurons in the VTA of *En1*+/− and WT mice is similar. Between 110 and 162 neurons were counted in each condition.

(D) Perinucleolar and perinuclear H3K27me3 staining is decreased in the *En1*+/− SNpc mDA neurons. Scale bar, 10 μm.

(E) H3K27me3 perinucleolar staining is quantified by measuring fluorescence intensity (left) along the dotted lines (Figure 1D). The percentage of cells with dense staining drops (right) in *En1*+/− mice (n = 3; Student's t test; 129 and 159 neurons counted in WT and *En1*+/− mice, respectively).

(F) Perinuclear H3K27me3 staining in *En1*+/− TH+ neurons is reduced, as shown by the decreased nuclear lamina/nucleoplasm fluorescence intensities (n = 3; Student's t test; 30 and 30 neurons counted in sham and 6-OHDA conditions).

(G) Surface quantification of DAPI-dense regions. Frequency distribution indicates a shift toward smaller DAPI-dense areas in *En1*+/− mice (n = 162–211; Kolmogorov-Smirnov test; three WT and *En1*+/− mice were analyzed). See also Figure S1 and Table S1.

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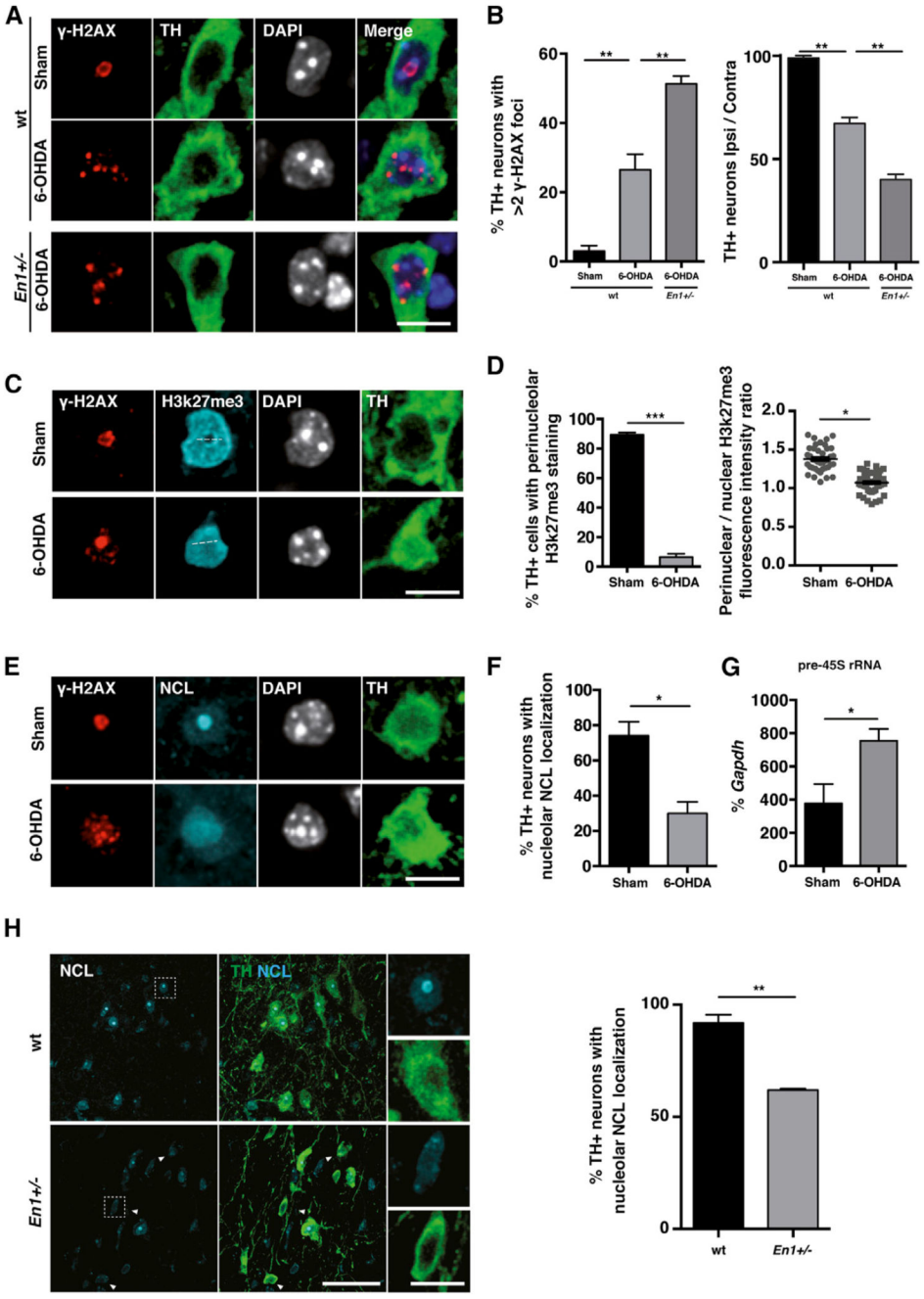

Figure 2. DNA Damage and Chromatin Alteration in SNpc TH+ Neurons upon 6-OHDA Injection

(A and B) 6-OHDA injected in the SNpc of WT mice leads 6 hr later to the appearance of γ-H2AX foci in about 25% of TH+ neurons (n = 3–6; Student's t test). *En1*+/− mice are more sensitive with 50% of neurons showing multiple γ-H2AX foci (n = 3–6; Student's t test; 130, 210, and 146 neurons were counted for each condition, respectively). Scale bar, 10 μm. 6-OHDA injection provokes the loss of about 30% and 60% of TH+ neurons in WT and *En1*+/− mice, respectively (n = 3; Student's t test). The contralateral non-injected side is taken as a reference. In each condition between 1,534 and 2,034 neurons were counted.

(C) Midbrain sections stained for γ-H2AX, H3k27me3, and TH and analyzed by confocal microscopy. Perinucleolar and perinuclear H3K27me3 staining is decreased upon 6-OHDA injection. Mice were analyzed 6 hr post-injection. Scale bar, 10 μm.

(D) The percentage of TH+ neurons displaying dense H3K27me3 perinucleolar and perinuclear staining, quantified as in Figure 1E and 1F, drops dramatically (left) in 6-OHDA-injected mice (n = 3; Student's t test; 148 and 91 neurons were counted in sham and 6-OHDA conditions, respectively). Perinuclear H3K27me3 staining in TH+ neurons also decreases upon 6-OHDA injection (right) (n = 3; Student's t test; 40 and 47 neurons were counted in sham and 6-OHDA conditions, respectively).

(E) Midbrain sections stained for γ-H2AX, nucleolin, and TH and analyzed by confocal microscopy. Nucleolin nucleolar localization in sham-injected mice was lost 6 hr after 6-OHDA injection. NCL, nucleolin. Scale bar, 10 μm.

(F) The percentage of TH+ neurons with nucleolar nucleolin is significantly decreased upon 6-OHDA injection (n = 3; Student's t test; 161 and 97 neurons were counted in sham and 6-OHDA conditions, respectively). NCL, nucleolin.

(G) The pre-45S rRNA analyzed by qRT-PCR is upregulated following 6-OHDA injection in SNpc purified nuclei (n = 3; Student's t test).

(H) Midbrain sections from 1-year-old animals stained for nucleolin and TH were analyzed by confocal microscopy. Nucleolin presents a nucleolar localization in WT TH+ neurons, whereas 40% of TH+ neurons in *En1*+/− mice present a diffuse staining pattern (arrows). Scale bar, 50 μm. Higher magnification images of dotted square areas are shown (right). Scale bar, 10 μm. The percentage of TH+ neurons with nucleolar nucleolin is significantly decreased in *En1*+/− mice (n = 3; Student's t test; 99 and 87 neurons were counted in WT and *En1*+/− mice, respectively). NCL, nucleolin. See also Figure S2.

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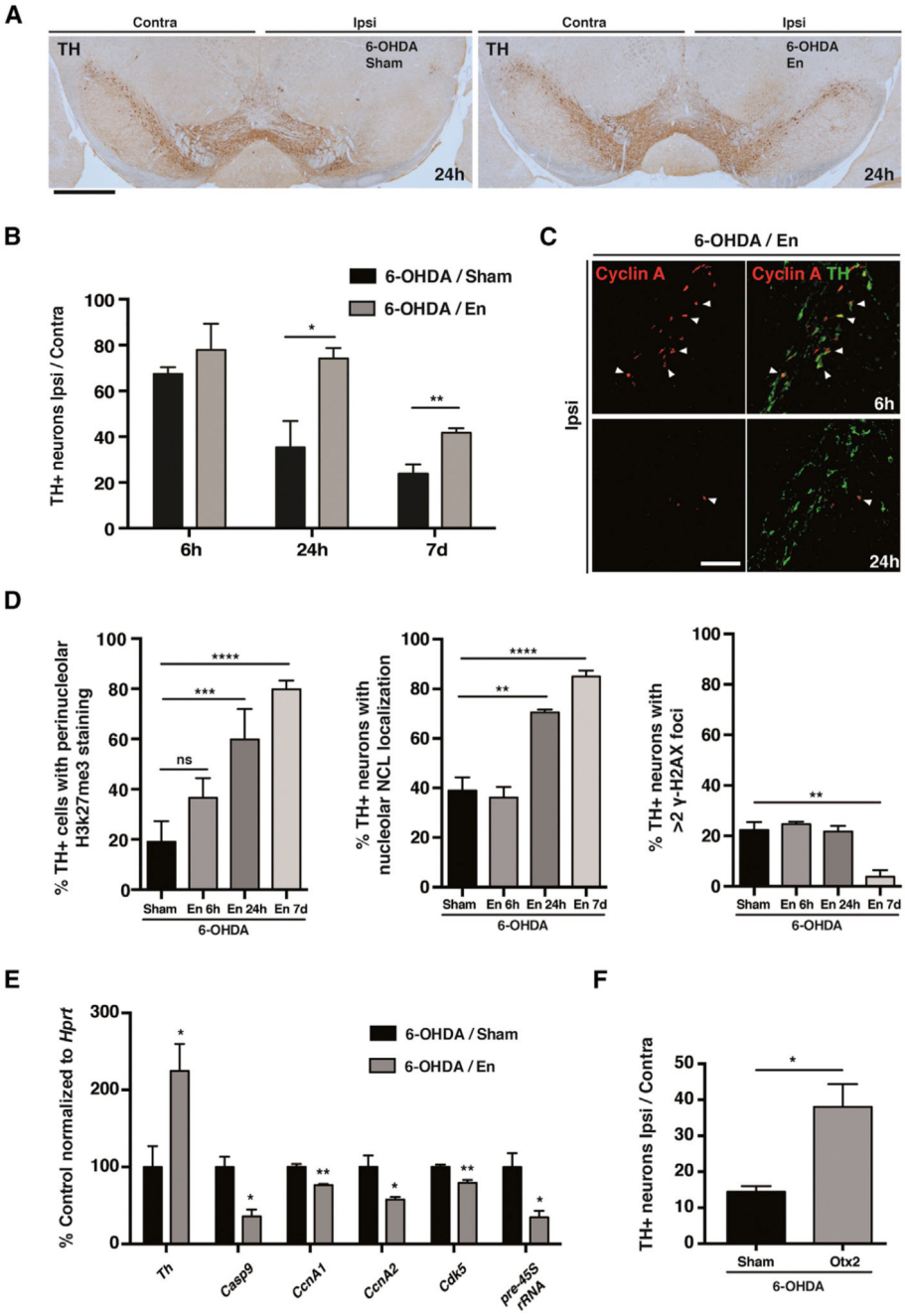

Figure 3. Engrailed Rescues TH+ Cells from 6-OHDA-Induced Cell Death

(A) Mice injected with 6-OHDA in the SNpc were re-injected 30 min later with either sham or En2 and analyzed at 24 hr. Compared to sham, En2 injection prevents the 6-OHDA-induced TH cell loss in the ipsilateral SNpc. Scale bar, 500 μm.

(B) Protective effect of En2 assessed by the ratio of TH+ cell counts in ipsilateral versus contralateral SNpc 6 hr, 24 hr, and 7 days post-injection (n = 3–5; Student's t test). The number of neurons counted ranged between 1,670 and 2,275 per condition.

(C) The rescue of TH+ neurons by En2 in 6-OHDA-injected mice is paralleled by a disappearance of cyclin A at 24 hr. Scale bar, 100 μm.
(D) Immunostaining for H3K27me3 shows a progressive recovery following En2 injection. Recovery of nucleolar nucleolin is almost complete at 24 hr. The percentage of TH+ neurons with γ-H2AX foci returns to normal between 24 hr and 7 days after injection (for each analysis, n = 3; one-way ANOVA followed by Dunetts's test [versus sham]). The number of neurons counted ranged between 102 and 150 for each condition.
(E) The expression of selected genes related to apoptosis and cell cycle in the SNpc of 6-OHDA/sham and 6-OHDA/En2 mice analyzed 6 hr post-injections (n = 5; Student's t test).
(F) Otx2 protects TH+ neurons against 6-OHDA-induced cell death. Otx2 was injected 30 min after 6-OHDA injection and mice were analyzed 24 hr later. Protective effect was assessed as for En2 injections (n = 3; Student's t test). The number of neurons counted ranged between 1,268 and 1,495 for each condition. See also Figure S3.

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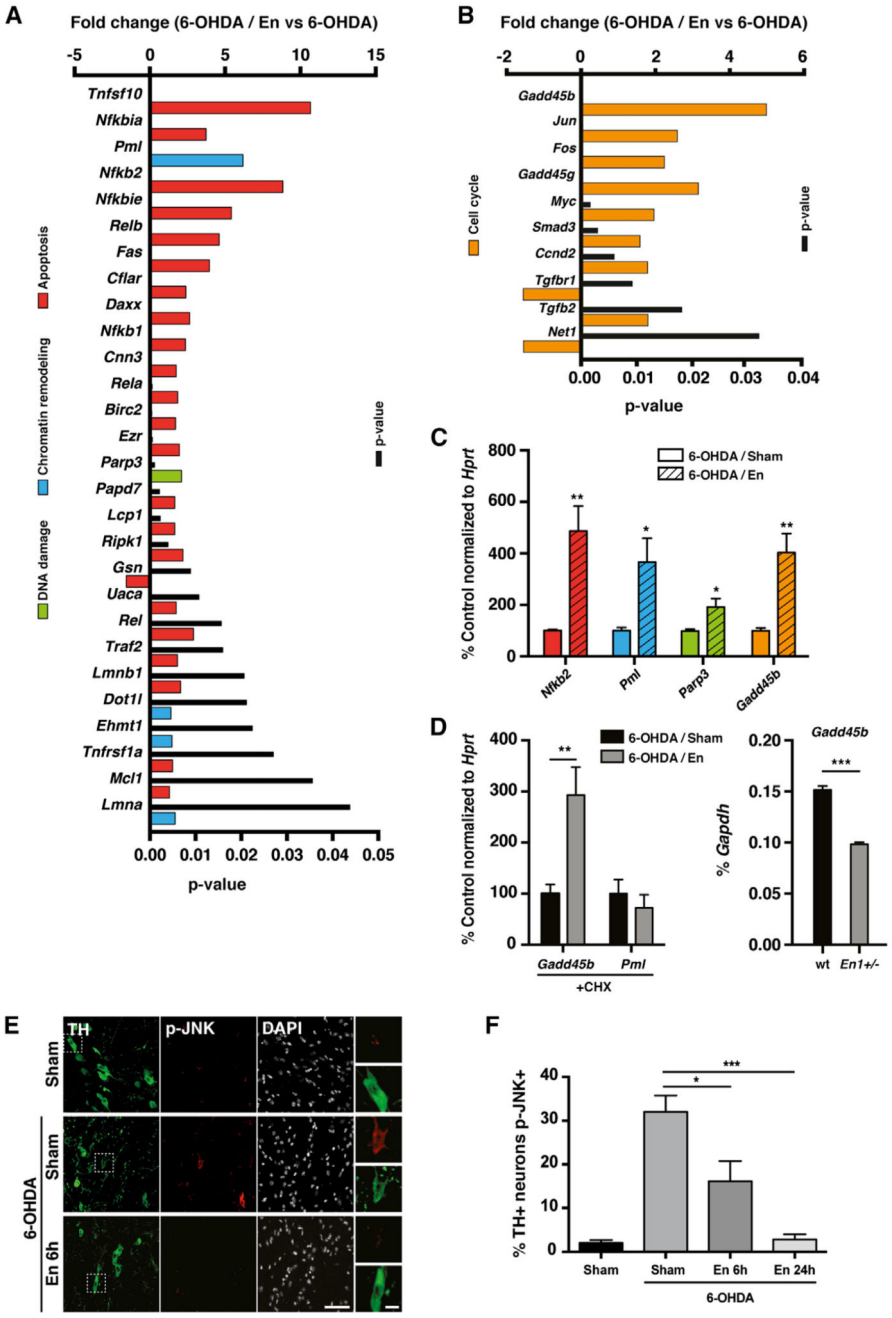

Figure 4. RNA-Seq Analysis Reveals Engrailed Anti-apoptotic Activity

(A and B) Differentially expressed genes in the SNpc of 6-OHDA versus 6-OHDA/En2 related to (A) DNA damage, chromatin remodeling, apoptosis, and (B) cell cycle are ranked by p values.

(C) The expression of selected genes in the SNpc of 6-OHDA and 6-OHDA/En2 is confirmed by qRT-PCR (n = 5; Student's t test).

(D) (Left) *Gadd45b* and *Pml* transcripts were measured by qRT-PCR in the SNpc of 6-OHDA and 6-OHDA/En2 at 6 hr (injection with cycloheximide [CHX]; n = 5; Student's t

test). (Right) *Gadd45b* transcripts in the SNpc of 8-week-old WT and *En1*+/− mice are shown (n = 4; Student's t test).

(E) Midbrain sections from sham, 6-OHDA/sham, and 6-OHDA/En2 stained for p-JNK and TH and analyzed by confocal microscopy are shown. Scale bar, 50 μm. Higher magnification images of dotted squares are shown (right). Scale bar, 10 μm.

(F) En2 significantly decreases the percentage of SNpc TH+ neurons with p-JNK staining (n = 3; one-way ANOVA followed by Tukey's multiple comparisons test; number of neurons analyzed ranged from 227 to 351 for each condition). See also Figure S4.