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(54) Title: HOMEOPROTEINS FOR USE IN THE TREATMENT OF NEURODEGENERATIVE DISORDERS

(57) Abstract: The present invention relates to the use of a homeoprotein or a recombinant vector encoding said protein for treating or preventing DNA damage and/or cellular aging. In particular, the invention concerns the use of Engrailed for the treatment of Parkinson disease.

HOMEOPROTEINS FOR USE IN THE TREATMENT OF NEURODEGENERATIVE DISORDERS

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The present invention relates to the use of a homeoprotein or a recombinant vector encoding said protein for treating or preventing conditions associated with DNA damage and/or cellular aging, and more particularly for the prevention and/or
10 treatment of DNA-damage related diseases or disorders and age-related diseases or disorders, especially neurodegenerative disorders. In particular, the present invention concerns the use of Engrailed in the treatment of Parkinson disease.

BACKGROUND OF THE INVENTION

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Study of aging processes is important, in part because many diseases or conditions become more prevalent among aged people, for example, cancers, Alzheimer's disease, Parkinson's disease, stroke, glaucoma, cardiovascular disease, just to name a few, among which many still lack effective preventive or treatment
20 methods. Although abundant literature has contributed to the understanding of aging processes, full understanding of the processes remains a major challenge. Given the increasing population of aged people around the world and the rising health care burden and cost associated therewith, studies of the aging processes leading to effective discovery of anti-aging agents for the prevention or treatment of
25 age-related diseases or disorders are becoming increasingly important.

Cellular DNA is under constant challenge by exogenous and endogenous genotoxic stresses (e.g. oxidative stress, toxic byproducts, reduced mitochondrial function) that modify DNA through base modification or mis-incorporation resulting in DNA damage. DNA damage is physical abnormality in the DNA structure such as
30 single and double strand breaks. DNA damage can be recognized by specific enzymes, and thus can be correctly repaired through the DNA Damage Response (DDR). The loss of this natural DNA repair capacity results in genetic instability that

may lead to cellular dysfunction and cell death. It has been further shown that chromatin remodeling and DDR pathways are interconnected as DNA damage induces chromatin changes, themselves necessary to give access to the DNA repair machinery (Soria et al., 2012, *Mol Cell* 46, 722-734).

5 Accumulation of DNA damage has been linked to the process of aging and to the onset of age-related diseases including neurodegenerative disorders, such as ataxias, Alzheimer's, amyotrophic lateral sclerosis, Huntington's and Parkinson's diseases (Canugovi et al., 2013, *12*, 578-587; Madabhushi et al., 2014, *Neuron* 83, 266-282).

10 Additionally, in neurodegenerative disorders, such as ischemia and Alzheimer's disease, neurons manifest aberrant cell cycle activity, expressing cell cycle markers such as Cyclin-A, and undergo a limited extent of DNA remodeling. This behavior is remarkable considering that in humans neurons terminally differentiate during development and remain quiescent for decades prior to the onset of these
15 events. While the underlying mechanisms are poorly understood, multiple lines of evidence suggest that these activities play an early and contributory role in neuronal death (Andorfer et al., 2005, *J. Neurosci.*, 1,25, 5446-54).

 It has further been shown that said DNA damage and re-entry into the cell cycle may constitute a common pathway in apoptosis in neurological diseases (Kim
20 and Tsai, 2009, *Ann N Y ACAD Sci.*, 1170, 674-9 or Pizarro et al., 2009, *Free Radic. Res.*, 43, 985-994).

 Therefore, there is a strong need to develop new therapeutic agents that promote DNA repair, that are able to prevent and/or treat DNA damage and/or cellular aging related disorders and/or regulate the cell cycle.

25 Homeoproteins, or homeodomain proteins, are transcription factors that play a major role in cell migration and differentiation processes involved in morphogenesis. They are characterized by the presence of a sequence of 60 amino acids, the homeodomain, which is a DNA binding domain with a helix/turn/helix structure. It has been shown that the isolated domain of Antennapedia protein of

Drosophila crosses the membrane of neurons in culture, accumulates in the nucleus and promotes neurite growth (EP0485578). The homeodomain is highly conserved and confers the internalization property to a large number of homeoproteins (Spatazza et al., 2013, Pharmacol. Rev. 65, 90-104).

5 Engrailed proteins (Engrailed-1 and Engrailed-2) are homeoproteins with similar biological activity that are designated collectively hereafter with the general term Engrailed (EN for the human or En1/2 for mice). In neonates and in adults, Engrailed is expressed in cerebellar granule cells and in mesencephalic dopaminergic (DA) nuclei, including the substantia nigra pars compacta (SNpc that degenerates in
10 Parkinson's disease), and the Ventral Tegmental Area (VTA). En1/2 plays important roles in the development of the midbrain and, in the midbrain, of the mesencephalic dopaminergic (mDA) neurons (Joyner, 1996, Trends Genet 12, 15-20). En1/2 also plays redundant roles in the survival of adult mDA neurons that are located in the SNpc and the VTA in the ventral part of the mesencephalon (Alberi *et al.*, 2004,
15 Development 131, 3229-3236), and therefore it was proposed for preventing or treating the loss of DA neurons in Parkinson disease. In WO 2013/128239, it was reported that local administration of En1/2 by infusion in the midbrain increases DA synthesis by DA neurons and associated motor activity. Prochiantz et al. (2011, FEBS Letters, 278, 52 (Abstract, Brunet *et al.*, Nature 438 : 94-98, 2005 and Alvarez-Fisher
20 *et al.*, Nature Neurosci 14 : 1260-1266, 2011) have reported that Engrailed is not only a transcription factor but also a translation regulator that enhances the translation of mitochondrial mRNAs transcribed in the nucleus and it was shown that Engrailed transduction up-regulates the translation of Ndufs1 and Ndufs3, two proteins of mitochondrial complex I and increases ATP synthesis (see also Alvarez-Fischer *et al.*,
25 2011, Nature Neuroscience, 14, 1260-1266, Stettler *et al.* 2012). Alternatively, in WO 2007/099227, it was shown that systemic administration of En1/2 to mice induces an increase in DA turnover in the striatum, reflected by an increase in the production of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) without modification of dopamine levels.

Otx2 (orthodenticle homolog 2) is another homeoprotein containing a bicoid-type homeodomain (Simeone *et al.*, 1993, EMBO J 12, 2735-2747). It belongs to the Otx protein family, which plays fundamental roles during brain embryogenesis (Acampora *et al.*, 1995; Simeone *et al.*, 2002). It has also been shown that Otx2 is
5 involved in the formation of retina and in adult retinal ganglion cell survival and physiology (Bernard *et al.*, 2013; Torero Ibad *et al.*, 2011). It was thus proposed to use homeoproteins of the bicoid family, in particular of the Otx family, such as Otx2, for improving survival of adult retinal ganglionic neurons (RGCs) in culture *in vitro*, and also for preventing or treating, *in vivo*, RGC degeneration (WO 2009/106767).

10 The Inventors have now shown that homeoproteins are able to protect cells from DNA damage and chromatin remodeling, to decrease the number of DNA strand breaks, to restore all nuclear and nucleolar heterochromatin marks, to regulate the cell cycle and to protect cell against aging-like excitotoxicity, including apoptosis. In particular, they showed that Engrailed, when administered into the
15 SNpc region, is able to repair the damages leading to cell death provoked by a strong oxidative stress. In particular, they activate anti-apoptotic pathways and promote long-term survival by restoring heterochromatin marks, including nucleolus integrity, and by abolishing double-strands breaks in a model of acute oxidative stress. These results allow to propose to use homeoproteins, and in particular Engrailed, in
20 pathologies wherein DNA damage occurs, such as in Parkinson disease.

According to the present Invention, the term "DNA damage" covers collectively DNA damage, DNA strand breaks (single and double strand), and nuclear and nucleolar heterochromatin disruption.

25 Therefore the present Invention relates to the use of at least one homeoprotein, or peptide derivatives thereof, for the treatment or prevention of DNA damage and/or cellular aging. The Invention further concerns the use of at least one homeoprotein, or peptide derivatives thereof, for the treatment or prevention of conditions associated with DNA damage and/or cellular aging, and more

particularly for the prevention and/or treatment of DNA-damage related diseases or disorders and age-related diseases or disorders.

Alternatively, the present invention relates to the use of a recombinant vector encoding said proteins or peptides for the treatment or prevention of DNA
5 damage and/or cellular aging and for the treatment or prevention of conditions associated with DNA damage and/or cellular aging, and more particularly for the prevention and/or treatment of DNA-damage related diseases or disorders and age-related diseases or disorders.

As used herein, the terms "homeoprotein " and " homeodomain proteins " are synonymous and refer to gene products, proteins and polypeptides commonly
10 known under these designations in the art. The terms encompass such gene products, proteins and polypeptides of any organism where found, and particularly of animals, preferably vertebrates, more preferably mammals, including non-human mammals, and even more preferably of humans. More particularly, "homeoprotein"
15 relates to a protein comprising a homeodomain. Homeoproteins include natural homeoproteins and recombinant homeoproteins or synthetic homeoproteins.

The present invention encompasses native homeoprotein, including allelic as well as non-naturally occurring variants, synthetic homeoprotein, fragments thereof, as well as fusion proteins involving all or part of homeoprotein, fragment or variant
20 thereof. Preferably, the homeoprotein of the present invention comprises the full-length homeodomain. According to special embodiment, the homeoprotein according to the Invention comprises an isolated homeodomain fused to a transcriptional repressor domain (fusion protein) or, in some cases to an activator domain. The repressor or activator domain may be from the same protein as the
25 homeodomain, another protein of the homeoprotein family or a protein that is not from the homeoprotein family. The fusion protein is different from a full-length homeoprotein.

In addition to naturally-occurring allelic variants of homeoprotein entities that may exist in the population, the skilled artisan will further appreciate that

changes (i.e. one or more deletions, additions and/or substitutions of one or more amino acid) can be introduced by mutation using classic or recombinant techniques to effect random or targeted mutagenesis. A suitable variant in use in the present invention preferably has an amino acid sequence having a high degree of homology with the amino acid sequence of the corresponding native homeoprotein. In one
5 embodiment, the amino acid sequence of the variant homeoprotein in use in the invention is at least 70%, at least about 75%, at least about 80%, at least about 90%, preferably at least about 95%, more preferably at least about 97% and even more preferably at least about 99% identical to the corresponding native sequence.

10 Percent identities between amino acid (or nucleic acid) sequences can be determined using standard methods known to those skilled in the art. For instance for determining the percentage of homology between two amino acid sequences, the sequences are aligned for optimal comparison purposes. The amino acid residues at corresponding amino acid positions are then compared. Gaps can be introduced in
15 one or both amino acid sequence(s) for optimal alignment and non-homologous sequences can be disregarded for comparison purposes. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the sequences are identical at that position. The percent identity between the two sequences is a function of the number of identical
20 positions shared by the sequences, taking into account the number of gaps that are introduced for optimal alignment and the length of each gap. The comparison of sequences and the percent identity and similarity between two sequences can be determined using a mathematical algorithm.

Suitable variants of homeoprotein for use in the present invention are
25 biologically active and retain at least one of the activities described herein in connection with the corresponding native homeoprotein. Preferably, the effect on DNA damage and/or chromatin alteration is preserved, although a given function of the native homeoprotein may be positively or negatively affected to some degree, e.g. with variants exhibiting reduced biological activity. Amino acids that are essential

for a given function can be identified by methods known in the art, such as by site-directed mutagenesis. For example, in one class of functional variants, one or more amino acid residues are conservatively substituted. A "conservative amino acid substitution" is one in which the amino acid residue in the native polypeptide is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. Typically, substitutions are regarded as conservative when the replacement, one for another, is among the aliphatic amino acids Ala, Val, Leu, and Ile; the hydroxyl residues Ser and Thr; the acidic residues Asp and Glu; the amide residues Asn and Gln; the basic residues Lys and Arg; or the aromatic residues Phe and Tyr.

The protein for use according to the invention can be prepared by the conventional techniques known to those skilled in the art, in particular by expression of a recombinant DNA in a suitable cell system (eukaryotic or prokaryotic) or by solid-phase or liquid-phase synthesis. More specifically, the protein and its derivatives are usually produced from a DNA polynucleotide comprising a sequence coding for the protein, obtained by any means known to those skilled in the art, including amplification of a nucleic sequence by PCR or RT-PCR, screening genomic DNA libraries by hybridization with a homologous probe, or else total or partial chemical synthesis. The recombinant vectors are constructed and introduced into host cells by conventional recombinant DNA and genetic engineering techniques, which are known in the art. The DNA polynucleotide is cloned into a eukaryotic or prokaryotic expression vector and the protein produced in the cells modified with the recombinant vector is purified by any suitable means, in particular by affinity chromatography. The peptide and its derivatives are usually solid-phase synthesized, according to the Fmoc technique, originally described by Merrifield *et al.* (J. Am. Chem. Soc., 1964, **85**: 2149-2154) and purified by reverse-phase high performance liquid chromatography.

According to more preferred embodiment, the terms designate proteins and polypeptides comprising the following amino acid sequence (homeodomain)

RRRKRTA-YTRYQLLE-LEKEFLF-NRYLTRRRRIELAHSL-NLTERHIKIWFQN-RRMK-WKKEN
 [typical insertion sites are noted with dashes] (SEQ ID NO:1) (see
<http://homeobox.biosci.ki.se/>).

Non-limitative examples of homeoproteins which can be used in the present
 5 invention include all homeoproteins expressed in different subsets of adult neurons
 such as: Engrailed proteins (Engrailed-1 or Engrailed-2), homeoproteins from the
 bicoïd family, in particular the Otx sub-family like Otx2, proteins from the paired
 family (Pax), the Lhx family, for example Lhx9, and Gbx2.

Homeoprotein gene sequences and deduced amino acid sequences are well
 10 known in the art and available in the sequence databases.

Exemplary En1/2 protein or polypeptide includes without limitation mouse
 En1/2 protein or polypeptide having primary amino acid sequence (SEQ ID NO: 2) :

musEn1: NP_034263 (401aa)

15 MEEQQPEPKSQRDSGLGAVAAAAPSGLSLSLSPGASGSSGSDGDSVPVSPQPAPPSPPAAPCLP
 PLAHHPHLPPHPPPPPPPPPPPPQHAAAPAHQPQPAQLHRTTNFFIDNILRPDFGCKKEQPLP
 QLLVASAAAGGGAAAGGGSRVERDRGQTGAGRDPVHSLGTRASGAASLLCAPDANCGPPDGS
 QPATAVGAGASKAGNPAAAAAAAAAAAAAAAAAVAAAAAAAAASKPSDSGGGSGGNAGSPGAQGA
 KFPEHNPAILLMGSANGGPVVKTDSSQQLVWPWVYCTRYSDRPSSGPRTRKLLKKKNEKEDK
 20 RPRTAFTAELQLRLKAEFQANRYITEQRRQTLAQELSLNESQIKIWFQNKRAKIKKATGIKINGLAL
 HLMAQGLYNHSTTTVQDKDESE

Exemplary Gbx2 protein or polypeptide includes without limitation mouse
 Gbx2 protein or polypeptide having primary amino acid sequence (SEQ ID NO: 3) :

musGbx2: NP_034392 (longest variant, 348aa)

25 MSAAFPPSLMMMQRPLGSSTAFSIDSIGSPPQSPGHFVYTGYPMFMPYRPVVLPPPPPPPP
 ALPQAALQPALPPAHPHHQIPSLPTGFCSSLAQGMALTSTLMATLPGGFSASPQHQEAAAARK
 FAPQLPGGGNFDKAEALQADAEDGKAFLAKEGSLAFSAAEAVQASLVGAVRGQGKDESKVE
 DDPKGKEESFSLESDVDYSSDDNLPGQTAHKEEDPGHALEETPQSGGAAGSTTSTGKNRRRRRTA
 30 FTSEQLLELEKEFHCKKYLSTERSQIAHALKLSEVQVKIWFQNRRAKWKRKAGNANSKTGEP
 RNPKIVVPIPVHVSRAIRSQHQLEQARP

Exemplary Lhx9 protein or polypeptide includes without limitation mouse Lhx9 protein or polypeptide having primary amino acid sequence (SEQ ID NO: 4) :

musLhx9: NP_001036042 (longest variant, 397aa)

5 MEIVGCRAENNSCFRPPAMLFHGISGGHIQGIMEEMERRSKTEARLTGKTQLNGRDAGMPPL
SPEKPALCAGCGGKISDRYLLAVDKQWHLRCLKCCECKLALESELTCFAKDGSIYCKEDYYRRFS
VQRCARCHLGISASEMVMRARDSVYHLSCFTCSTCNKTLTTGDHFGMKDSLVCRAHFETLLQ
GEYPPQLSYTELAASKGGLALPYFNGTGTVQKGRPRKRKSPALGVDIVNYSNGCENEADHLDR
DQQPYPPSQKTKRMRTSFKHHQLRTMKSYFAINHNPDAKDLK

10 Exemplary En1 protein or polypeptide includes without limitation human EN1
protein or polypeptide having primary amino acid sequence as annotated under
Genbank accession number AAA 53502.2 or NCBI NP_001417.3.

Exemplary En2 protein or polypeptide includes without limitation human EN2
protein or polypeptide having primary amino acid sequence as annotated under
15 Genbank accession number AAA 53504.2 or NCBI NP_001418.2.

Exemplary Gbx2 protein or polypeptide includes without limitation human
Gbx2 protein or polypeptide having primary amino acid sequence as annotated
under Genbank accession number EAW71084.1.

Exemplary Lhx9 protein or polypeptide includes without limitation human
20 Lhx9 protein or polypeptide having primary amino acid sequence as annotated under
Genbank accession number NP_001014434.1 or NP_064589.2.

According to one preferred embodiment of the invention, said homeoprotein
is Engrailed protein (Engrailed-1 or Engrailed-2) or a fusion protein including a
homeodomain thereof fused to a repressor or activator domain. The fused domain
25 may be from Engrailed protein or another protein, for example a homeoprotein as
defined above. The Engrailed repressor domain may be the domain situated from
positions 1 to 298 of Engrailed-1 amino acid sequence or a subdomain thereof such
as the eh1 domain (positions 46 to 67 of Engrailed-2 amino acid sequence).

According to another preferred embodiment of the invention, said
30 homeoprotein is different from Engrailed protein (Engrailed-1 or Engrailed-2) and a

homeoprotein of the bicoïd family. According to another preferred embodiment of the invention, said homeoprotein is a fusion protein comprising a homeodomain fused to a repressor domain, as defined above.

According to another preferred embodiment of the invention, said
5 homeoprotein is a homeoprotein of a vertebrate.

The homeoprotein, or related encoding nucleic acid sequence, for use according to the invention is capable of protecting cells *in vivo* from DNA damage, in particular DNA strand breaks, in particular DNA double strand breaks (DSBs), formation and chromatin remodeling, in particular chromatin relaxation, especially
10 those that are induced by stress factors or ageing. Stress factors refer in particular to oxidative or exocytotoxic stress.

The effect of the homeoprotein toward DNA damage is not restricted to neurons or a particular type of neuron (e.g. mesencephalic DA neurons or RGCs) and can thus apply to other type of cells, such as for example motoneurons, fibroblasts,
15 epithelial cells.

As used herein, the term "DNA-damage-related disorders" includes neurodegenerative disorders and diseases, such as cancer, aging and other disorders caused by damage to DNA due to exposure to oxidative stress, carcinogens, toxins, free radicals such as oxygen radicals, or DNA damaging radiation such as ionizing
20 radiation and UV radiation. Although an understanding of mechanism is not required for practice of the invention, it is believed that administration of homeoprotein according to the present Invention prevents DNA damage, inhibits the effects of DNA damage, reduces the number of DNA strand breaks and/or stimulates a cellular repair response to DNA damage.

25 According to special embodiment, DNA damage may take the form of chromosomal dysfunction such as DNA strand breaks, or chemical modification of DNA (e.g. alkylation). According to preferred embodiment, DNA damage means DNA

strand breaks, including single and double-strand breaks, preferably double-strand breaks.

According to preferred embodiment, the term "DNA-damage-related disorders" relates to neurodegenerative conditions including ataxias together with
5 Alzheimer's, amyotrophic lateral sclerosis, Huntington's and Parkinson's diseases. However, the important pathological feature is DNA damage. Thus, suppressing or limiting DNA damage and/or decreasing the amount of DNA strand breaks provides a method for decreasing neuronal cell death and/or treating /preventing neurological disorders associated to DNA damages.

10 Therefore according to special embodiment, the present Invention relates to the use of at least one homeoprotein, or peptide derivatives thereof, or a recombinant vector encoding said protein/peptide, for the production of a medicament for the suppression of DNA damage in neuronal cells and the treatment of neurological disorders associated to DNA damages. It further relates to said
15 method of treatment of neurological disorders associated with DNA damage.

According to another special embodiment of the invention, said degenerative disease is a neurodegenerative disease, in particular Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, Huntington disease, and degenerative diseases affecting the eyes or ears (*i.e.*, the vision or audition) such as glaucoma, for example.
20 In a preferred embodiment, said neurodegenerative disorder is Parkinson disease.

According to another special embodiment of the invention, said degenerative disease is not associated or does not result from a loss of any specific type of neuron or even of neurons. For example, said degenerative disease may be selected from the group consisting of muscular dystrophies or progeria.

25 According to another preferred embodiment, the term "age-related diseases or disorders" include, for example, renal dysfunction, kyphosis, herniated intervertebral disc, frailty, hair loss, hearing loss, vision loss (blindness or impaired vision), muscle fatigue, skin conditions, skin nevi, diabetes, metabolic syndrome, and

sarcopenia. Vision loss refers to the absence of vision when a subject previously had vision. Various scales have been developed to describe the extent of vision and vision loss based on visual acuity. Age-related diseases and conditions also include dermatological conditions, for example without limitation, treating one or more of the following conditions: wrinkles, including superficial fine wrinkles; hyperpigmentation; scars; keloid; dermatitis; psoriasis; eczema (including seborrheic eczema); rosacea; vitiligo; ichthyosis vulgaris; dermatomyositis; and actinic keratosis.

According to another special embodiment, the present Invention relates to the use of at least one homeoprotein, or peptide derivatives thereof, for the production of medicine for decreasing the amount of DNA strand breaks in cells, more particularly in neuronal cells.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Engrailed protein and said DNA-damage related disease or disorder is Parkinson disease.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Engrailed protein and said DNA-damage related disease or disorder is amyotrophic lateral sclerosis disease.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Gbx2 protein and said DNA-damage related disease or disorder is Huntington disease.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a

medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Gbx2 protein and said DNA-damage related disease or disorder is Parkinson disease.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Lhx9 protein and said DNA-damage related disease or disorder is Huntington disease.

According to another special embodiment, the present Invention relates to the use of a homeoprotein, or peptide derivatives thereof, for the manufacture of a medicament for the treatment and/or prevention of DNA-damage related and age-related disease or disorder wherein said homeoprotein is Lhx9 protein and said DNA-damage related disease or disorder is Parkinson disease.

Alternatively, the present Invention relates to the use of a recombinant vector encoding the said homeoprotein instead of the protein or peptide itself. The term "vector" as used herein refers to a vehicle, preferably a nucleic acid molecule or a viral particle that contains elements necessary to allow delivery, propagation and/or expression of one or more nucleic acid molecule(s) within a host cell or organism. This term encompasses vectors for maintenance (cloning vectors) or vectors for expression in various host cells or organisms (expression vectors), extrachromosomal vectors (e.g. multicopy plasmids) or integration vectors (e.g. designed to integrate into the host cell genome and produce additional copies of the nucleic acid molecules when the host cell replicates) as well as shuttle vectors (e.g. functioning in both prokaryotic and/or eukaryotic hosts) and transfer vectors (e.g. for transferring nucleic acid molecule(s) in a viral genome). For the purpose of the invention, the vectors may be of naturally occurring genetic sources, synthetic or artificial, or some combination of natural and artificial genetic elements.

In the context of the invention, the term "vector" has/is to be understood broadly as including plasmid and viral vectors. A "plasmid vector" as used herein refers to a replicable DNA construct. Usually plasmid vectors contain selectable marker genes that allow host cells carrying the plasmid vector to be selected for or against in the presence of a corresponding selective drug. A variety of positive and negative selectable marker genes are known in the art. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be selected in the presence of the corresponding antibiotic. The term "viral vector" as used herein refers to a nucleic acid vector that includes at least one element of a virus genome and may be packaged into a viral particle or to a viral particle. The terms "virus", "virions", "viral particles" and "viral vector particle" are used interchangeably to refer to viral particles that are formed when the nucleic acid vector is transduced into an appropriate cell or cell line according to suitable conditions allowing the generation of infectious viral particles. In the context of the present invention, the term "viral vector" has/is to be understood broadly as including nucleic acid vector (e.g. DNA viral vector) as well as viral particles generated thereof. The term "infectious" refers to the ability of a viral vector to enter into and infect a host cell or organism. Viral vectors can be replication-competent or -selective (e.g. engineered to replicate better or selectively in specific host cells), or can be genetically disabled so as to be replication-defective or replication-impaired. Vectors which are appropriate in the context of the present invention, include, without limitation, bacteriophage, plasmid or cosmid vectors for expression in prokaryotic host cells such as bacteria (e.g. *E. coli*, *Bacillus subtilis* or *Listeria*); vectors for expression in yeast (e.g. *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Pichia pastoris*); baculovirus vectors for expression in insect cell systems (e.g. Sf 9 cells); viral and plasmid vectors for expression in plant cell systems (e.g. Ti plasmid, cauliflower mosaic virus CaMV; tobacco mosaic virus TMV); as well as viral and plasmid vectors for expression in higher eukaryotic cells or organisms. Typically, such vectors are commercially available (e.g. in Invitrogen, Stratagene, Amersham Biosciences, Promega, etc.) or available from depositary

institutions such as the American Type Culture Collection (ATCC, Rockville, Md.) or have been the subject of numerous publications describing their sequence, organization and methods of producing, allowing the artisan to apply them. Representative examples of suitable plasmid vectors include, without limitation, pREP4, pCEP4 (Invitrogen), pCI (Promega), pVAX (Invitrogen) and pgWiz (Gene Therapy System Inc). Representative examples of suitable viral vectors are generated from a variety of different viruses (e.g. retrovirus, adenovirus, adenovirus-associated virus (AAV), poxvirus, herpes virus, measles virus, foamy virus, alphavirus, vesicular stomatis virus, etc). As described above, the term "viral vector" encompasses vector DNA, genomic DNA as well as viral particles generated thereof. The present invention also encompasses vectors (e.g. plasmid DNA) complexed to lipids or polymers to form particulate structures such as liposomes, lipoplexes or nanoparticles. In accordance with the present invention, the nucleic acid molecule(s) comprised in the vector of the invention is in a form suitable for expression in a host cell or organism, which means that the nucleic acid molecule is placed under the control of appropriate regulatory sequences. As used herein, the term "regulatory elements" refers to any element that allows, contributes or modulates the expression of a nucleic acid molecule in a given host cell or organism, including replication, duplication, transcription, splicing, translation, stability and/or transport of the nucleic acid or its derivative (i.e. mRNA). It will be appreciated by those skilled in the art that the choice of the regulatory sequences can depend on such factors as the vector itself, the host cell, the level of expression desired, etc. Promoters suitable for constitutive expression in mammalian cells include but are not limited to the cytomegalovirus (CMV) immediate early promoter, the RSV promoter, the adenovirus major late promoter, the phosphoglycero kinase (PGK) promoter, the thymidine kinase (TK) promoter of herpes simplex virus (HSV)-1 and the T7 polymerase promoter. Those skilled in the art will appreciate that the regulatory elements controlling the expression of the nucleic acid molecule of the invention may further comprise additional elements for proper initiation, regulation and/or termination of transcription (e.g. polyA transcription termination sequences), mRNA

transport (e.g. nuclear localization signal sequences), processing (e.g. splicing signals), and stability (e.g. introns and non-coding 5' and 3' sequences), translation (e.g. an initiator Met, tripartite leader sequences, IRES ribosome binding sites, Shine-Dalgarno sequences, etc.) into the host cell or organism and purification steps (e.g. a tag).

In another aspect, the invention provides a method for treating DNA-damage-related disorder in a subject, the method comprising administering to a subject in need a composition comprising at least one homeoprotein, or peptide derivatives thereof, or a recombinant vector encoding said protein, and pharmaceutically acceptable vehicle.

As used herein, a "pharmaceutically acceptable vehicle" is intended to include any and all carriers, solvents, diluents, excipients, adjuvants, dispersion media, coatings, antibacterial and antifungal agents, and absorption delaying agents, and similar, compatible with pharmaceutical administration.

The composition of the invention is suitably buffered in order to be appropriate for human use at a physiological or slightly basic pH (e.g. from approximately pH 7 to approximately pH 9). Suitable buffers include without limitation phosphate buffer (e.g. PBS), bicarbonate buffer, HEPES and PIPES buffers and/or Tris buffer. The composition of the invention can further comprise a diluent appropriate for human or animal use. It is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength. Representative examples include sterile water, physiological saline (e.g. sodium chloride), Ringer's solution, glucose, trehalose or saccharose solutions, Hank's solution, and other aqueous physiologically balanced salt solutions (see for example the most current edition of Remington: The Science and Practice of Pharmacy, A. Gennaro, Lippincott, Williams & Wilkins). Pharmaceutically acceptable vehicles included in the composition of the invention must also allow the preservation of its stability under the conditions of manufacture and long-term storage (i.e. at least one month with a preference for at least one year) at freezing (e.g. -70 deg. C., -20 deg. C.), refrigerated (e.g. 4 deg. C.) or ambient

temperatures. Additional pharmaceutically acceptable excipients may be used for providing desirable properties, including for example modifying or maintaining the pH, osmolarity, viscosity, clarity, colour, sterility, stability, rate of dissolution of the formulation, modifying or maintaining release or absorption into an the human or
5 animal organism, promoting transport across the blood barrier or penetration in a particular organ (e.g. brain).

A composition comprising any one of the homeoproteins described herein may be formulated for sustained or slow release (also called timed release or controlled release). Such compositions may generally be prepared using well-known
10 technology and administered by, for example, oral, rectal, intradermal, intranasal, or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain the compound dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane.

The composition may advantageously comprise at least another
15 pharmaceutical drug, such as for example: growth factor, complex sugar (glycosaminoglycans, polysialic acid), sirtuin or sirtuin activators, anticholinesterase, or reverse transcriptase inhibitors, in particular nucleosidic transcriptase inhibitors

Alternatively, the present Invention concerns a method for treating or preventing DNA-damage related disorder in a subject treated for cancer.

20 The present Invention further concerns a method for treating or preventing DNA-damage response pathways or chromatin changes or alterations.

The present Invention further concerns a method for treating or preventing DNA-damage in response to exposure to sunlight or exposure to DNA damaging chemicals. Said method according to the Invention may reduce skin aging, prevent
25 skin cancer and improve cosmetic appearance.

The present Invention further concerns a method for preventing or reducing the effects of cellular aging in a subject in need thereof, comprising administering to a subject in need thereof an effective amount of at least one homeoprotein, or

peptide derivatives thereof or a recombinant vector encoding said protein. Homeoprotein according to the present Invention may also be useful for treating or preventing (i.e. reducing the likelihood of occurrence) of "age-related diseases and disorders" or "aging" that occurs as part of the natural aging process or that occurs
5 when the subject is exposed to inducing agents or factors (e.g., irradiation, chemotherapy, smoking tobacco, high-fat/high sugar diet, other environmental factors).

As understood by a person skilled in the medical art, the terms, "treat" and "treatment," refer to medical management of a disease, disorder, or condition of a
10 subject (i.e., patient). In general, an appropriate dose and treatment regimen provide the homeoprotein in an amount sufficient to provide therapeutic and/or prophylactic benefit. Therapeutic benefit for subjects to whom the homeoprotein described herein are administered, includes, for example, an improved clinical outcome, wherein the object is to prevent or slow or retard an undesired
15 physiological change associated with the disease, or to prevent or slow or retard the expansion or severity of such disease. As discussed herein, effectiveness of the one or more homeoprotein(s) may include beneficial or desired clinical results that comprise, but are not limited to, abatement, lessening, or alleviation of symptoms that result from or are associated with the disease to be treated; decreased
20 occurrence of symptoms; improved quality of life; longer disease-free status (i.e., decreasing the likelihood or the propensity that a subject will present symptoms on the basis of which a diagnosis of a disease is made); reduction of disease extent; stabilized (i.e., not worsening) state of disease; delay or slowing of disease progression; amelioration or palliation of the disease state; and remission (whether
25 partial or total), whether detectable or undetectable; and/or overall survival. The effectiveness of the homeoprotein described herein may also mean prolonging cell survival, more particularly of neurons, when compared to expected cell survival if a subject were not receiving the homeoprotein according to the Invention.

Subjects in need of treatment include those who already have the disease or disorder as well as subjects prone to have or be at risk of developing the disease or disorder, and those in which the disease, condition, or disorder is to be treated prophylactically. A subject may have predisposition for developing a disease or disorder that would benefit from the method of the Invention or may be of a certain age wherein receiving the method of the Invention would provide clinical benefit delaying development or reducing severity of a disease, including DNA damage-related disease or disorder and/or an age-related disease or disorder.

The dose of homeoprotein according to the Invention for treating DNA damage-related disease or disorder and/or an age-related disease or disorder may depend upon the subject's condition, that is, stage of the disease, severity of symptoms caused by the disease, general health status, as well as age, gender, and weight, and other factors apparent to a person skilled in the medical art. Pharmaceutical compositions may be administered in a manner appropriate to the disease to be treated as determined by persons skilled in the medical arts. In addition to the factors described herein and above related to use of homeoprotein for treating DNA damage-related disease or disorder and/or an age-related disease or disorder, suitable duration and frequency of administration of the homeoprotein may also be determined or adjusted by such factors as the condition of the patient, the type and severity of the patient's disease, the particular form of the active ingredient, and the method of administration. Optimal doses of the homeoprotein may generally be determined using experimental models and/or clinical trials. The optimal dose may depend upon the body mass, weight, or blood volume of the subject. The use of the minimum dose that is sufficient to provide effective therapy is usually preferred. Design and execution of pre-clinical and clinical studies for a homeoprotein (including time of administration for prophylactic benefit) described herein are well within the skill of a person skilled in the relevant art. When two or more homeoproteins are administered, the optimal dose of each homeoprotein may be different, such as less, than when either agent is administered alone as a single agent therapy. Typically, said homeoprotein may be used at concentrations ranging

from 0.001 to 100 μM , or more preferable from 0.005 to 100 μM , advantageously from 0.005 to 10 μM , and particularly advantageously from 0.001 to 0.10 μM . An amount of homeoprotein that may be administered per day may be, for example, between about 0.01 mg/kg and 100 mg/kg (e.g., between about 0.1 to 1 mg/kg, 5 between about 1 to 10 mg/kg, between about 10-50 mg/kg, between about 50-100 mg/kg body weight). In other embodiments, the amount of an homeoprotein that may be administered per day is between about 0.01 mg/kg and 1000 mg/kg, between about 100- 500 mg/kg, or between about 500-1000 mg/kg body weight. For intracerebral injection, the homeoprotein can be administered at at a dose 10 comprised between 10 pmol and 100 pmol, for example at 25 pmol especially for Engrailed, as described in Rekaik *et al.*, (2015, Cell Reports). In another embodiment, the homeoprotein can be administered by intraocular injection, especially for Otx2.

The optimal dose (per day or per course of treatment) may be different for the DNA damage–related disease or disorder and/or an age-related disease or 15 disorder to be treated and may also vary with the route of administration and/or therapeutic regimen.

The pharmaceutical composition is formulated for administration by a number of routes, including but not limited to oral, parenteral and local. The pharmaceutically acceptable carriers are those conventionally used.

20 A pharmaceutical composition may be delivered to a subject in need thereof by any one of several routes known to a person skilled in the art. By way of non-limiting example, the composition may be delivered orally, intravenously, intraperitoneally, by infusion (e.g., a bolus infusion), subcutaneously, enteral, rectal, intranasal, by inhalation, buccal, sublingual, intramuscular, transdermal, intradermal, 25 topically, intraocular, vaginal, rectal, or by intracranial injection, or any combination thereof. In certain particular embodiments, administration of a dose, as described above, is via intravenous, intraperitoneal, directly into the target tissue or organ, or subcutaneous route. In certain embodiments, a delivery method includes drug-

coated or -permeated stents for which the drug is the homeoprotein or nucleic acid encoding it.

For the treatment of neurodegenerative diseases, the homeoprotein may be administered locally, in particular by injection or infusion into the targeted cerebral area. It can also be administered using a controlled-release device, for example an osmotic minipump connected to a canula implanted in the brain.

According to a preferred embodiment of the invention, the homeoprotein is directly administered into the SNpc region, especially for treatment of Parkinson disease. In a particularly preferred embodiment, the homeoprotein administered through this procedure is Engrailed. In a particularly preferred embodiment, a single injection of Engrailed is administered to an individual in need thereof.

Thus, in a particular embodiment, the invention concerns the use of Engrailed as a medicament for the treatment of Parkinson disease wherein Engrailed is administered once in the SNpc region of a subject in need thereof.

In addition, the administration of the homeoprotein may be combined with a non-pharmacological therapy. For example, it can be combined with subthalamic nucleus stimulation, for treating Parkinson Disease.

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication or entry were specifically and individually indicated to be incorporated by reference.

In addition to the above arrangements, the invention also comprises other arrangements, which will emerge from the description, which refers to exemplary embodiments, with reference to the Figures in which:

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, 5 10 μ m.
- (C) The percentage of TH⁺ neurons with >2 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 counted in each condition.
- 10 (D) Perinucleolar and perinuclear H3K27me3 staining is decreased in the *En1*^{+/-} SNpc mDA neurons. Scale bar, 10 μ m.
- (E) H3K27me3 perinucleolar staining is quantified by measuring fluorescence intensity (left) along the dotted lines. The percentage of cells with dense staining drops (right) in *En1*^{+/-} mice (n = 3; Student's t test; 129 and 159 neurons counted in 15 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 20 shift towards smaller DAPI-dense areas in *En1*^{+/-} mice (n=162-211; Kolmogorov-Smirnov test; 3 wt and *En1*^{+/-} mice analyzed). See also **Figure S1** and **Table S1**.

Figure 2. DNA damage and chromatin alteration in SNpc TH⁺ neurons upon 6-OHDA injection

- (A,B) 6-OHDA injected in the SNpc of wt mice leads 6h later to the appearance of γ -H2AX foci in about 25% of TH⁺ neurons (n = 3-6; Student's t test). *En1*^{+/-} mice are 25 more sensitive with 50% of neurons showing multiple γ -H2AX foci (n = 3-6; Student's t test; 130, 210 and 146 neurons counted for each condition, respectively). Scale bar, 10 μ m. 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 1534 and 2034 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
5 upon 6-OHDA injection. Mice were analyzed 6h post injection. Scale bar, 10 μ m.

(D) The percentage of TH+ neurons displaying dense H3K27me3 perinucleolar and perinuclear staining quantified as in **Figures 1E,F** drops dramatically (left) in 6-OHDA-injected mice (n = 3; Student's t test; 148 and 91 neurons counted in sham and 6-OHDA conditions, respectively). Perinuclear H3K27me3 staining in TH+ neurons also
10 decreases upon 6-OHDA injection (right) (n = 3; Student's t test; 40 and 47 neurons 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 is lost 6 h after 6-OHDA-injection. NCL, nucleolin. Scale bar, 10 μ m.

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

20 (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 μ m. Higher magnification images of dotted square areas are shown in the right most panels. Scale bar, 10 μ m. The percentage of TH+
25 neurons with nucleolar nucleolin is significantly decreased in *En1*^{+/-} mice (n = 3; Student's t test; 99 and 87 neurons counted in wt and *En1*^{+/-} mice, respectively). NCL, nucleolin. See also **Figure S2**.

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 24h. Compared to sham, En2 injection prevents the 6-OHDA-induced TH cell loss in the ipsilateral SNpc. Scale bar, 500 μ m.
- (B) Protective effect of En2 assessed by the ratio of TH+ cell counts in ipsilateral versus contralateral SNpc 6h, 24h and 7 days post-injection (n = 3-5; Student's t test).
5 The number of neurons counted ranged between 1670 and 2275 per condition.
- (C) The rescue of TH+ neurons by En2 in 6-OHDA-injected mice is paralleled by a disappearance of cyclin A at 24h. Scale bar, 100 μ m.
- (D) Immunostaining for H3K27me3 shows a progressive recovery following En2
10 injection. Recovery of nucleolar nucleolin is almost complete at 24h. The percentage of TH+ neurons with γ -H2AX foci returns to normal between 24h and 7 days after injection. For each analysis, n = 3. One way ANOVA followed by Dunetts's test (vs 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
15 6-OHDA/sham and 6-OHDA/En2 mice analyzed 6h 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 analyzed 24h later. Protective effect was
20 assessed as for En2 injections (n = 3; Student's t test). Number of neurons counted ranged between 1268 and 1495 for each condition. See also **Figure S3**.

Figure 4. RNA-seq analysis reveals Engrailed anti-apoptotic activity

- (A,B) Differentially expressed genes in the SNpc of 6-OHDA versus 6-OHDA/En2 related to DNA damage, chromatin remodeling, apoptosis and cell cycle are ranked
25 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; Students t test).
- (D) Left panel: *Gadd45b* and *Pml* transcripts were measured by qRT-PCR in the SNpc of 6-OHDA and 6-OHDA/En2 at 6h (injection with cycloheximide, CHX; n = 5;

Student's t test). Right panel: *Gadd45b* transcripts in the SNpc of 8 week-old wt and *En1*^{+/-} mice (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. Scale bar, 50 μ m. Higher magnification
5 images of dotted squares are shown in the right most panels. Scale bar, 10 μ m.

(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 and 351 for each condition). See also
Figure S4.

10

Figure S1 (related to Figure 1). Altered genes expression in mDA neurons of *En1*^{+/-} mice

(A) SNpc from wt and *En1*^{+/-} mice was laser microdissected for RNA-seq analysis. Total number of reads and differentially expressed genes (p < 0.05) are indicated.
15 Decreased expression of *En1* in *En1*^{+/-} SNpc was confirmed by RNA-seq.

(B) RNA-seq data analysis (Pathway Studio) reveals a significant enrichment (p < 0.005) in Ontology gene subgroups involved in DNA damage and chromatin remodeling between wt and *En1*^{+/-} littermates. Analysis using Cell Process Pathways also showed differential expression of apoptosis-related genes.

20 (C) Analysis of RNA-seq data from *Engrailed* gain of function experiments by Pathway Studio. The gene subgroups enriched (p < 0.01) are indicated (n = 5).

(D) Differentially expressed transcription factors in RNA-seq analysis by Pathway Studio of wt and *En1*^{+/-} SNpc are ranked by p-value.

(E) Altered expression of a selection of genes related to DNA damage, chromatin
25 remodeling and apoptosis is confirmed by qRT-PCR using SNpc RNAs from 6 week-old wt and *En1*^{+/-} mice (n = 3-6; Student's t test).

(F) The percentage of TH+ neurons with >2 γ -H2AX foci in the SNpc increases from 5% in wt to 17% in 24 week-old *En1*^{+/-} mice (n = 3; Student's t test; 107 and 94 neurons were counted in wt and *En1*^{+/-} conditions, respectively).

(G) LINE-1 transcripts in the SNpc of *En1*^{+/-} mice analyzed by qRT-PCR increase,
5 compared to wt (n = 5; Student's t test).

(H) Activated caspase-3 positive TH+ neurons are detected in the SNpc of *En1*^{+/-} mice (8 week-old). Scale bar, 50 μ m.

Figure S2 (related to Figure 2). TH cell death and chromatin changes in 6-OHDA injected mice

10 (A) Western blot analysis shows a 50% decrease in TH protein levels at 6h in the 6-OHDA injected ipsilateral SNpc (n = 3).

(B) The levels of mRNAs for TH and *En1* are decreased (at 6h) by 70 and 50%, respectively in 6-OHDA injected SNpc (n = 6; Student's t test).

(C) Activated caspase-3 is detected at 6h in TH+ neurons in the 6-OHDA-injected side
15 but not in the contralateral side. Scale bar, 100 μ m.

(D) Triple immunostaining of midbrain sections for γ -H2AX, H3K9me3 and TH shows that H3K9me3 colocalization with DAPI in sham condition is lost in 6-OHDA-injected mice. Similarly, MeCP2 colocalization with DAPI in TH+ neurons of sham-injected mice is lost in 6-OHDA-injected mice. Scale bar, 10 μ m.

20 (E) The percentage of TH+ neurons with dense H3K9me3 or MeCP2 marks is significantly decreased in 6-OHDA-injected mice (n = 3; Student's t test). The numbers of neurons counted were 161 (sham) and 97 (6-OHDA) for H3K9me3 and 150 (sham) and 128 (6-OHDA) for MecP2.

(F) Loss of perinuclear lamin B2 staining in TH+ neurons in the SNpc of 6-OHDA-
25 injected mice. Scale bar, 20 μ m.

(G) Surface quantification of DAPI-dense regions in TH+ neurons in sham or 6-OHDA-injected mice. Compared to sham-injected controls, the distribution of the relative frequencies indicates a shift towards smaller DAPI-dense areas in TH+ neurons of 6-OHDA-injected mice (n = 137-177; Kolmogorov-Smirnov test; 3 mice per condition).

5 (H) *p53* and *p21* mRNAs were quantified by qRT-PCR using total RNA from the SNpc of 6-OHDA or sham injected mice (n = 6; Student's t test).

(I) Higher expression of repeat elements in 6-OHDA injected SNpc. The levels of LINE-1 transcripts analyzed by qRT-PCR using total SNpc RNA increase in 6-OHDA-injected mice. The level of LINE-1 RNA was also analyzed using RNAs from SNpc purified
10 nuclei (n = 3-5; Student's t test).

Figure S3 (related to Figures 2-3). Engrailed transcription activity involved in TH neuron protection

(A) Mice were unilaterally infused with sham or EnHD-VP64 in the SNpc and analyzed at 7 days. Immunostaining of midbrain sections for TH shows that EnHD-VP64
15 infusion (but not sham) provokes TH cell loss in the ipsilateral SNpc. Scale bar, 500 μm .

(B) EnHD-VP64 induced TH cell loss was assessed by comparing the ratio of TH+ cell counts in ipsilateral versus contralateral SNpc at 7 days post infusion (n = 6; Student's t test). The number of neurons counted ranged between 1902 and 2118 per
20 condition

(C) Quantification of γ -H2AX staining shows that the percentage of TH+ neurons with >2 γ -H2AX foci in the SNpc increases upon EnHD-VP64 infusion (n = 3; Student's t test; 138 and 129 neurons were counted in sham and EnHD-VP64 conditions, respectively).

25 (D) Analysis of RNA-seq data also reveals differentially expressed genes in Pathway Studio Ontology subgroups related to nucleolar organization and biogenesis in the SNpc of *En1*^{+/-} mice (p < 0.1).

(E) The level of pre-45S rRNA in the SNpc of wt and *En1*^{+/-} mice (8 week-old animals) was analyzed by qRT-PCR (n = 4; Student's t test).

(F) Engrailed promotes TH⁺ neurons survival in the 6-OHDA model. The survival effect of Engrailed was not observed if Engrailed was injected 24h after 6-OHDA injection (when the majority of TH⁺ neurons are lost) and recovery assessed 6h later (n = 3).

Figure S4 (related to Figure 4). Detection of cell cycle markers in TH⁺ neurons upon acute oxidative stress *in vivo* and Engrailed-mediated protection against H₂O₂-induced DSBs *in vitro*

10 (A) Detection of PCNA and p3 cell cycle markers in TH⁺ neurons in the 6-OHDA-injected side. Scale bar, 20 μm.

(B) Immunostaining of midbrain sections reveals TH⁺ neurons expressing cyclin A only in the ipsilateral side of 6-OHDA-injected mice. Mice were analyzed 6h post injection. Scale bar, 100 μm.

15 (C) Detection of activated caspase-3 in TH⁺ neurons staining positive for p3. Scale bar, 50 μm.

(D) The percentage of TH⁺ neurons expressing cyclin A upon 6-OHDA injection was quantified (left, n = 3; Student's t test) and confirmed by Western blot analysis (right, n = 4; Student's t test).

20 (E) Engrailed treatment protects midbrain neurons against H₂O₂-induced DSBs. Primary mesencephalic neuron cultures of E14.5 mouse embryos were treated with H₂O₂ at 5 μM after a 24h incubation with NaCl or En1 (15 nM). Cells were fixed 1h after H₂O₂ treatment, stained for γ-H2AX and the number of foci was quantified (n = 68-137; one way ANOVA followed by Tukey's multiple comparisons test). En2-
25 mediated protection against H₂O₂-induced DSBs using midbrain neuron cultures was assessed by the comet assay performed according to Trevigen CometAssay kit instructions and analyzed with OpenComet plugin, scale bar, 100 μm. The number of

neurons with a comet tail was quantified (n = 39-46; one way ANOVA followed by Tukey's multiple comparisons test).

(F) Differentially expressed genes in the SNpc of En2 versus sham injected mice. Genes related to DNA damage, chromatin remodeling, apoptosis are ranked by p-values.

Figure 5 : En1/GBX2 /LHX9

5.1, Dose response for Engrailed ; 5.2, Dose response for Gbx2 ; 5.3, Dose response for Lhx9 .

10 EXAMPLES

EXPERIMENTAL PROCEDURES

Animals

Mice were treated as per the guidelines for the care and use of laboratory animals (US National Institute of Health) 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 Science 269, 679-682) were maintained in conventional animal facility. Experimental groups consisted of 6 to 9 week-old mice.

In vivo treatments

20 For 6-OHDA injections, mice were placed in a stereotaxic instrument and a burr hole 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%) injections were performed over 4 min. This procedure targets the homeoprotein to the SNpc tissue. 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 J Neurosci 27, 1063-1071) 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

of the protein was used. Mice were killed at 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 5 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 μ l containing En-VP64 (400 nM, 0.9% NaCl or the equivalent volume of an empty-plasmid-containing bacterial extract) and colominic acid (1.5 μ g/ μ l).

Image quantification

10 Images were analyzed with ImageJ. For immunofluorescence, all quantifications were performed using 60X magnification and 0.7 μ m-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 15 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 20 expressed as means \pm SEM. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ in all experiments.

RNA-seq analysis

mDA neurons in the SNpc of wt and *En1*^{+/-} mice labeled using the quick TH-staining protocol (Chung et al., 2005, Hum Mol Genet 14, 1709-1725) were isolated by Laser 25 Capture Microdissection (LMD7000, Leica). Samples from 4 animals per group were pooled and total RNA was extracted using the AllPrep DNA/RNA Micro Kit (Qiagen) followed by DNase I using the RNeasy MinElute Cleanup protocol for on-column DNase I treatment. Construction of cDNA libraries (Ovation RNA-seq System V2) and

Illumina RNA-seq were performed by the Ecole Normale Supérieure Genomic platform (Paris). p-values of differentially expressed genes between wt and *En1+/-* samples were computed using DESeq package (Anders et al., 2010, Genome Biol 11, R106).. Normalized read counts were injected into the Gene Set Enrichment Analysis algorithm (Pathway Studio, Elsevier) to test for statistical enrichment. Pathway Studio Ontology and Cell Process Pathways collections were selected as Gene Set Categories for the analysis. RNA-seq analysis was also performed using RNA extracted from Laser Capture microdissected mDA neurons after Engrailed infusion (Alvarez-Fischer et al., 2011, Nat Neurosci 14, 1260-1266) in the SNpc of wt mice and from SNpc tissue punches collected 6h after 6-OHDA/sham and 6-OHDA/*En2* injection.

qRT-PCR

Total RNA from SNpc tissues was extracted using the RNeasy Lipid Tissue kit (Qiagen) followed by DNase I (Thermo) digestion. RNA (200 ng) was transcribed using the QuantiTect Reverse Transcription kit (Qiagen). qRT-PCR was performed using SYBR-Green (Roche Applied Science) and values normalized to *Gapdh* and/or *Hprt*. Data were analyzed using the ddCt method. In some experiments, RNA was isolated specifically from SNpc nuclei (Subcellular Protein Fractionation Kit, Thermo).

20 Supplementary Methods Table : List of primers used for qRT-PCR analysis

(refers to Experimental Procedures)

Gene	Forward primer	Reverse primer
<i>Casp9</i>	TGGCTCCTGGTACATCGAGA SEQ ID NO. 5	AGCATTGGCAACCCTGAGAA SEQ ID NO.6
<i>CcnA1</i>	GGGCCTGCTCAGGCTGT SEQ ID NO. 7	GTTCTCTGTGGGGGATCCTG SEQ ID NO. 8

<i>CcnA2</i>	CTCGCTGCATCAGGAAGACC SEQ ID NO. 9	TAAGAGGAGCAACCCGTCG SEQ ID NO. 10
<i>Cdk5</i>	GCCAGACTATAAGCCCTACCC SEQ ID NO. 11	GCTGCACAGGGTTACACTTC SEQ ID NO. 12
<i>En1</i>	CCGGTGGTCAAGACTGACTC SEQ ID NO. 13	CTGGTGCGTGGACCAGAG SEQ ID NO. 14
<i>En2</i>	CCTTCTTCAGGTCCCAGGTC SEQ ID NO. 15	AACTCAGCCTTGAGCCTCTG SEQ ID NO. 16
<i>Fen1</i>	ATCCTCTTCGCCGCCATT SEQ ID NO. 17	GAGGCATCGATGGCCACTTT SEQ ID NO. 18
<i>Gadd45b</i>	TCTCTAGAGGAACGCTGAGACC SEQ ID NO. 19	GTAGGGTAGCCTTTGAGGGATT SEQ ID NO. 20
<i>Hdac7</i>	CCCACCTGTCAGACCCAAGT SEQ ID NO. 21	AGTCATAGACCAGCCCTGTAGCA SEQ ID NO. 22
LINE-1 5'UTR	CTGGGAACTGCCAAAGCAAC SEQ ID NO. 23	CCTCCGTTTACCTTTGCGCA SEQ ID NO. 24
LINE-1 ORF2	AATCGACAAATGGGACCTAATGA SEQ ID NO. 25	GTAAAGATCCTTTCCAATCTGTTG SEQ ID NO. 26
<i>Nfkb2</i>	GGTGGAAGACAAGGAGGAAG SEQ ID NO. 27	AGGAGGAGAAAAAGCCGAG SEQ ID NO. 28
<i>p21 (Cdkn1a)</i>	GGCCCGGAACATCTCAGG SEQ ID NO. 29	AAATCTGTCAGGCTGGTCTGC SEQ ID NO. 30
<i>p53</i>	GCGTAAACGCTTCGAGATG SEQ ID NO. 31	CTTCAGGTAGCTGGAGTGAGC SEQ ID NO. 32
<i>Parp3</i>	GACCCAGCTTGAAGAGTCC	CTGCCCATCCAGTTCGAGTT

	SEQ ID NO. 33	SEQ ID NO. 34
<i>Phf1</i>	CTATCCGGATGTTTCGCTCC	GAGGTGACCTATCTGGGGGT
	SEQ ID NO. 35	SEQ ID NO. 36
<i>Pml</i>	ATAGCAGCAGTGAGTCCAGC	GCTGGCTAATTTTCTGGGTTTCA
	SEQ ID NO. 37	SEQ ID NO. 38
<i>Polk</i>	CACAGCACTTGCAGGAAAGG	CCTGTCTGGGTGTGTCGATT
	SEQ ID NO. 39	SEQ ID NO. 40
pre-45S rRNA	CGTGTAAGACATTCTATCTCG	GCCCGCTGGCAGAACGAGAAG
	SEQ ID NO. 41	SEQ ID NO. 42
<i>Rad1</i>	TACTGCTTAGTGGCCAGCCT	AGGCATTTGCTTGCACACAC
	SEQ ID NO. 43	SEQ ID NO. 44
Th	GTACTGGACAGTCCTCACACCA	GGTGGTACCCTATGCATTTAGC
	SEQ ID NO. 45	SEQ ID NO. 46
Uaca	AGAGGTCAAGAAGGGCAAGC	TGCCACTCAACGCTCTTTCT
	SEQ ID NO. 47	SEQ ID NO. 48
<i>Xpa</i>	GCGTGGCCAGTGTA AAAAGCA	CGCATTCTTCACAGATGGTGT
	SEQ ID NO. 49	SEQ ID NO. 50

Immunostaining

Mice were perfused with 4% paraformaldehyde in PBS, brains were postfixed for 1h and cryoprotected in 15% sucrose. Tissues embedded in Tissue-Tek O.C.T. (Sakura Finetek) were frozen isopentane prior to storage at -80°C . Brains were cut at the level of the SNpc into 18 μm -thick sections. For immunofluorescence, sections were permeabilized in 1% Triton X-100 in PBS for 20 min and incubated at 100°C for 20 min in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). After blocking for 1h (10% normal goat serum, 0,05% % Triton X-100 in PBS), tissues were incubated

overnight at 4°C with primary antibodies diluted in the blocking solution (mouse anti- γ -H2AX, 1:200, Millipore; chicken anti-TH, 1:500, Abcam; rabbit anti-activated-caspase3, 1:200, Abcam; rabbit anti-nucleolin, 1:200, Sigma; rabbit anti-fibrillarin, 1:200, Cell Signaling; rabbit anti-H3k27me3, 1:200, Millipore; rabbit anti-H3k9me3, 1:200, Abcam; a generous gift from Edith Heard; rabbit anti-Lamin B2, 1:100, Santa Cruz; rabbit anti-Mecp2, 1:300, Abcam; rabbit anti-PCNA, 1:200, Cell Signaling; rabbit anti-cyclin A, 1:200, Santa Cruz; mouse anti-pH3, 1:100, Cell Signaling; mouse anti-p-JNK, 1:200, Santa Cruz). Sections were incubated with appropriate secondary antibodies (488 anti-chicken, 647 anti-chicken, 488 anti-mouse, 546 anti-mouse and 546 anti-rabbit Alexa Fluor, Life Technologies) for 1h at room temperature. Labeled sections were imaged by confocal microscopy (SP5, Leica). For TH immunohistochemistry, sections were permeabilized in 1% Triton X-100 and incubated overnight at 4°C with 10% normal goat serum in PBS containing a rabbit polyclonal antibody against TH (1:1000; Pel-Freez Biologicals). Sections were treated with biotinylated secondary antibody and incubated with avidin-biotinylated horseradish peroxidase complex (ABC system, Vectastain). Peroxidase was revealed using DAB peroxidase (HRP) substrate kit (Vectastain) and imaged with a Nikon Eclipse i90 microscope.

20 RESULTS

Example 1 : mDA neuron gene expression in the SNpc of *En1*^{+/-} mice

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**). Analysis was performed on 6 week-old animals when all neurons are still present in the in *En1*^{+/-} mice. 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 factors 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 very less so DNA damage response and chromatin modifying ones (**Figure S1C**) that were thus further investigated in the context of this study. Quantitative RT-PCR (**Figure S1E**) confirmed that 6 week-old *En1*^{+/-} mice display altered expression of several genes related to DNA damage, chromatin remodeling and apoptosis.

10

Example 2 : DNA strand breaks and modified chromatin marks in *En1*^{+/-} mDA neurons

mDA neurons in the SNpc of *En1*^{+/-} mice (between 6-8 weeks of age) were examined for signs of DNA damage by following the DNA strand breaks (DSBs) marker γ -H2AX (Lobrich et al., 2010 Cell Cycle 9, 662-669). This revealed the presence of multiple γ -H2AX foci in about 16% of TH⁺ neurons in the SNpc (**Figures 1B,C**). Of note, DSBs do not necessary lead to rapid cell death as shown by the absence of significant death, in the *En1*^{+/-} mutant, between 24 and 48 weeks 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, 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 **Figure 1D-F**, the pattern of perinucleolar and perinuclear H3K27me3 (K27 trimethylated histone H3) staining is changed in a significant fraction of *En1*^{+/-} TH⁺ neurons. The size distribution of the DAPI-dense regions of heterochromatin (Guenatri et al., 2004 J Cell Biol 166, 493-505) also shows a reduction in the percentage of large spots in the *En1*^{+/-} mouse (**Figure 1G**). Heterochromatin changes are often associated with

changes in the expression of Long Interdispersed Nuclear Elements (LINEs) accordingly LINE expression is increased in the SNpc of *En1*^{+/-} mutants (**Figure S1G**). The induction of apoptosis genes (**Figure 1A**) is confirmed by the TH/activated caspase-3 co-staining in the SNpc of *En1*^{+/-} mice, never seen in wt littermates (**Figure**
5 **S1H**).

Example 3 : 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
10 SNpc. This induced, within 6h, the loss of 35% of the TH⁺ neurons and the formation of multiple abnormal γ -H2AX foci in about 26% of the remaining ones (**Figure 2A,B**), only in the ipsilateral SNpc. Neuronal loss was paralleled by a reduction in TH protein and mRNA (*En1* mRNA also) (**Figures S2A,B**) and the presence of numerous activated caspase-3-positive mDA neurons (**Figure S2C**). Injecting 6-OHDA in *En1*^{+/-} mice led to
15 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%) (**Figure 2A, B**). EnHD-VP64 infusion indeed leads to mDA cell death (**Figure S3A,B**) and to an increase in the number of γ -H2AX foci (**Figures S3C**).

20 Example 4 : Oxidative stress induces changes in heterochromatin marks

Heterochromatin marks are also modified 6h after 6-OHDA injection in wt mice. The dense perinucleolar and perinuclear H3K27me3 staining in TH⁺ neurons of sham-injected mice is changed into diffuse nucleoplasmic staining in 6-OHDA-injected mice (**Figure 2C**). This change was quantified in the nucleolus by measuring perinucleolar
25 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 (**Figure**

S2D,E), 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.

Nucleolar stress, suggested by perinucleolar γ -H2AX loss upon 6-OHDA (**Figure 2A**), is
5 verified by the drop from 70% (sham) to 30% (6-OHDA) of mDA cells with dense nucleolin staining (**Figure 2E,F**). This change was accompanied by a strong up-regulation of ribosomal pre-45S RNA (**Figure 2G**) signing nucleolar damage. In comparison, nucleolin staining, normal in *En1*^{+/-} TH⁺ neurons at 6 weeks, shows signs of disruption at one year (**Figure 2H**). However, the expression of genes
10 involved in nucleolus organization (RNA-seq) and qRT-PCR analysis of pre-45S rRNA suggest a change in nucleolar physiology in 6-8 week-old *En1*^{+/-} mice (**Figure S3D,E**).

Example 5 : Engrailed decreases the number of DSBs induced by H₂O₂

15 To verify if Engrailed is able to regulate DNA damage induced by 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 6h, 24h or 7 days post-injections. The dramatic loss at 24h of TH⁺ cells in the SNpc of 6-OHDA/sham injected is highly reduced in 6-OHDA/En2 injected mice (**Figure 3A**). Protection is still visible at 7 days
20 with 40% and 20% of surviving neurons in En2-injected and sham mice, respectively (**Figure 3B**). En2 injection 24h (instead of 30 min) after 6-OHDA and analyzed 6h later showed no recovery, showing that TH staining corresponds to true survival and not to TH re-expression (**Figure S3F**).

6-OHDA-induced apoptosis is paralleled by the expression of cell cycle markers
25 (**Figure S4A-D**). Accordingly, Cyclin A expression detected in En2-treated TH⁺ cells 6h post injections had almost disappeared at 24h (**Figure 3C**). Neuronal rescue by En2 is associated with the reappearance of normal staining patterns for H3K27me3, nucleolin and γ -H2AX (**Figure 4D**). The percentage of TH⁺ cells with "wild-type"

perinucleolar H3K27me3 staining increased from 20% to 37% at 6h and reached 60% and 80% at 24h and 7 days, respectively. Wild-type nucleolin pattern took longer to reappear as recovery was observed only at 24h with little changes between 24h and one week. Finally the decrease in the number of γ -H2AX foci was slower with full
5 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 6h after En2 injection (**Figure 3E**). The expression of selected genes related to cell cycle and apoptosis, up-regulated in the *En1*^{+/-} mouse or upon 6-OHDA injection, was repressed in the SNpc of 6-OHDA/En2 mice (**Figure**
10 **3E**). Analyses were done at 6h when sham and En2-injected animals still have similar numbers of mDA neurons (**Figure 3B**).

E14.5 midbrain neurons expressing Engrailed but of which only 1-2% are dopaminergic were exposed to H₂O₂ with or without En2. **Figure S4E** illustrates that En2 decreases the number of DSBs induced by H₂O₂ in parallel with a decrease in the
15 formation of comet tails that sign DNA damage.

Example 6 : Engrailed activates short-and long-term survival pathways

Protection by En2 requires its injection before 24h (**Figure S3F**). To identify genes in this early survival pathway, SNpc RNA from 6-OHDA/sham or 6-OHDA/En2 mice (6h)
20 was sequenced and genes in the chromatin remodeling, DNA damage, apoptosis and cell cycle pathways were analyzed according to Pathways Studio. **Figure 4A,B** ranks 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 4 analyzed pathways. Genes in the apoptosis pathway
25 are more represented than in the *En1*^{+/-} mouse (**Figure 1**). This is not due to En2 injection per se (**Figure S4F**), suggesting that the rapid up-regulation 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 demonstrates that *Gadd45b*, as opposed to *Pml* for control, does 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
5 *NF- κ B* suggested a role for c-Jun N-terminal kinase (JNK) signaling, a pathway implied in several neurodegenerative diseases, including PD (Coffey, 2014, Nat Rev Neurosci 15, 285-299). **Figure 4E,F** confirms that the strong increase in p-JNK staining 6h after 6-OHDA is antagonized by En2 (**Figure 4F**).

10 **Example 7 : Engrailed as well as two other homeoproteins, Gbx2 and Lhx9, decrease the number of DSBs induced by H₂O₂**

Given the high structural and functional conservation of homeoproteins, the Inventors have expected that other homeoproteins would have effects similar to that of Engrailed. They thus have compared effect on DNA damage induced by H₂O₂ of
15 two other homeoproteins, Gbx2 (Lin et al., 1996, Genomics, 3, 335-342) and Lhx9 (Rétaux et al., 1999, J. of Neuroscience, 19(2), 783-793) compared to Engrailed.

E14.5 midbrain cells were dissociated by trypsin and cultured for 6 days in Neurobasal medium supplemented with glutamine, aspartate, glutamate and antibiotic/antimycotic mix on poly-D-lysine/laminin glass coverslips. Cells were then
20 treated with the homeoprotein at the indicated concentration for 24h and then challenged for 1h with 100 uM H₂O₂ and fixed in 4% paraformaldehyde. DNA breaks were visualized with γ H2AX immunofluorescence. Each data point in the graphs represents the number of medium to large foci in the nucleus of a cell. 5-10 cells per coverslip and there were 4 coverslips per condition. Cells without foci were not
25 included.

Differences between the groups were evaluated using the nonparametric Kruskal-Wallis followed by Dunn's Multiple Comparison Test.

7.1. Dose response for Engrailed 1 (figure 5.1)

Treatment with 100 μ M H₂O₂ significantly increased the number of foci from 1.9 to 8.3/cell ($p < 0.01$). Pretreatment with En1 reduced the number of foci in a dose-dependent. 1 ng/ml (0.25 nM) partially but significantly ($p < 0.01$) reduced the number of foci caused by H₂O₂. 5 ng/ml (1.25 nM) and 20 ng/ml (0.5 nM) reduced the number of foci even further ($p < 0.01$) but were still greater than vehicle treated control condition ($p < 0.01$). 100 ng/ml (2.5 nM) was indistinguishable from control treated cells and thus completely prevented DNA breaks caused by H₂O₂.

7.2. Dose response for Gbx2 (Figure 5.2)

10 Treatment with H₂O₂ significantly doubled the number γ H2AX foci compared to vehicle treated control ($p < 0.01$). 1 ng/ml (0.027 nM) partially but significantly reduced the number of foci ($p < 0.01$). 20 ng/ml (0.54 nM) reduced somewhat further the number of foci and 100 ng/ml (2.68 nM) blocked the effects of H₂O₂.

7.3. Dose response for Lhx9 (Figure 5.3)

15 Treatment with H₂O₂ significantly doubled the number γ H2AX foci compared to vehicle treated control ($p < 0.01$). 5 ng/ml (0.022 nM) significantly reduced the number of foci and this reduction was even greater but not complete at 20 ng/ml (0.54 nM). 100 ng/ml (2.68 nM) completely blocked the effects of H₂O₂.

20 Conclusion

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 inflicted 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. These results support the fact that Engrailed is not able only to prevent the damage of dopaminergic neurons present in dopaminergic neurons of the substantia nigra but also to rescue these neurons when damaged by oxidative stress.

High levels of reactive oxygen species (ROS) are toxic, in particular at the DNA level where they directly induce DDR. In consequence, neurons with high metabolic activity, such as SNpc mDA neurons, producing high amounts of ATP and ROS, are at risk for degeneration. Chromatin remodeling and DDR pathways are interconnected as DNA damage induces chromatin changes, themselves necessary to give access to the DNA repair machinery. 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, Engrailed dosage is important in the adult SNpc. Indeed, given that En1 and En2 are biochemically equivalent, 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, manifests 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. 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 in PD patients or models. For example, the loss of MeCP2 in SNpc mDA neurons compromises the

nigrostriatal dopaminergic pathway; JNK and cyclin pathways are implied in PD and nucleolin diffusion was reported in PD patients.

In a previous study, it was shown that Engrailed protects 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, in the previous study, Engrailed was infused in the SNpc 3 weeks before the striatal insult and not injected 30 min after 6-OHDA injection. Finally, the present 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 activity is very important as it suggests that Engrailed may have a long-lasting effect on mDA neuron survival. Further, the present study, contrary to the previous one, shows that Engrailed can be useful to rescue the dopaminergic, not only to prevent their oxidative stress-induced injury.

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; Stettler *et al.*, 2012) and, in particular but not only, the transcription of anti-apoptotic genes (this study) and the repression of apoptosis as already proposed (Alberi *et al.*, 2004; Beltran *et al.*, 2014), with a predominance, seemingly, of GADD45b/g, NF- κ B, and JNK pathways. We propose that long-lasting effects relate to transcriptional and epigenetic mechanisms allowing both chromatin restructuring 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.

CLAIMS

1. An homeoprotein, or peptide derivatives thereof, for use in the treatment and/or prevention of neurodegenerative disorders.
2. The homeoprotein, or peptide derivatives thereof, according to
5 claim 1 for use as defined in claim 1, wherein said homeoprotein is selected in the group consisting of Engrailed protein, Gbx2 and Lhx9.
3. The homeoprotein, or peptide derivatives thereof, according to claim 1 or 2 for use as defined in claim 1 or 2, wherein said neurodegenerative
10 disease is selected from the group consisting of Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, Huntington disease, amyotrophic lateral sclerosis, and degenerative diseases affecting the eyes or ears.
4. The homeoprotein, or peptide derivatives thereof, according to claim 3 for use as defined in claim 3, wherein said neurodegenerative disorder is Parkinson disease.
- 15 5. The homeoprotein, or peptide derivatives thereof, according to any one of claims 1 to 4 for use as defined in any one of claims 1 to 4, wherein said homeoprotein is Engrailed protein.
6. The homeoprotein, or peptide derivatives thereof, according to claim 5 for use as defined claim 5 wherein said Engrailed protein is administered
20 directly into the SNpc region.
7. The homeoprotein, or peptide derivatives thereof, according to any one of claims 1-5 for use as defined in any one of claims 1- 5 wherein said homeoprotein, or peptide derivatives thereof is administered once.
8. The homeoprotein, or peptide derivatives thereof, according to
25 claim 1 or 2 for use as defined in claim 1 or 2, wherein said homeoprotein is Engrailed protein and said DNA-damage related disease or disorder is amyotrophic lateral sclerosis.
9. The homeoprotein, or peptide derivatives thereof, according to claim 1 or 2 for use as defined in claim 1 or 2, wherein said homeoprotein is Gbx2
30 protein and said DNA-damage related disease or disorder is Huntington disease.

10. The homeoprotein, or peptide derivatives thereof, according to claim 1 or 2 for use as defined in claim 1 or 2, wherein said homeoprotein is Lhx9 protein and said DNA-damage related disease or disorder is Huntington disease.

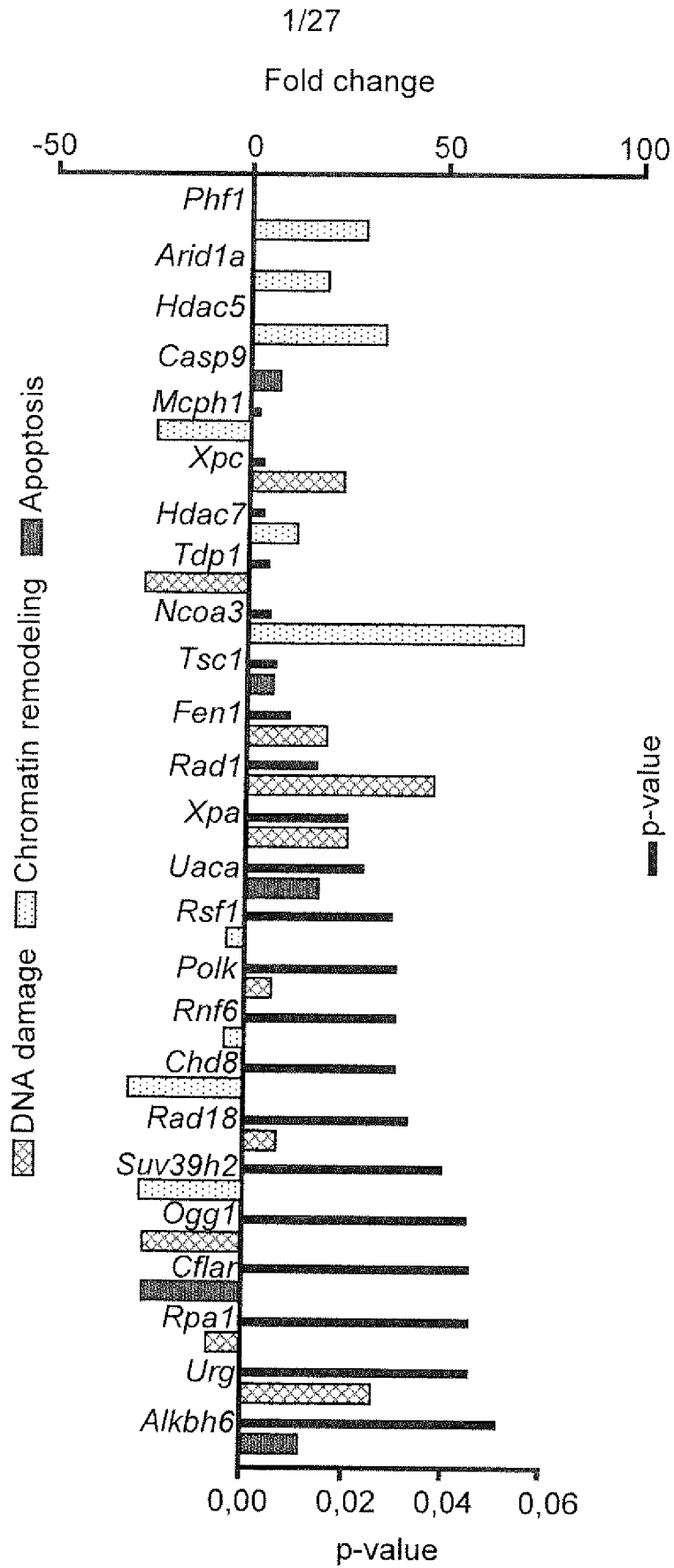


Fig. 1A

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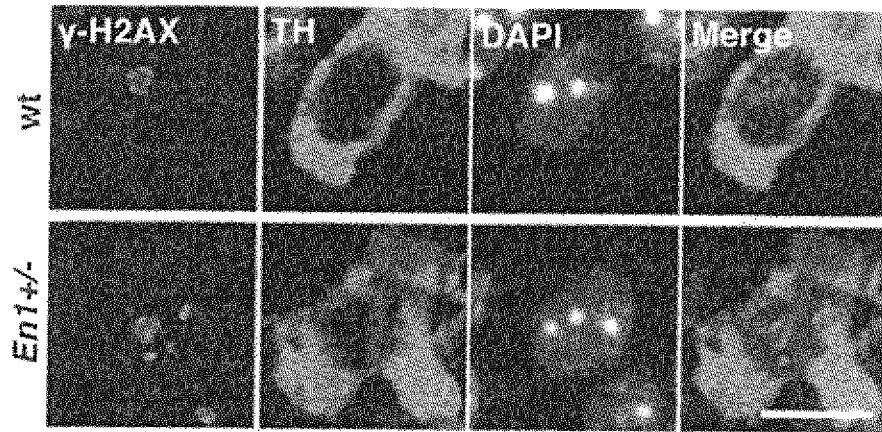


Fig. 1B

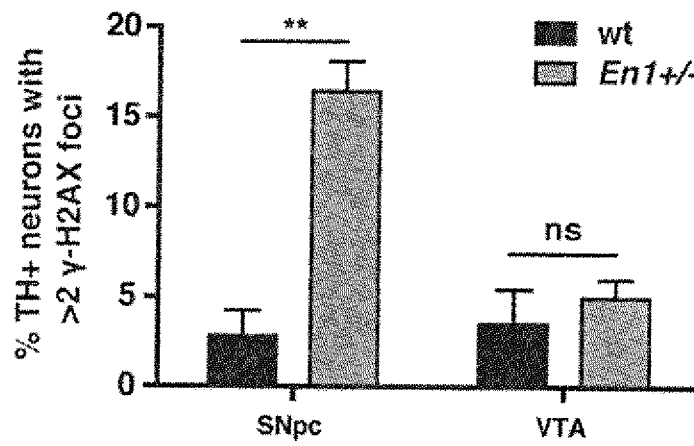


Fig. 1C

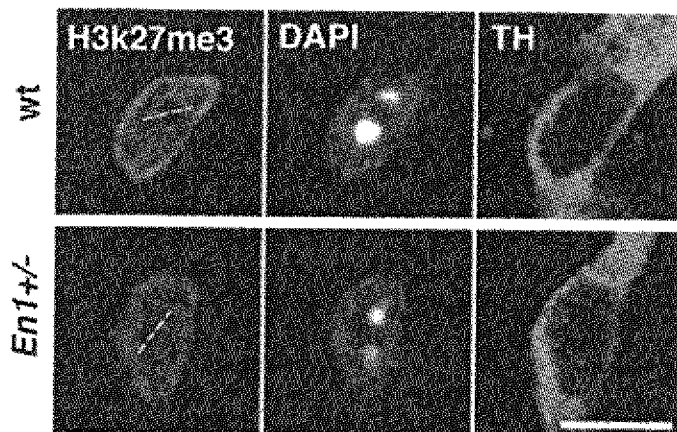


Fig. 1D

3/27

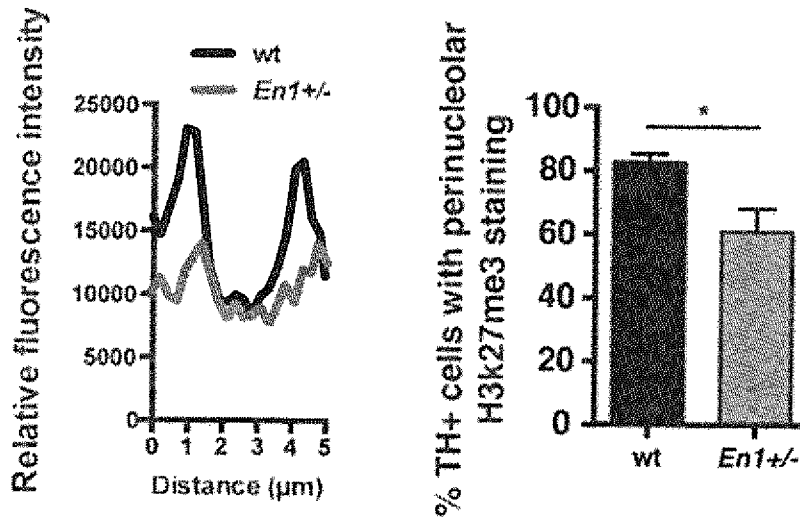


Fig. 1E

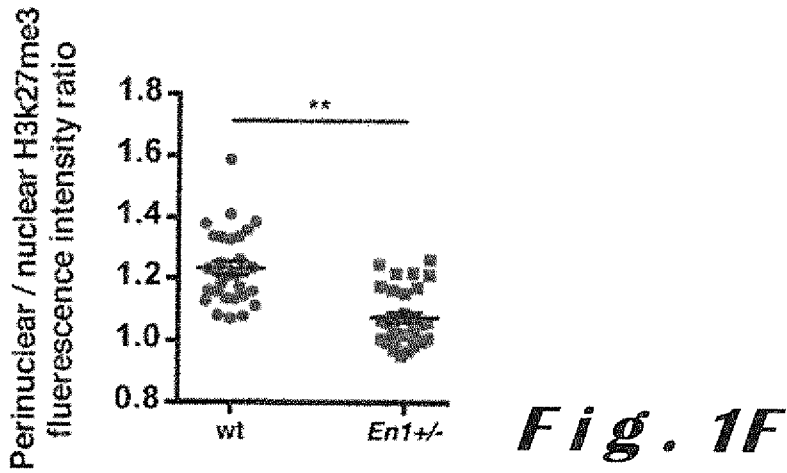


Fig. 1F

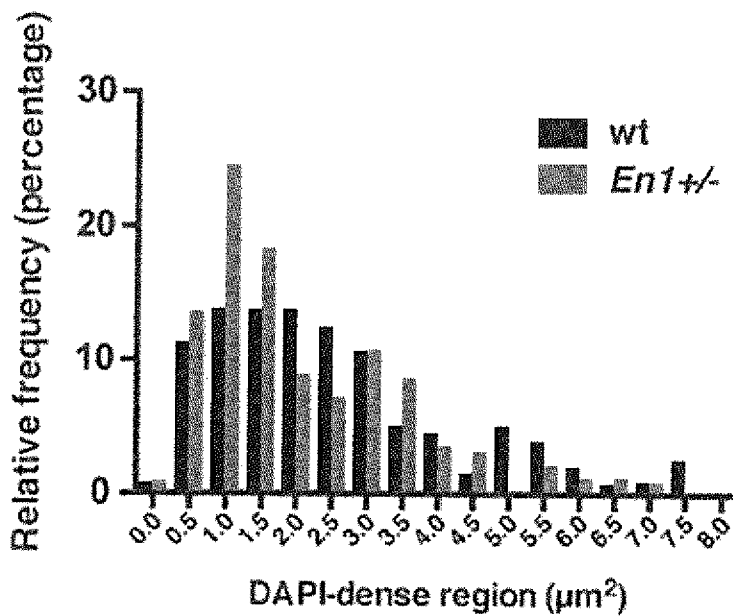


Fig. 1G

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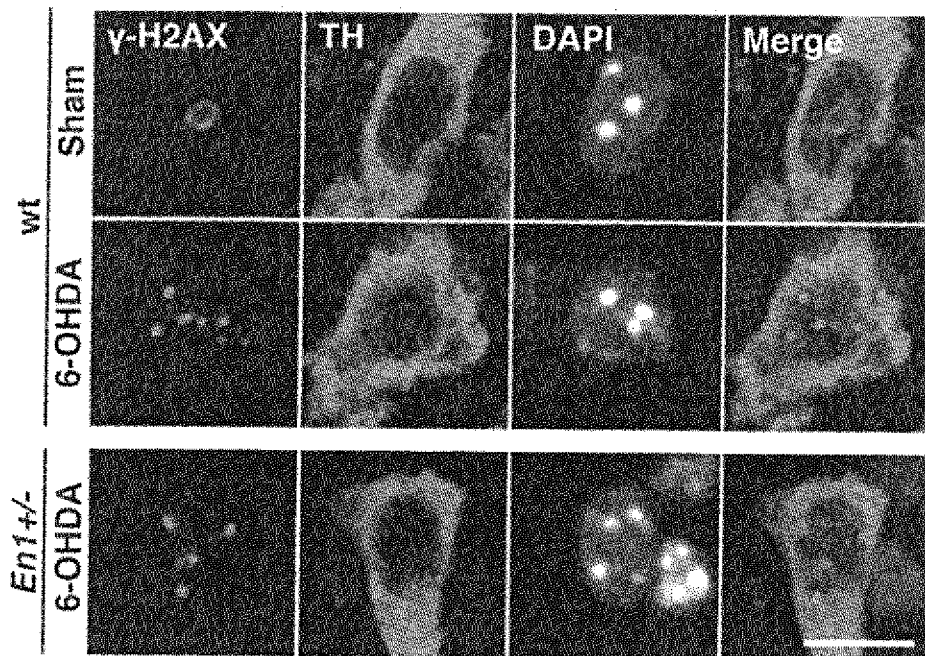


Fig. 2A

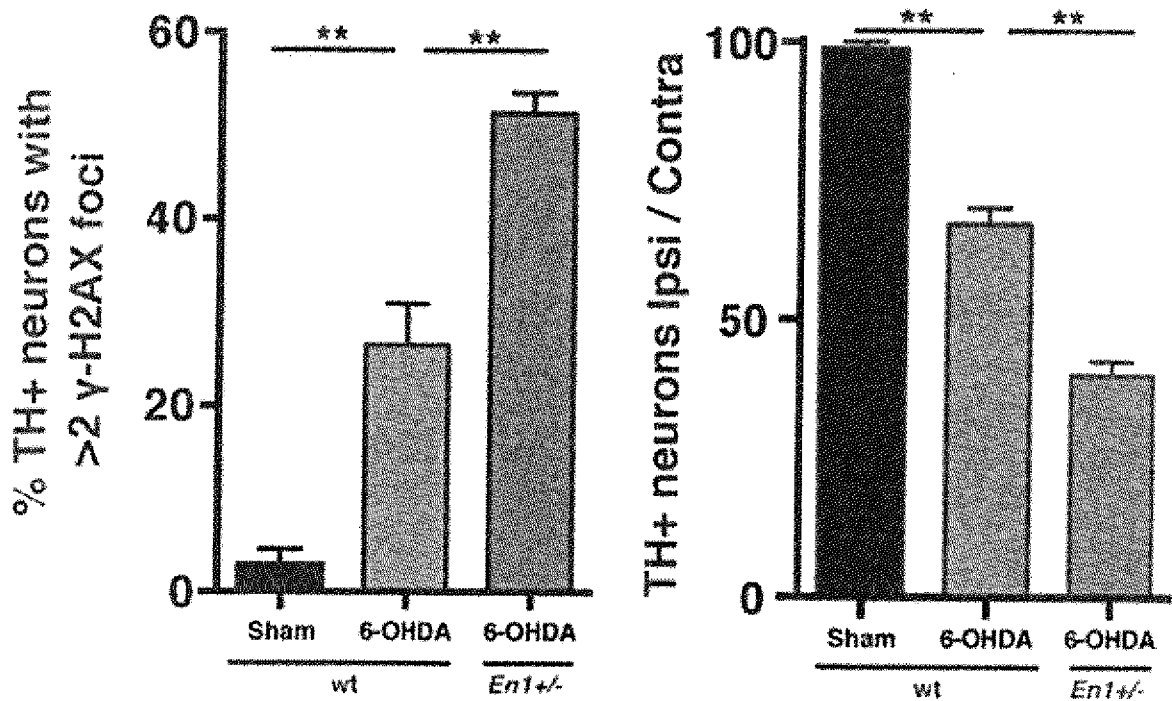


Fig. 2B

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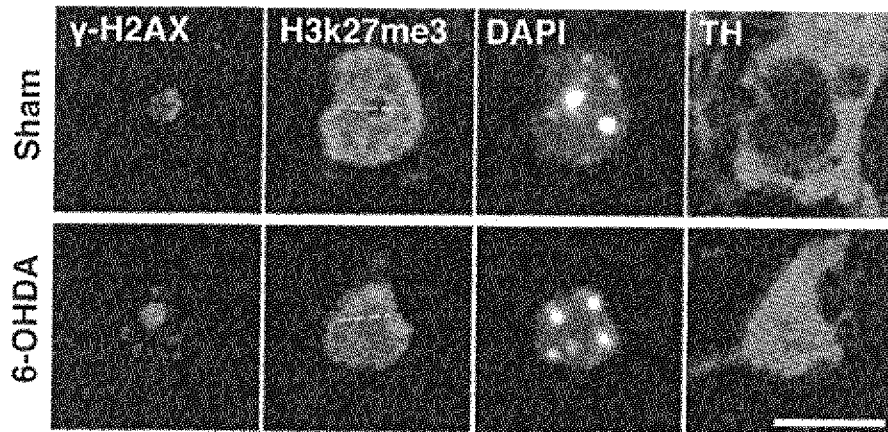


Fig. 2C

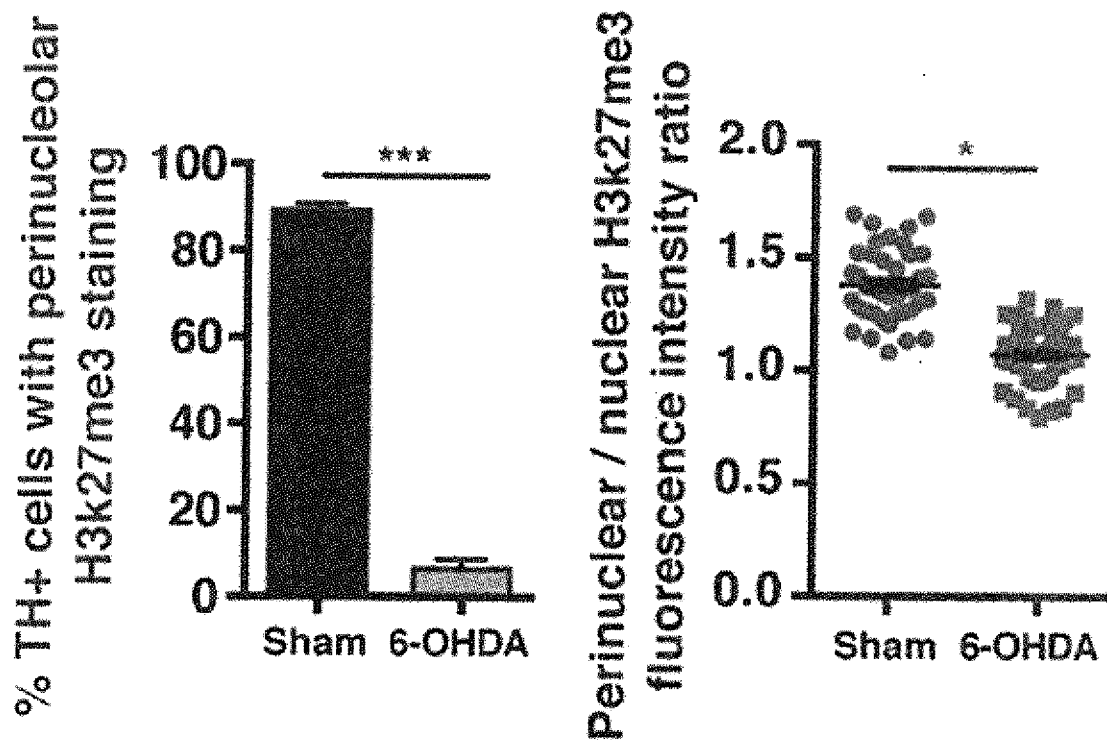


Fig. 2D

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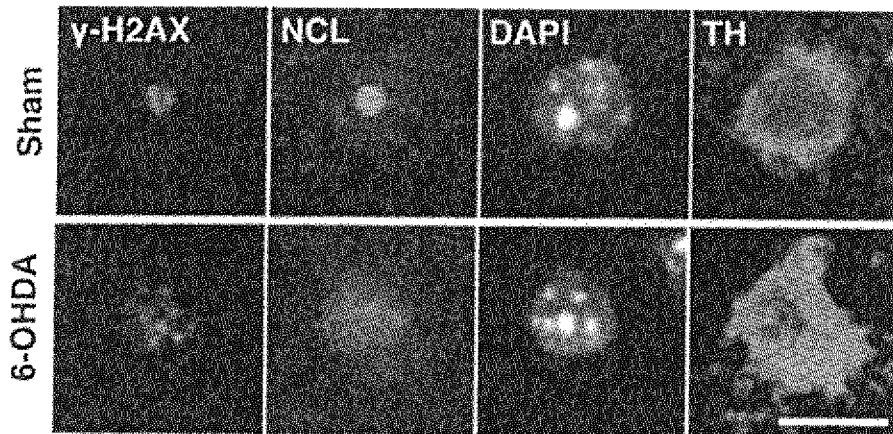


Fig. 2E

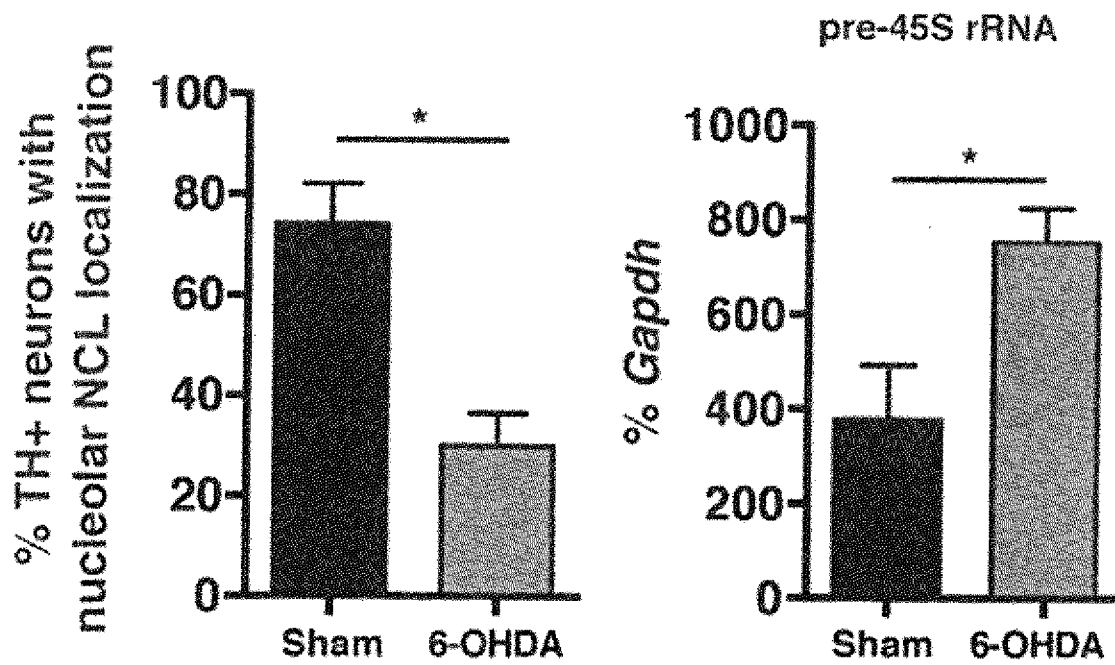


Fig. 2F

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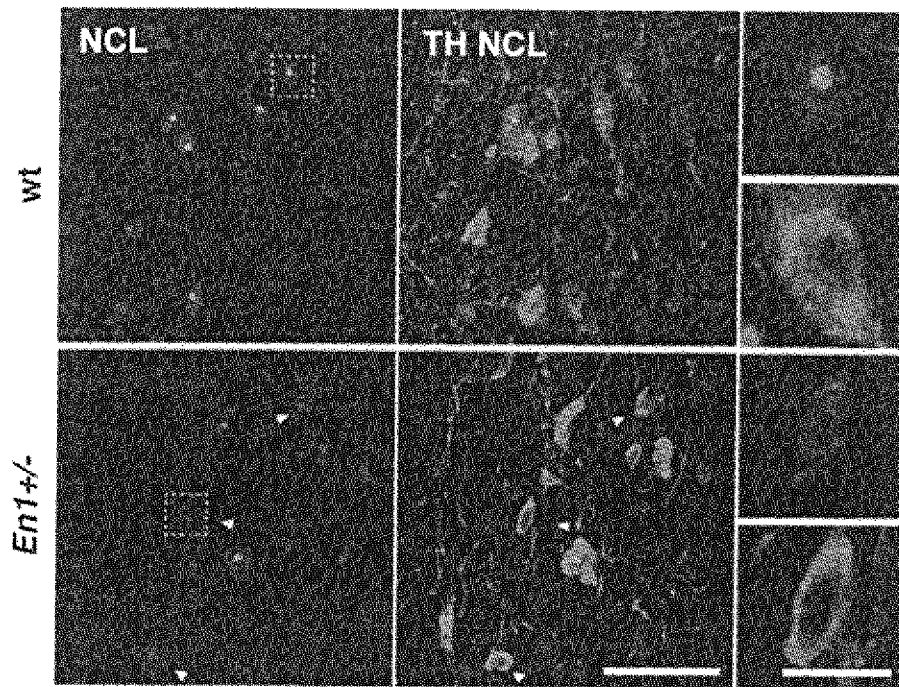


Fig. 2H

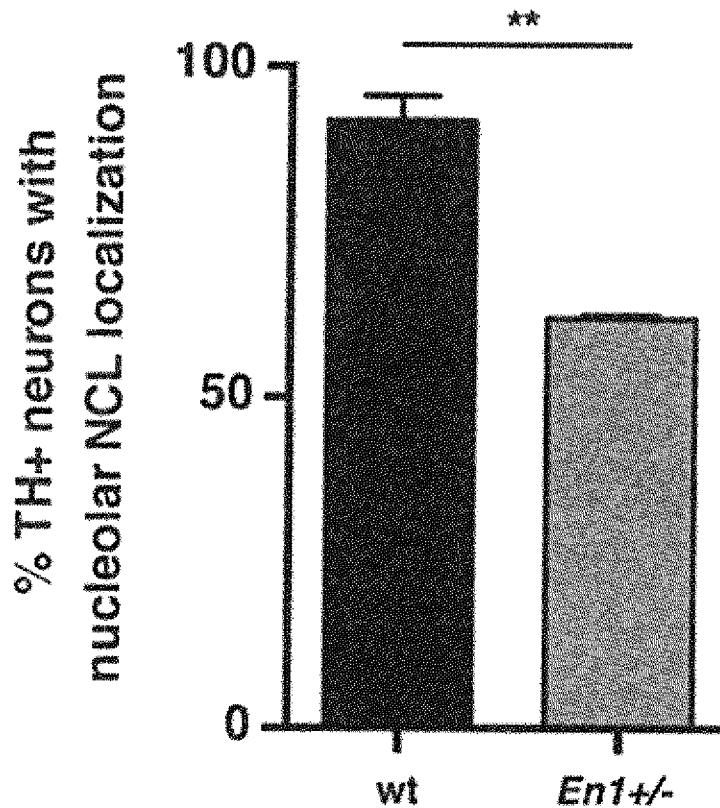


Fig. 2H

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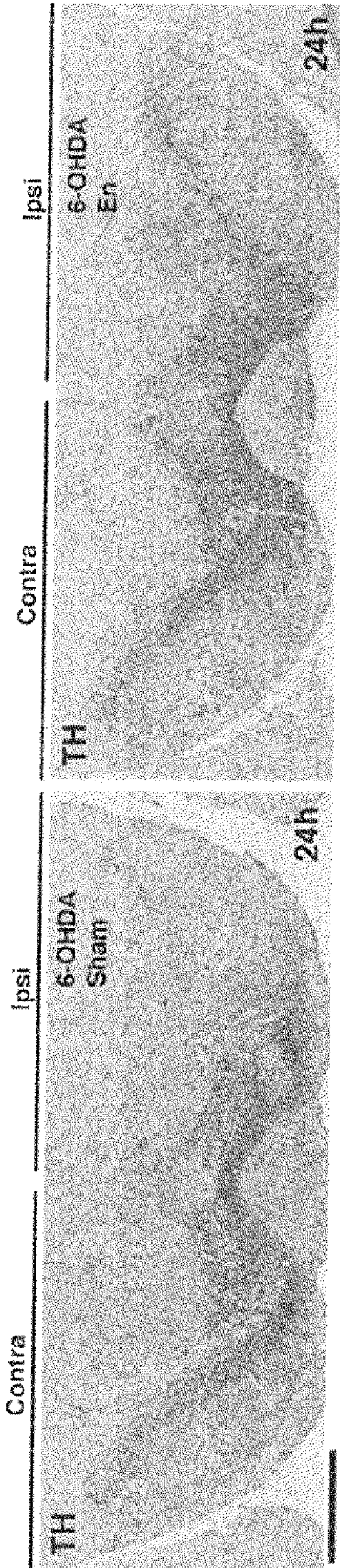


Fig. 3A

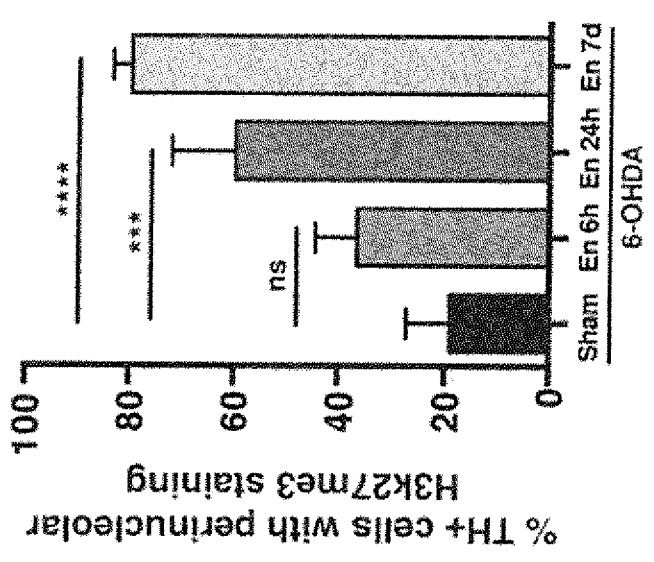
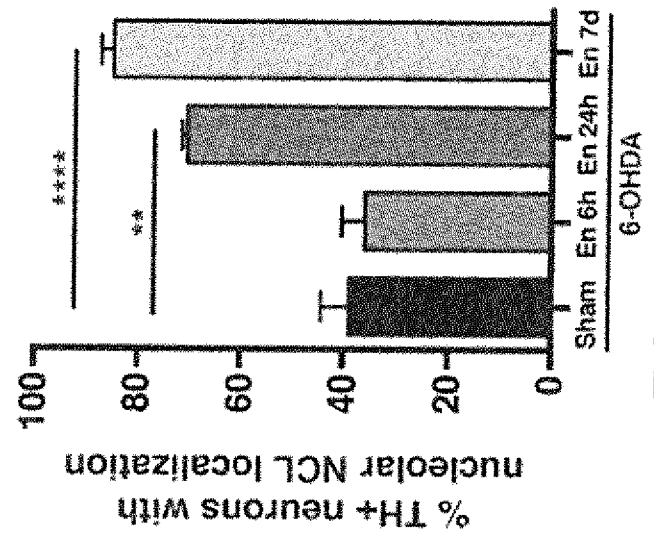
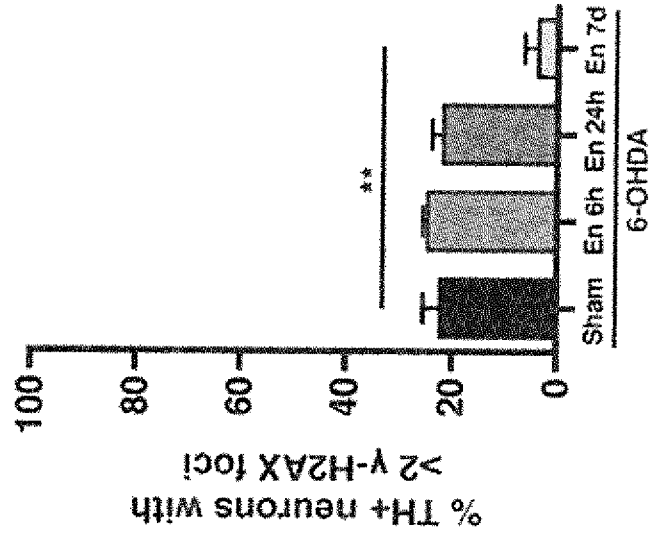


Fig. 3D

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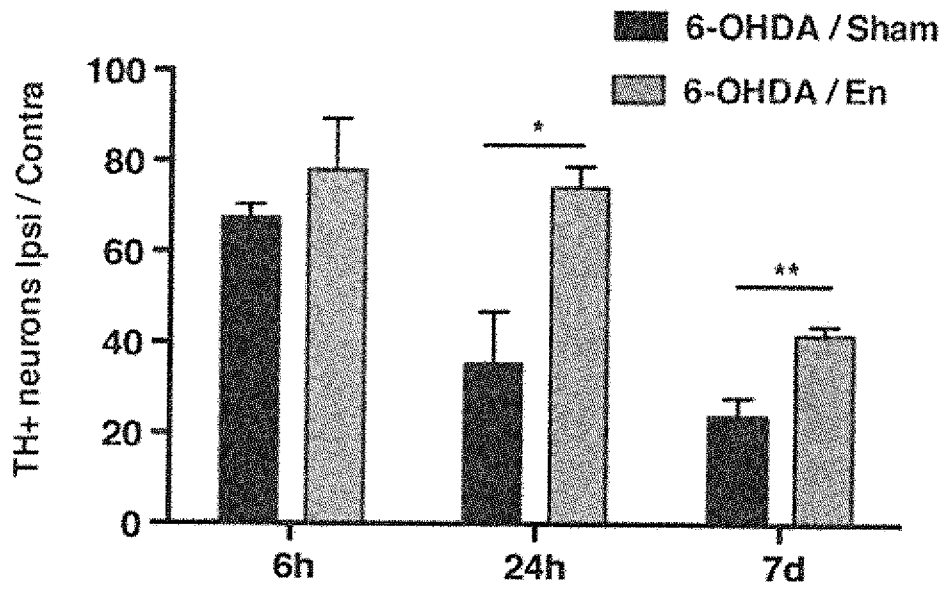


Fig. 3B

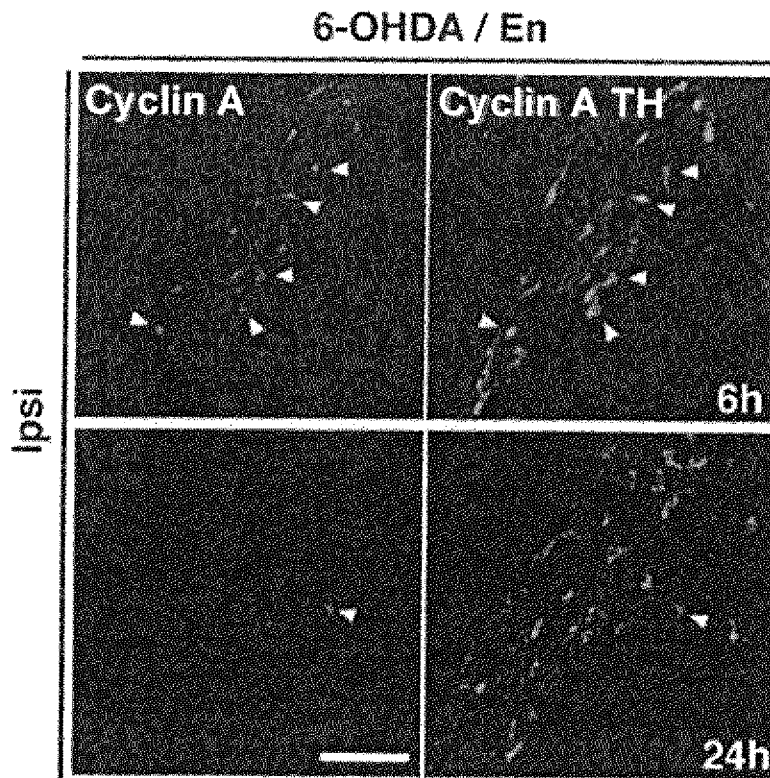


Fig. 3C

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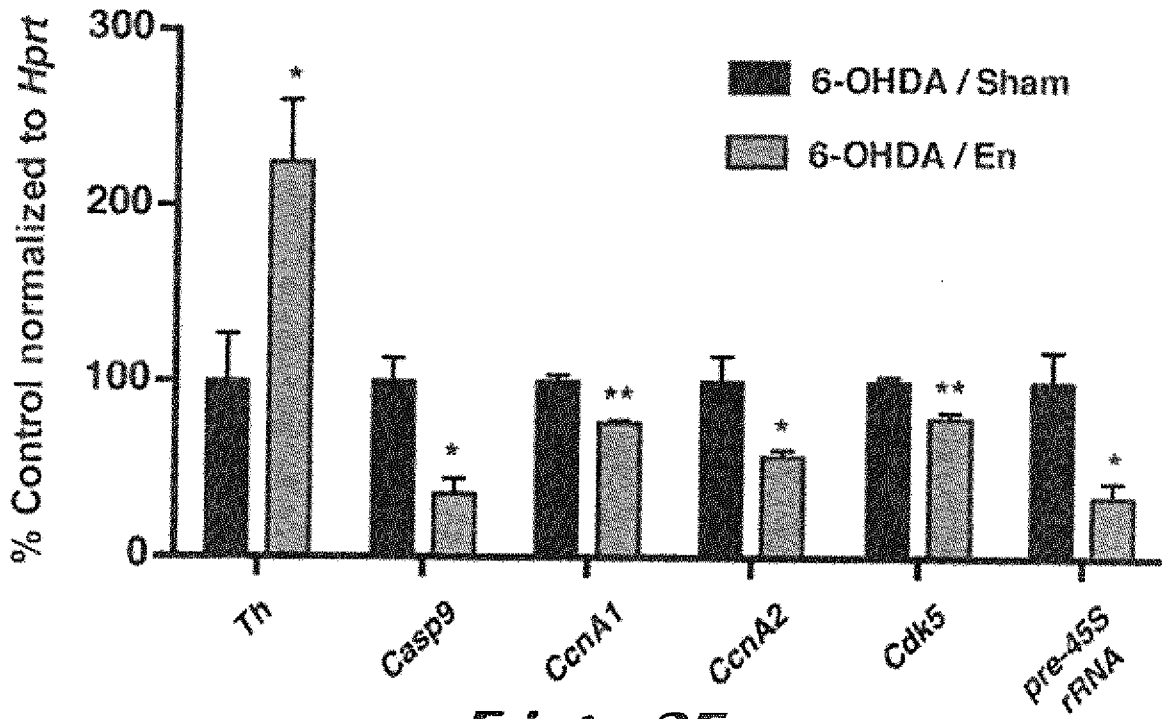


Fig. 3E

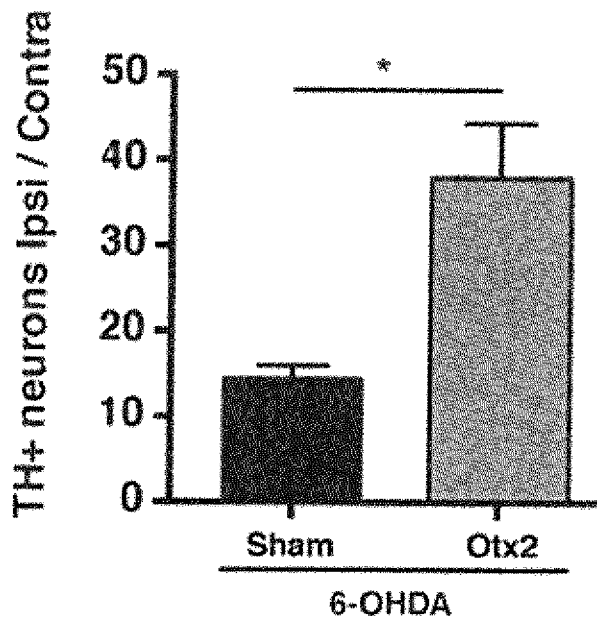


Fig. 3F

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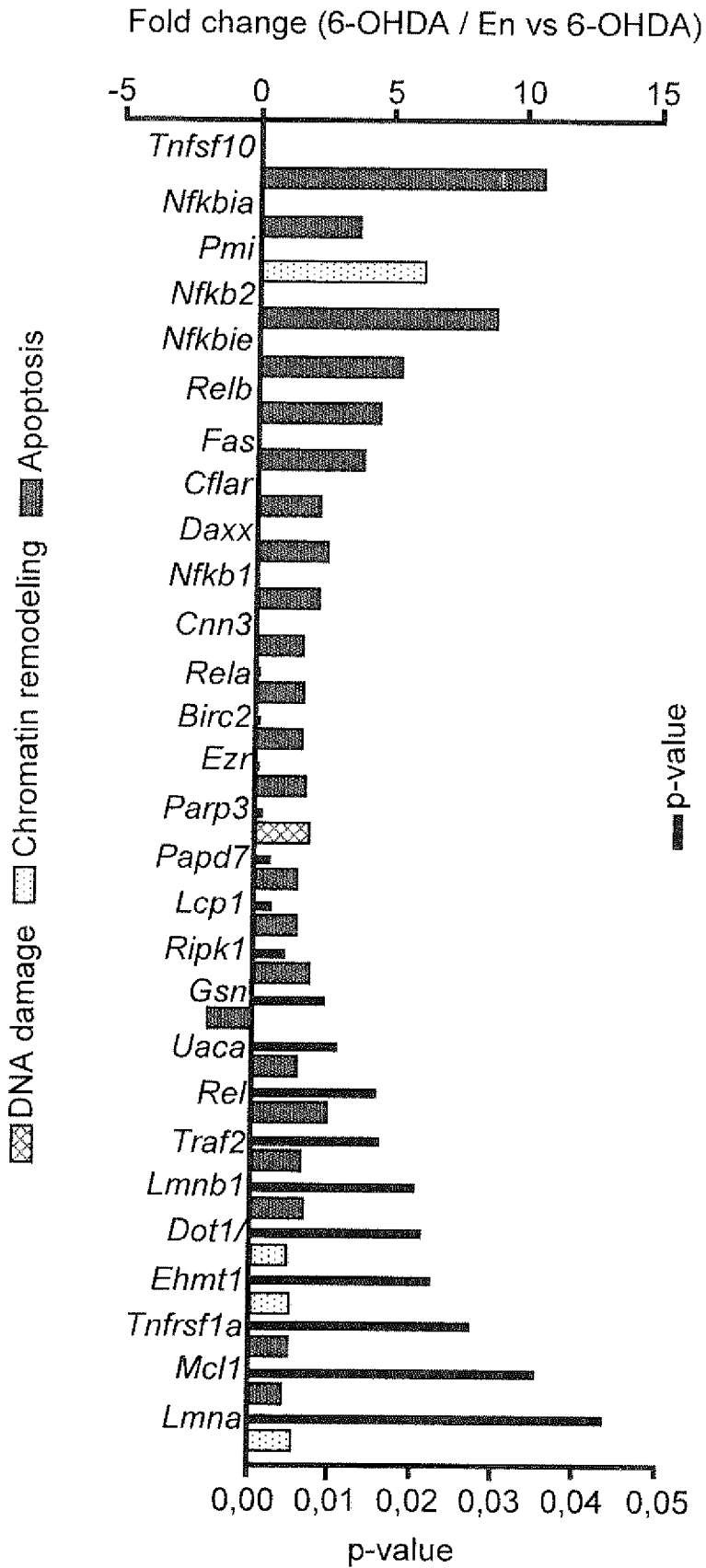


Fig. 4A

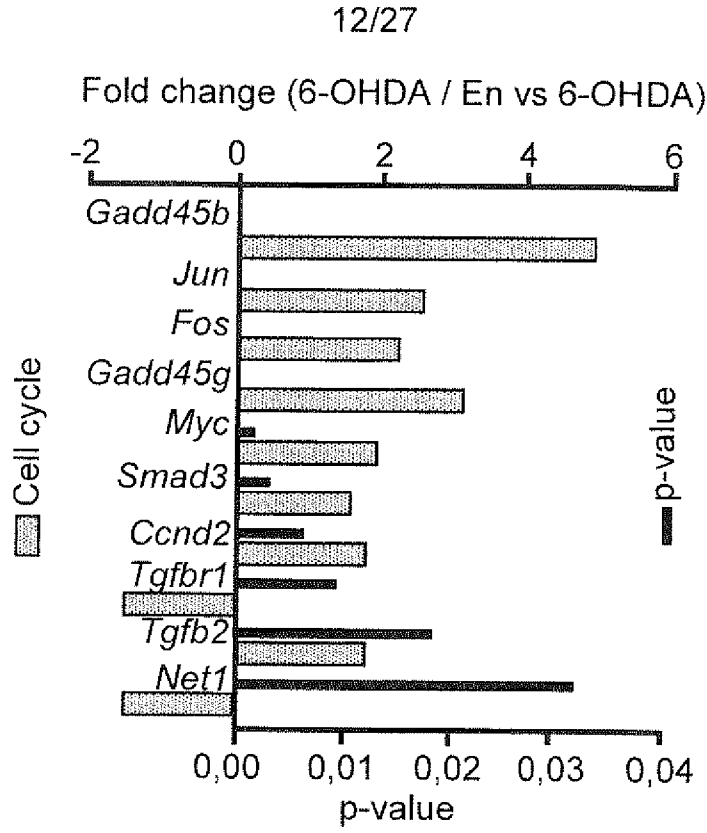


Fig. 4B

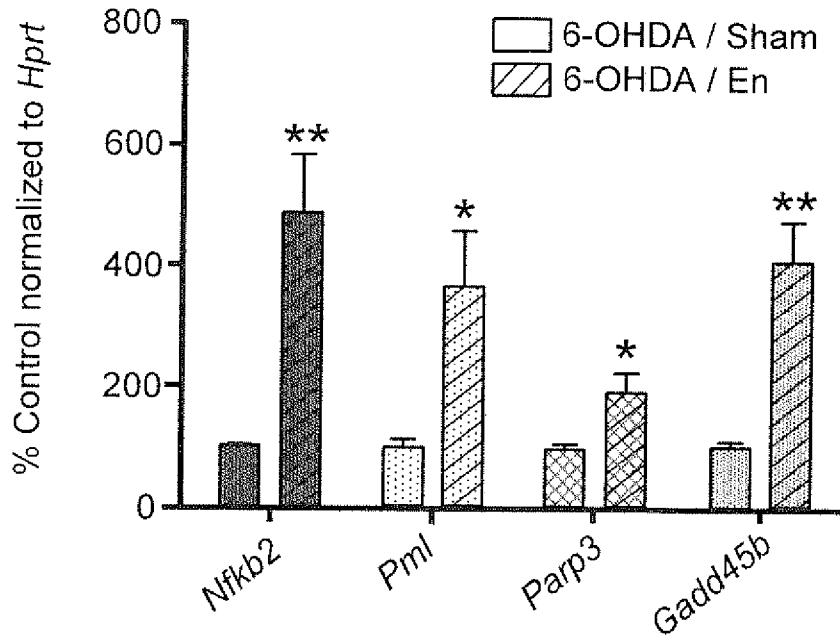


Fig. 4C

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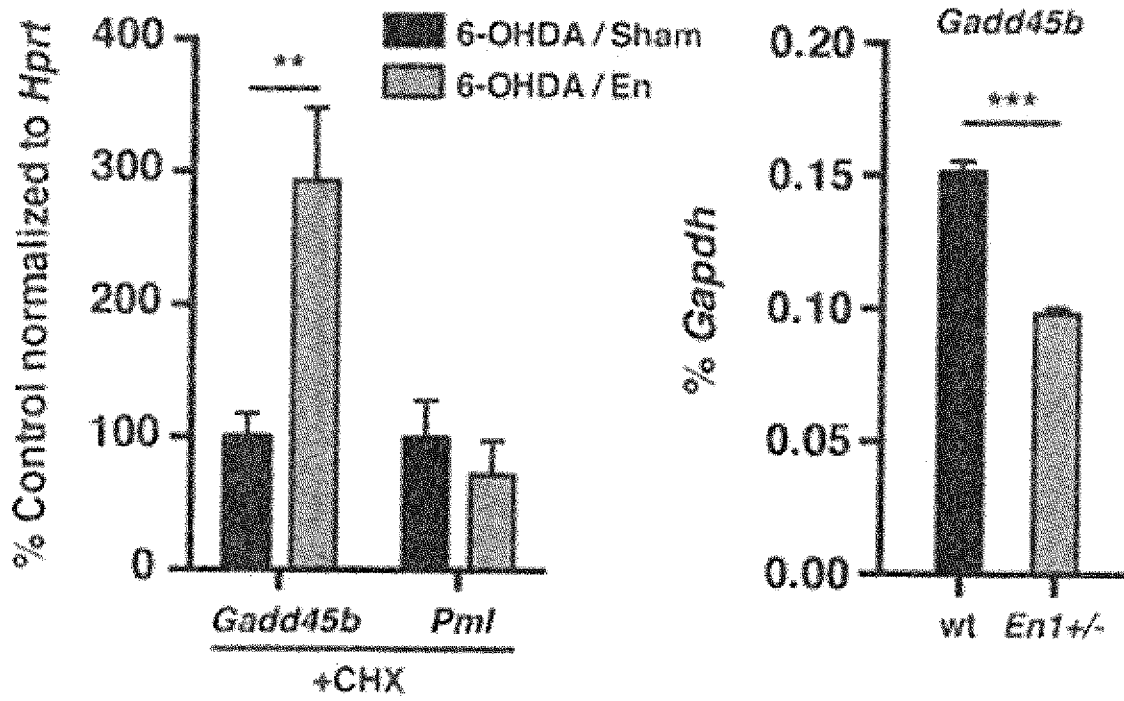


Fig. 4D

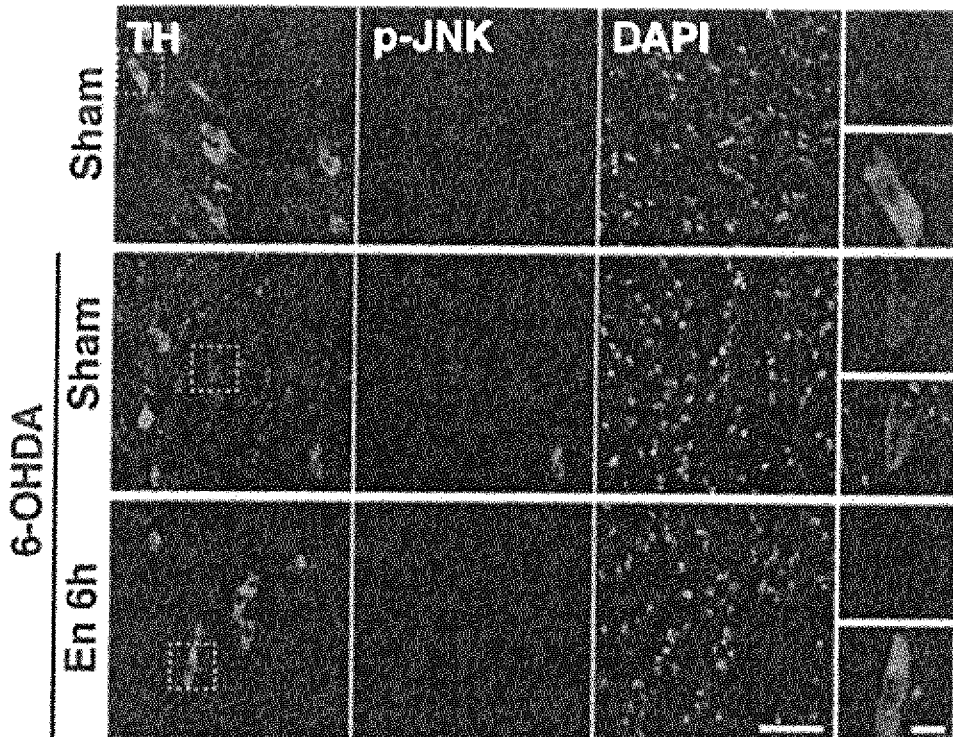


Fig. 4E

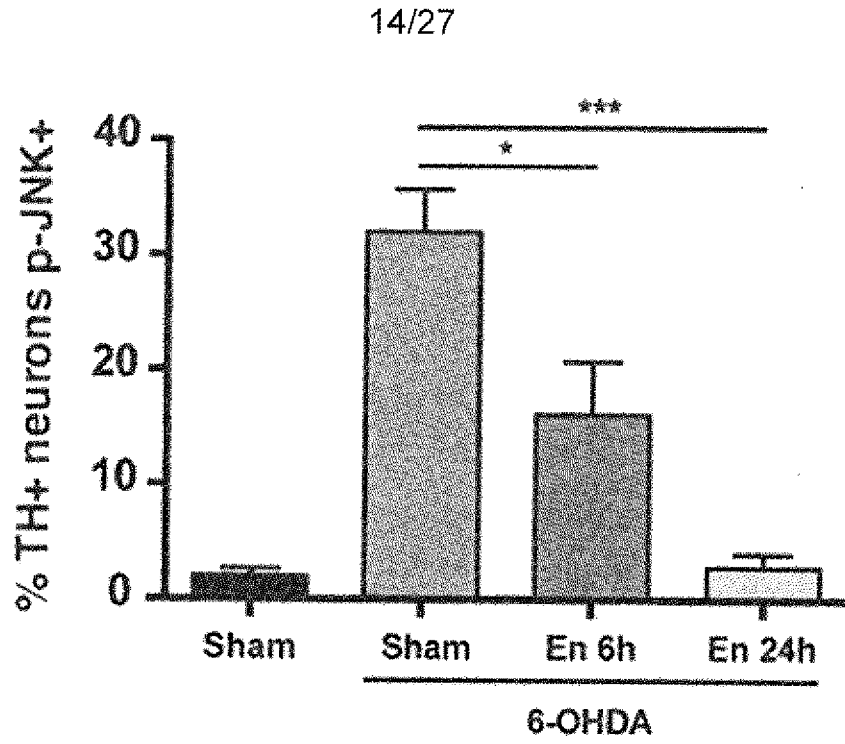


Fig. 4F

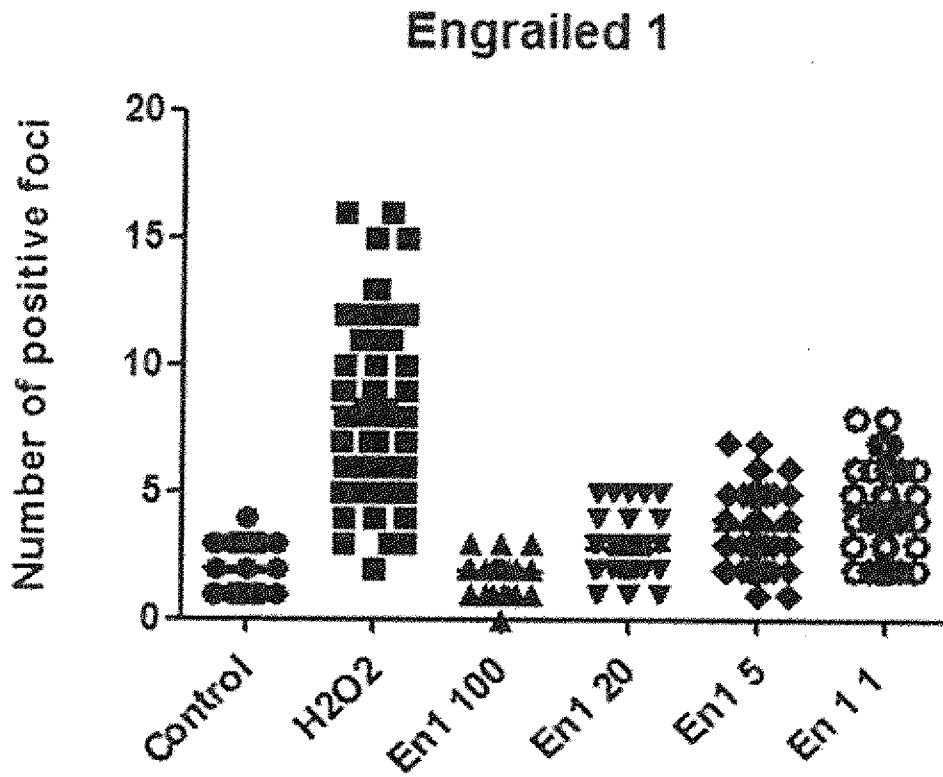


Fig. 5.1

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GBX

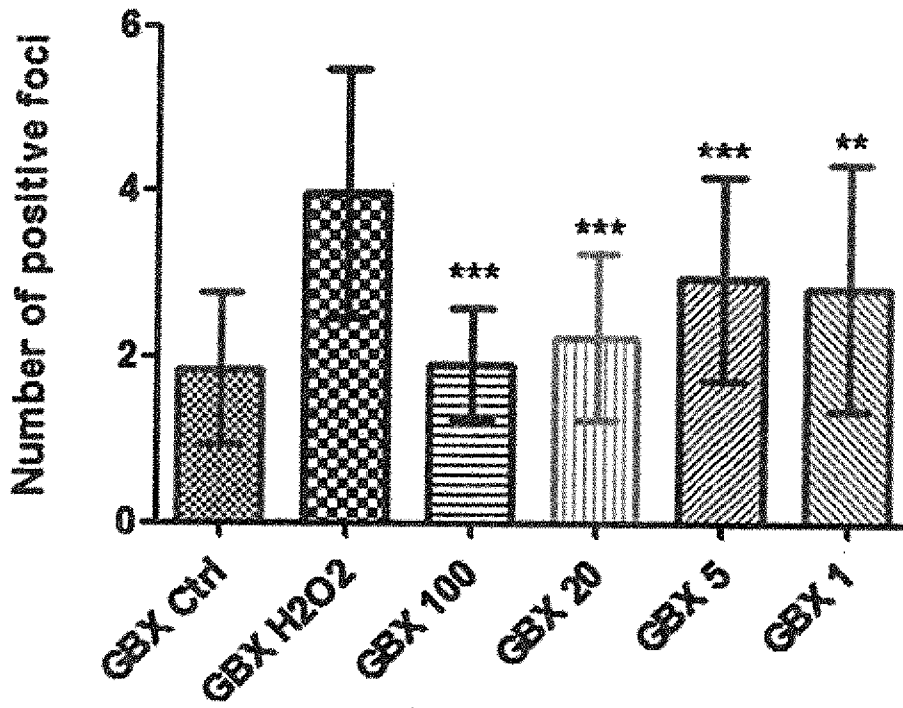


Fig. 5.2

LHX

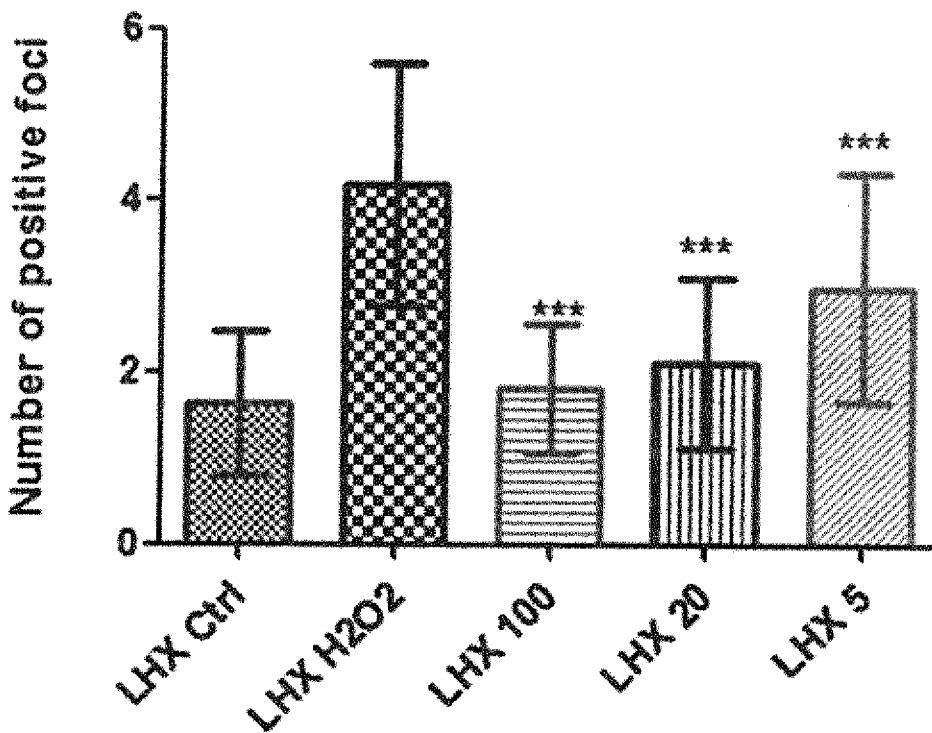


Fig. 5.3

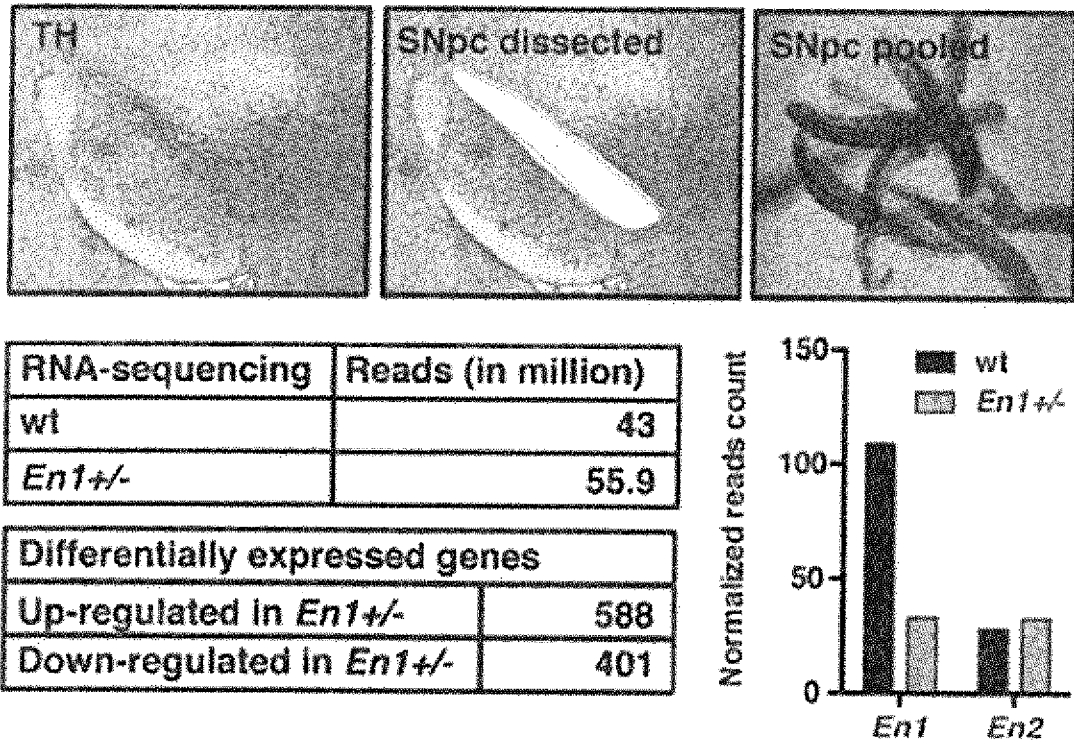


Fig. S1A

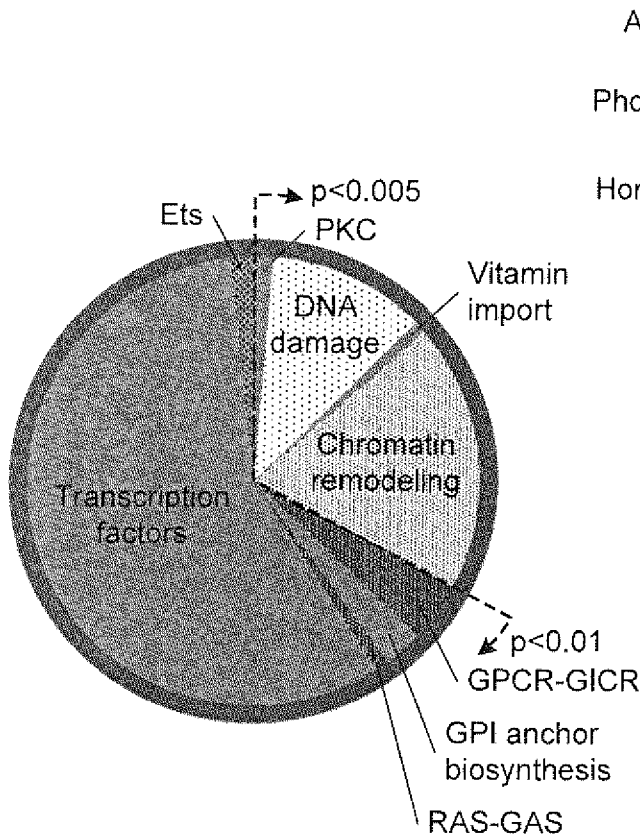


Fig. S1B

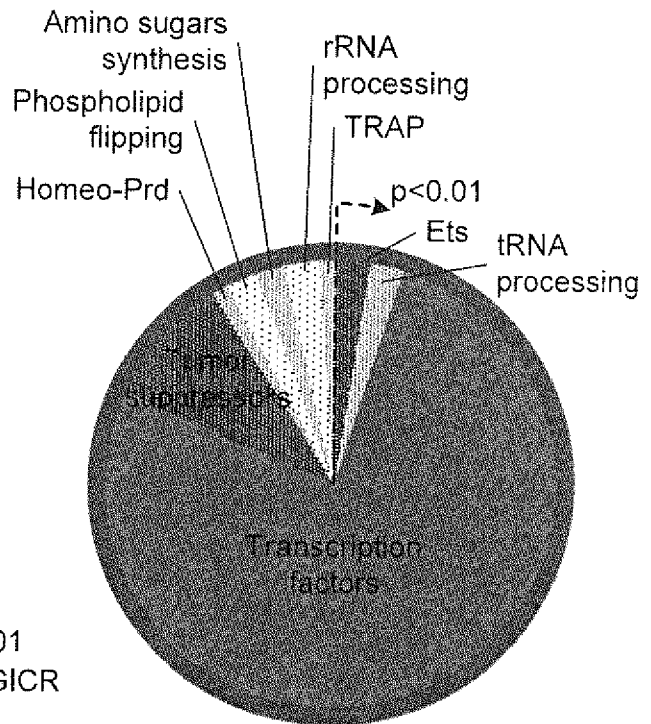


Fig. S1C

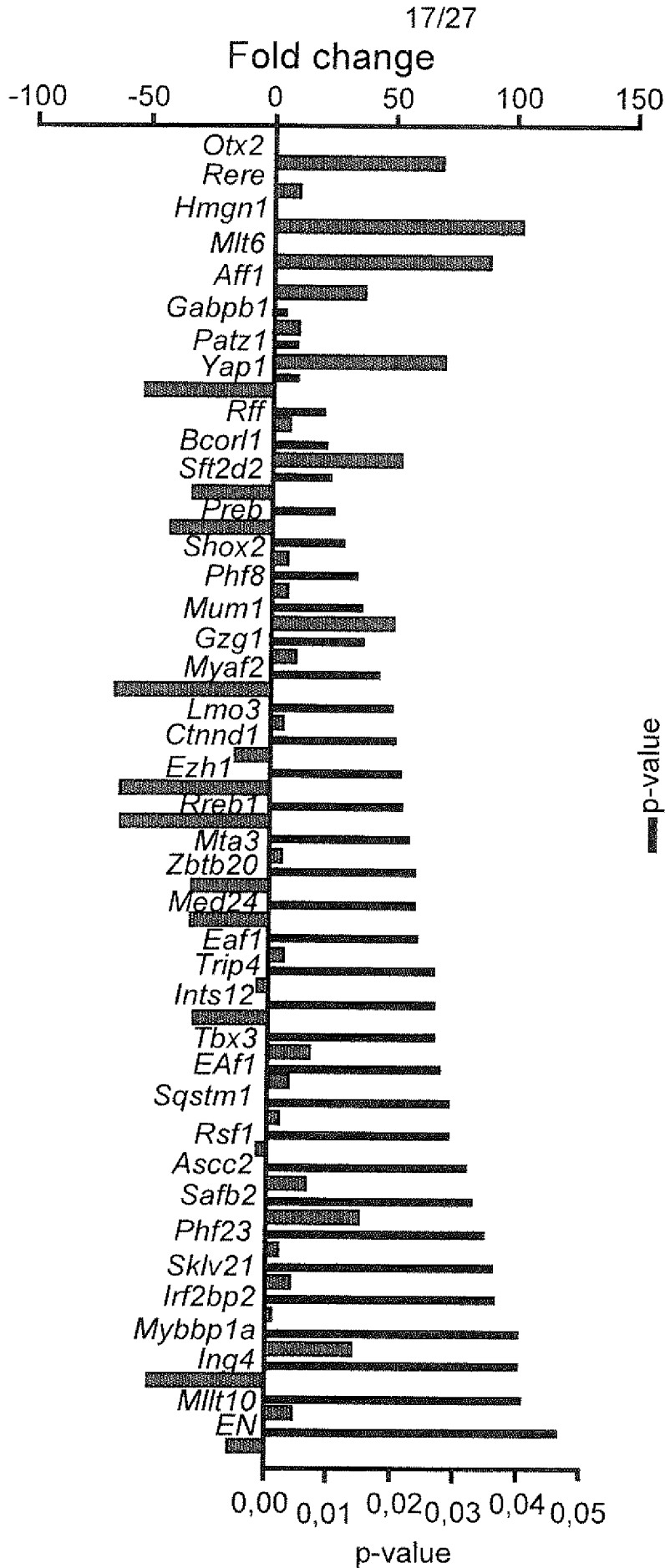


Fig. S1D

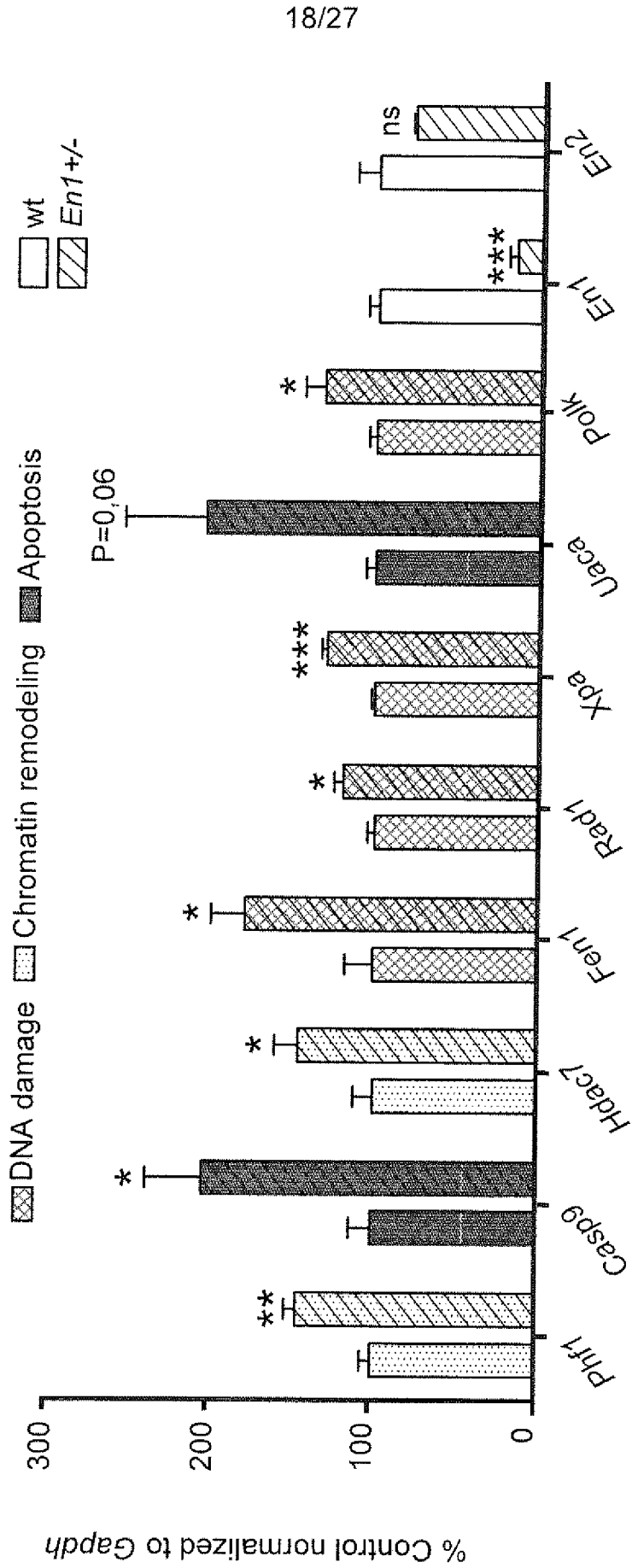
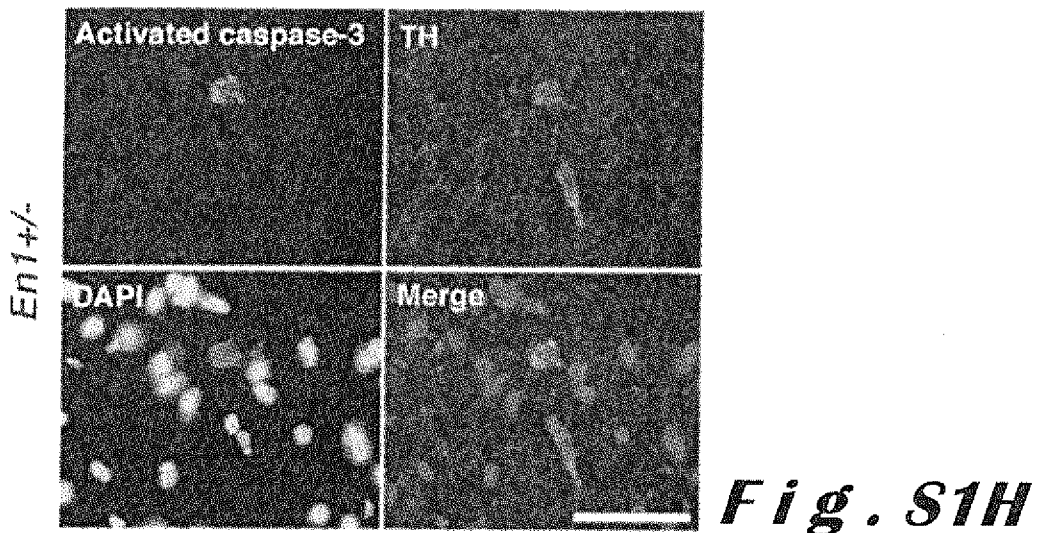
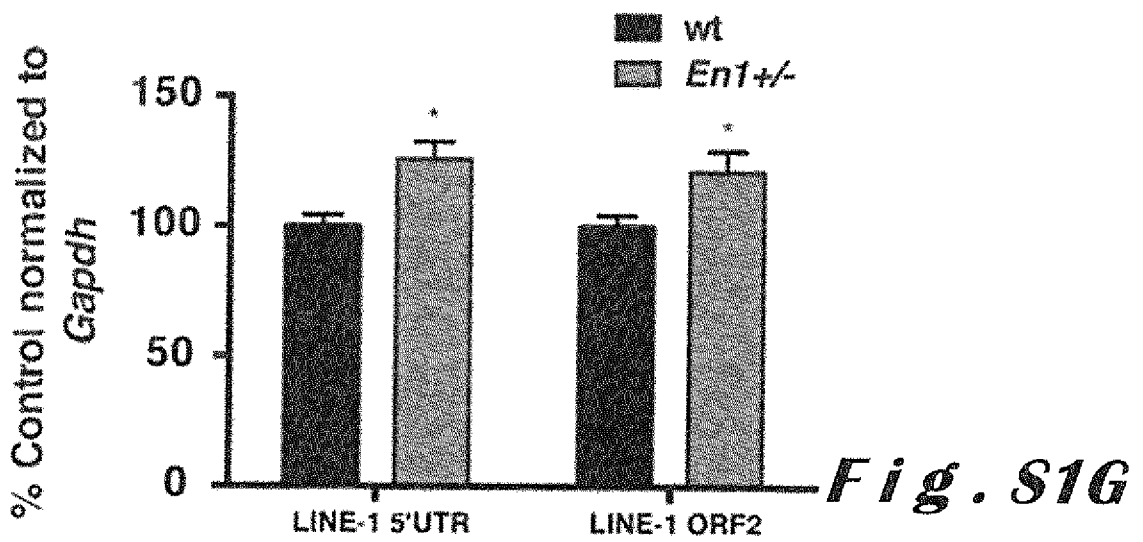
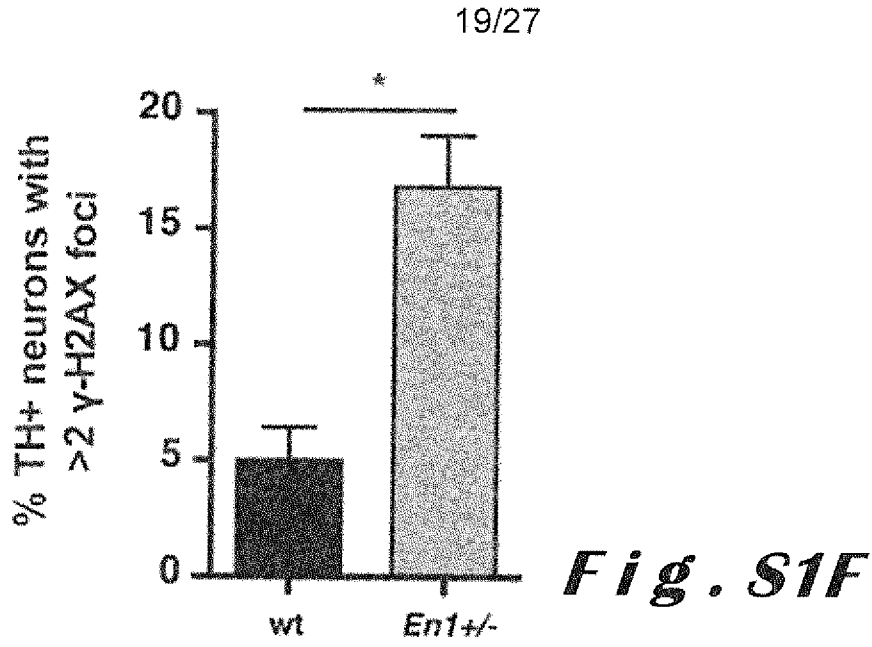


Fig. S1E



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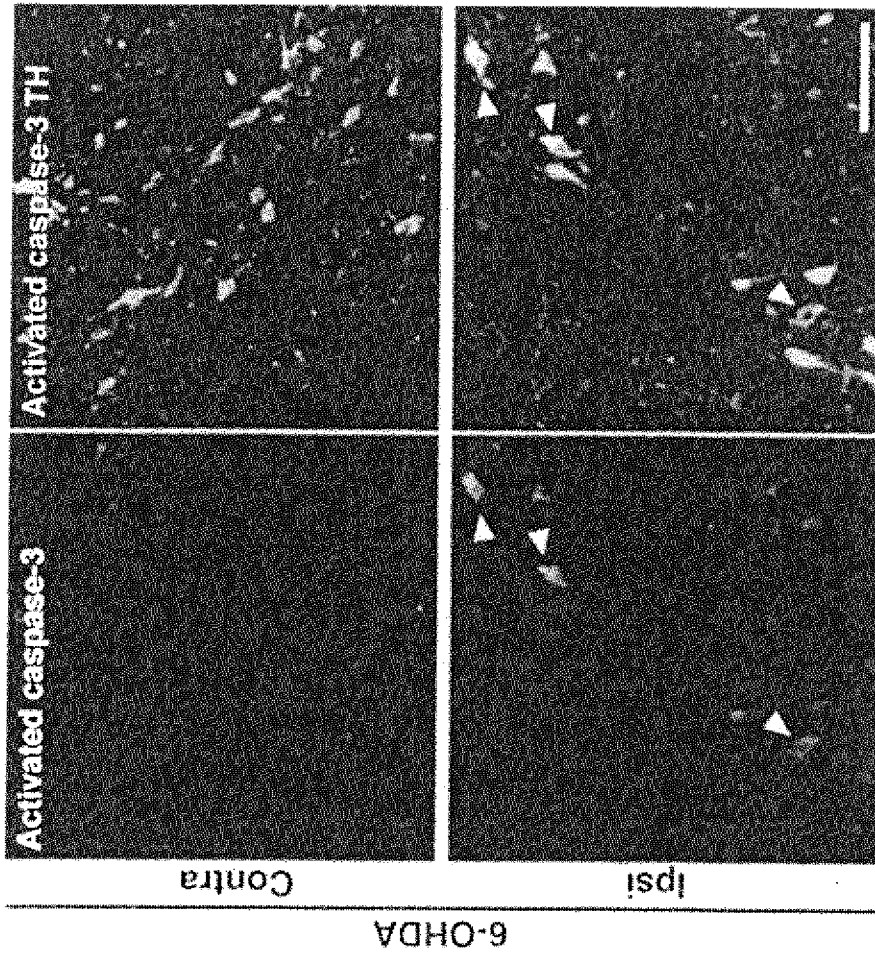


Fig. S2C

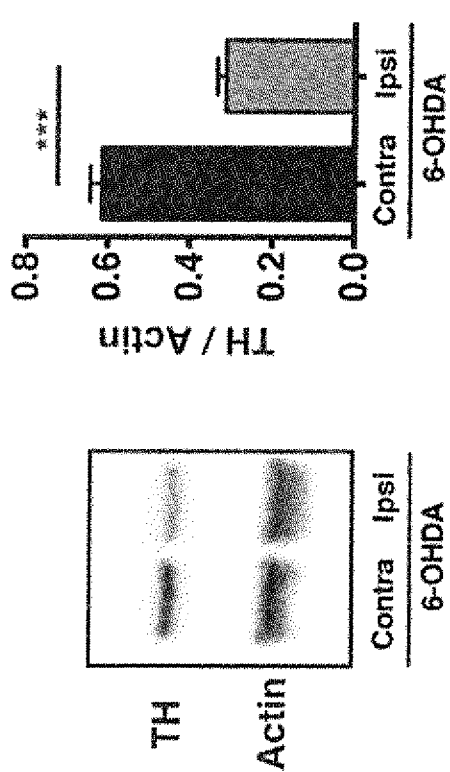


Fig. S2A

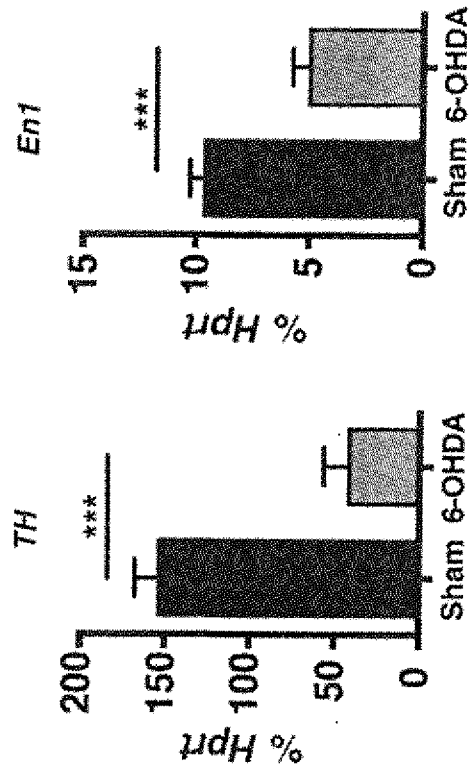


Fig. S2B

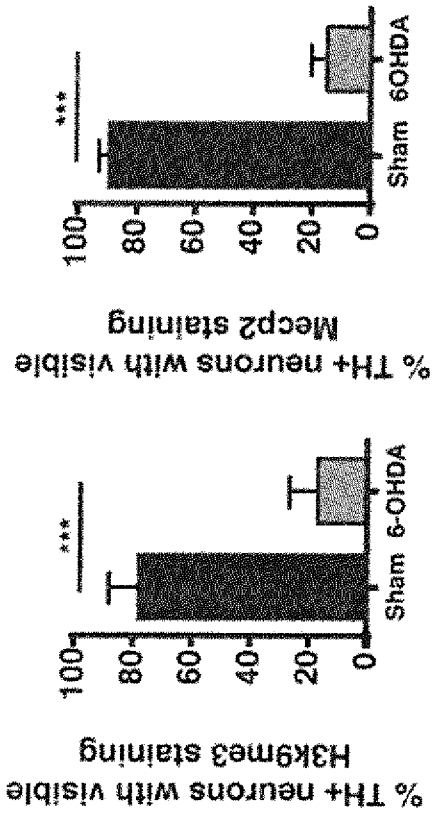


Fig. S2E

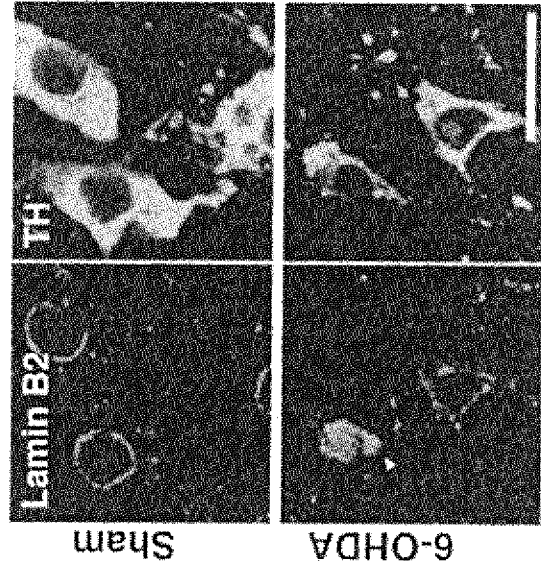


Fig. S2F

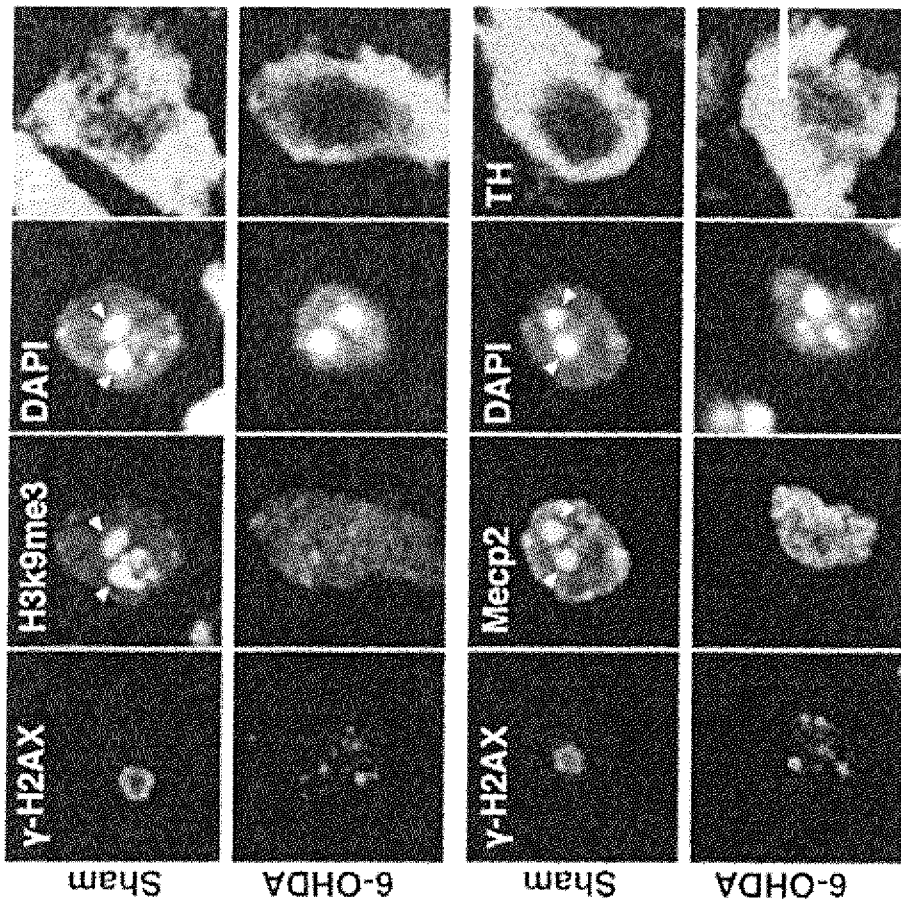


Fig. S2D

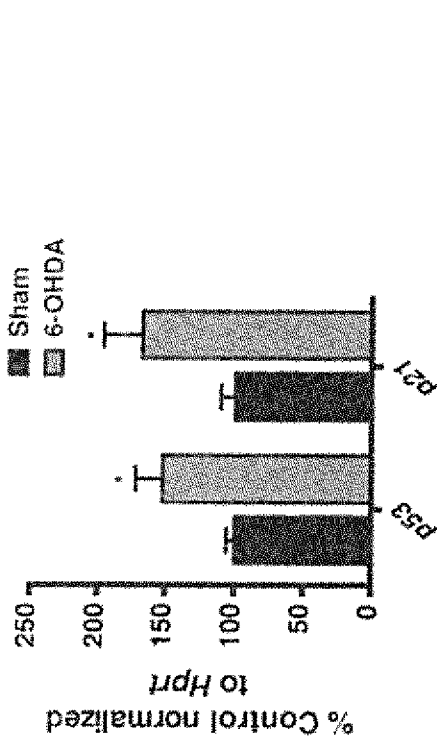


Fig. S2H

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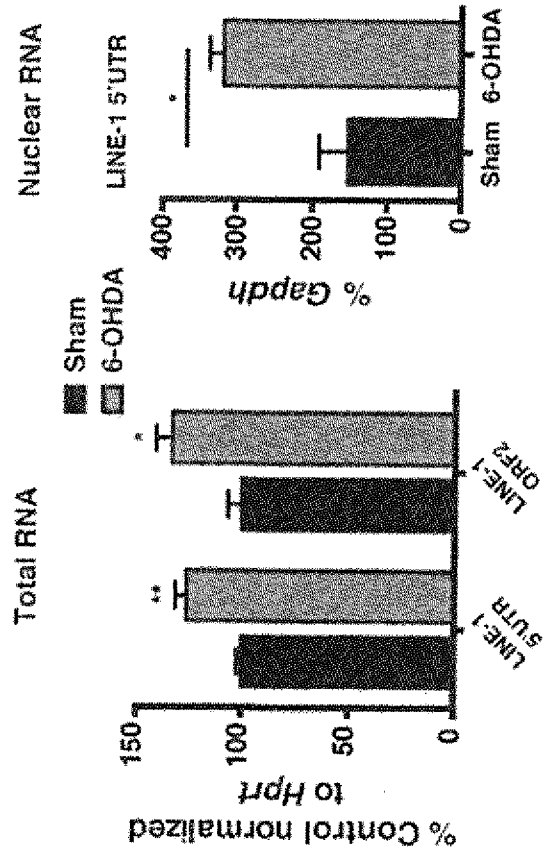


Fig. S2I

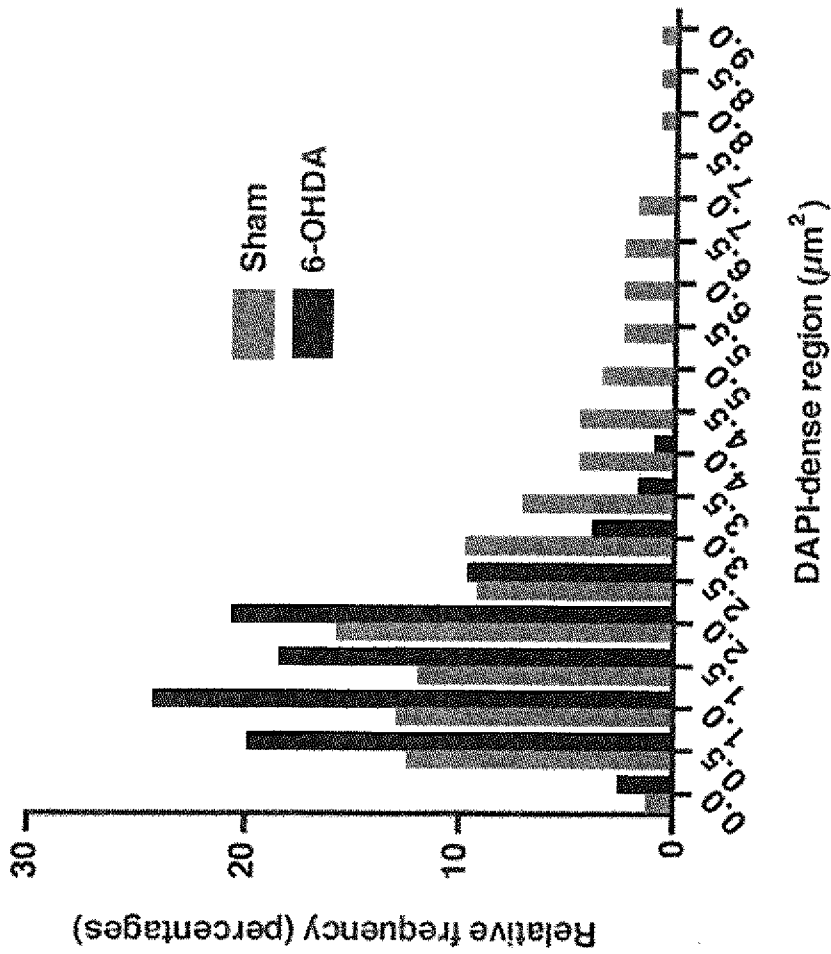


Fig. S2G

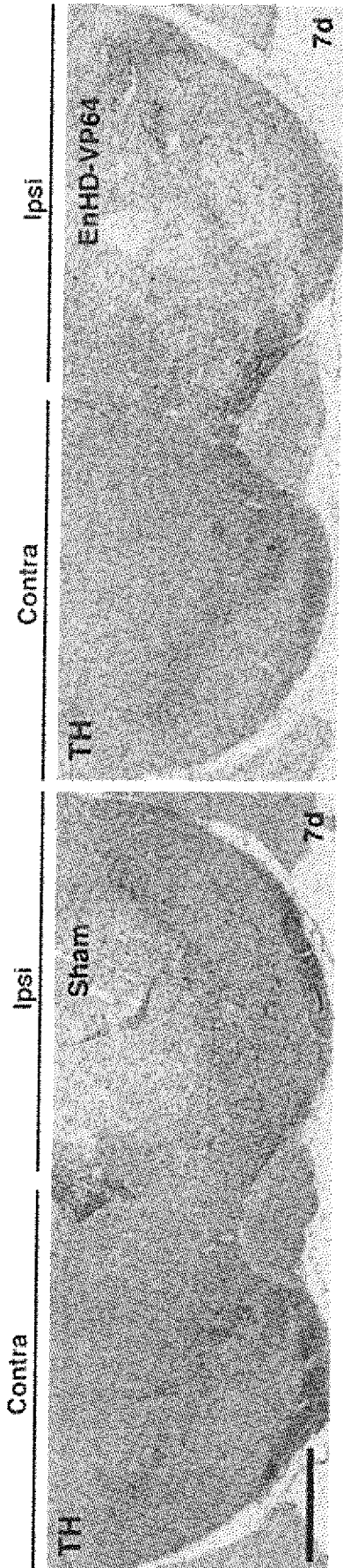


Fig. S3A

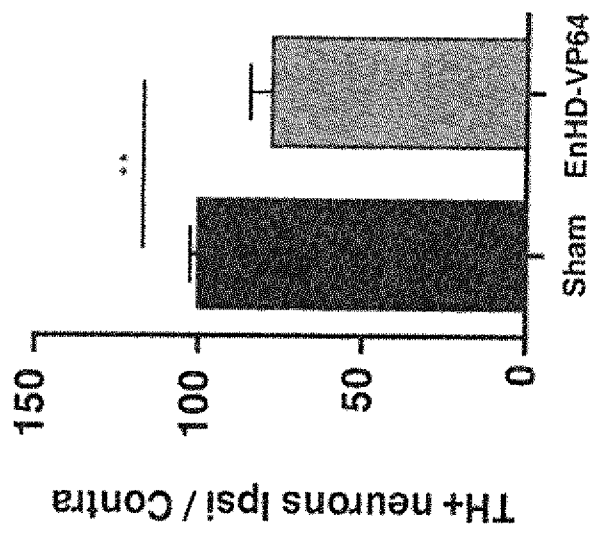


Fig. S3B

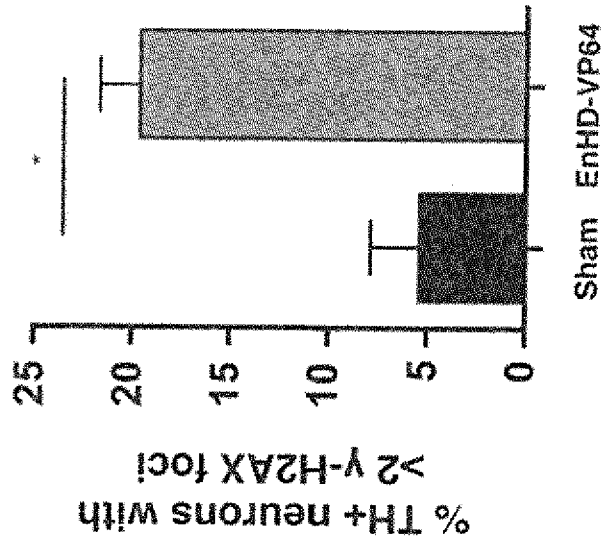


Fig. S3C

Nucleolus organization and biogenesis

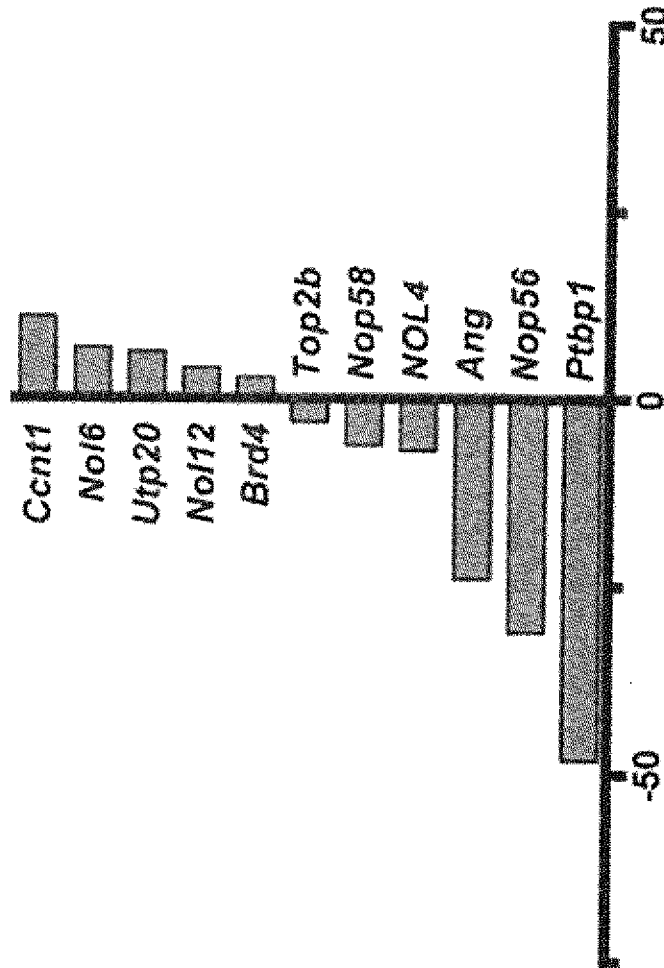
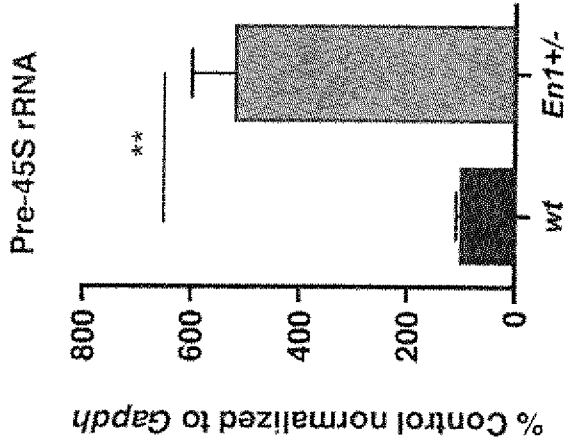


Fig. S3D



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Fig. S3E

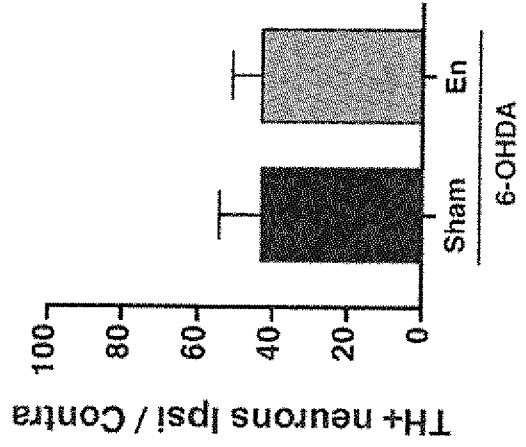


Fig. S3F



Fig. S4A

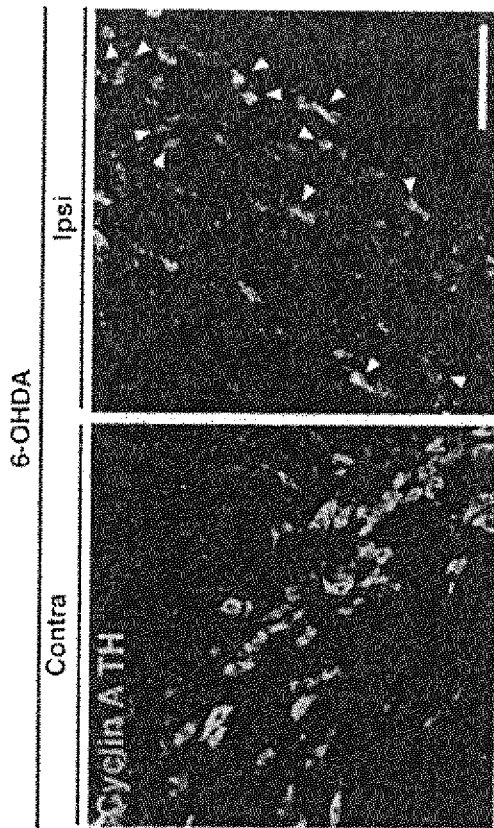


Fig. S4B

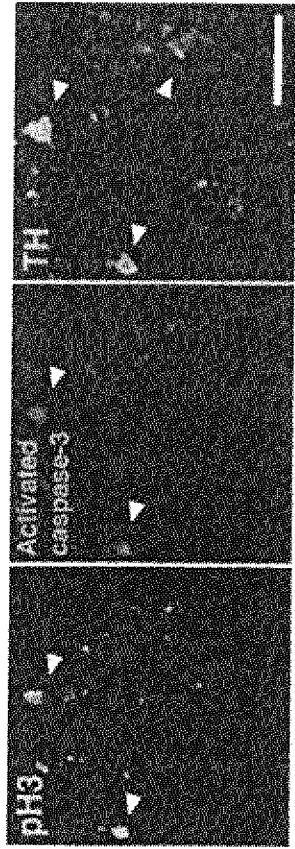


Fig. S4C

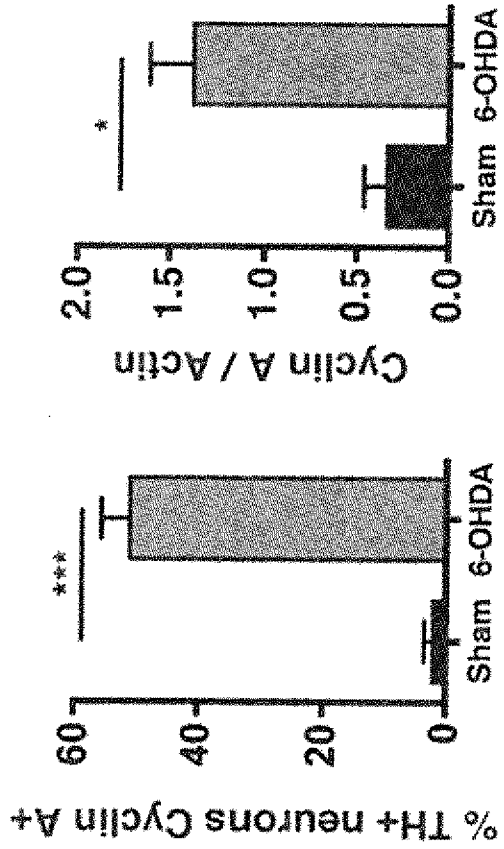


Fig. S4D

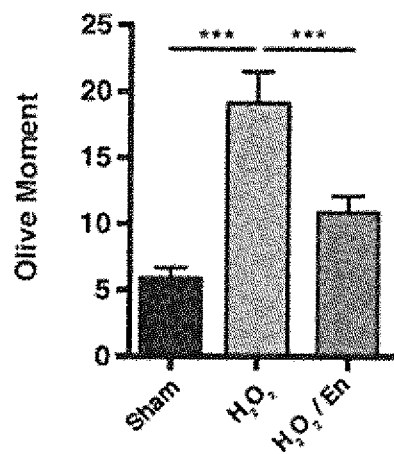
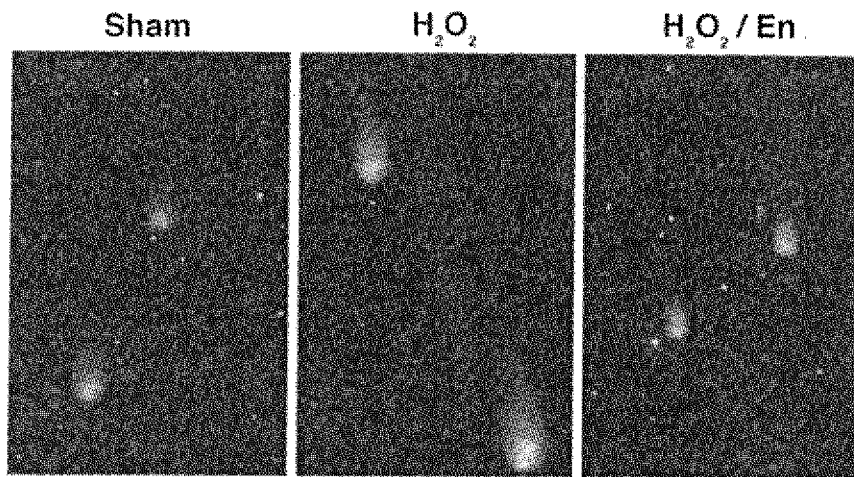
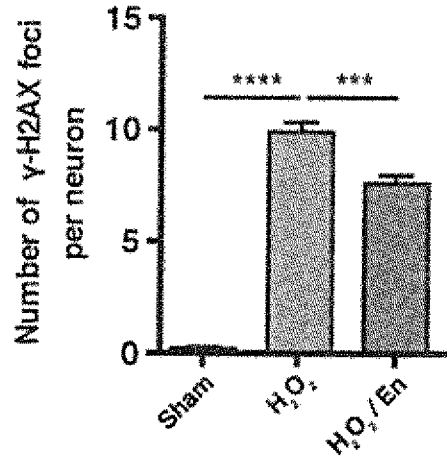
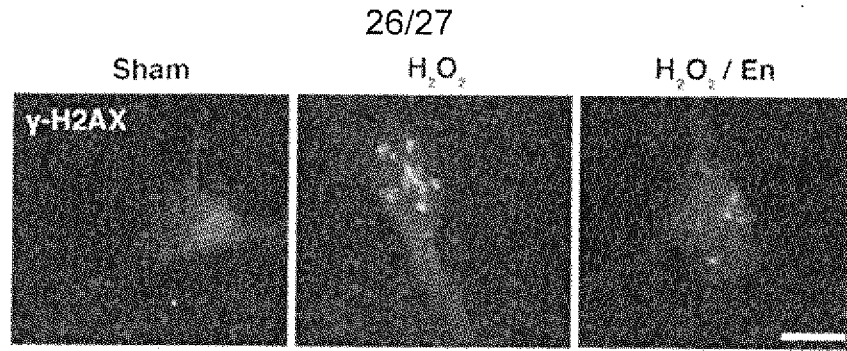


Fig. S4E

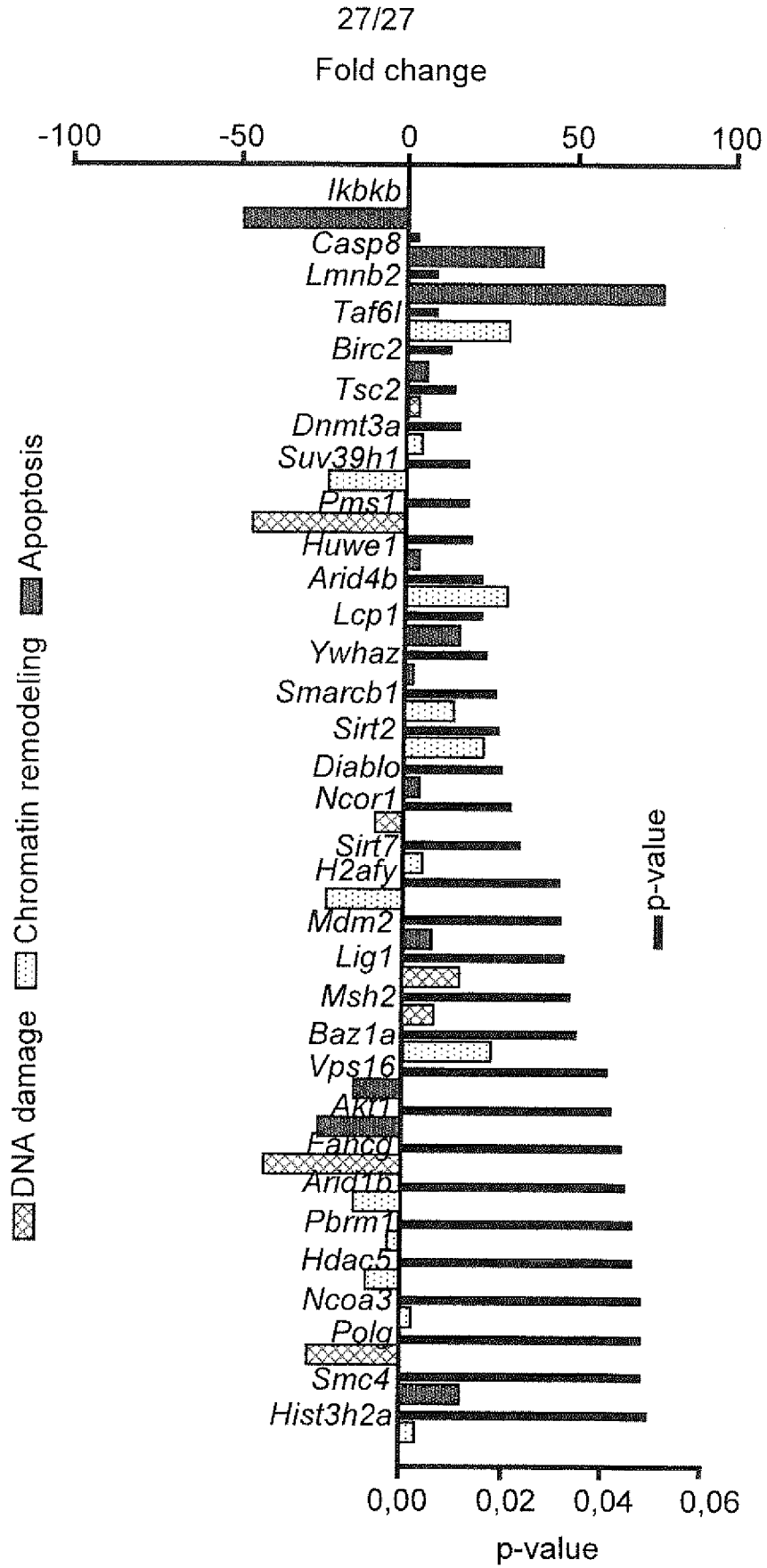


Fig. S4F

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/072675

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P25/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K A61P
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DANIEL ALVAREZ-FISCHER ET AL: "Engrailed protects mouse midbrain dopaminergic neurons against mitochondrial complex I insults", NATURE NEUROSCIENCE, vol. 14, no. 10, 1 January 2011 (2011-01-01), pages 1260-1266, XP055043540, ISSN: 1097-6256, DOI: 10.1038/nn.2916 cited in the application page 1261 ----- -/--	1-7

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 5 December 2016	Date of mailing of the international search report 17/02/2017
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Hars, Jesko
--	---------------------------------------

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/072675

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TORERO IBAD R ET AL: "Otx2 Promotes the Survival of Damaged Adult Retinal Ganglion Cells and Protects against Excitotoxic Loss of Visual Acuity In Vivo", JOURNAL OF NEUROSCIENCE, SOCIETY FOR NEUROSCIENCE, US, vol. 31, no. 14, 6 April 2011 (2011-04-06), pages 5495-5503, XP002699685, ISSN: 0270-6474, DOI: 10.1523/JNEUROSCI.0187-11.2011 -----	1-7
X	J. SPATAZZA ET AL: "Homeoprotein Signaling in Development, Health, and Disease: A Shaking of Dogmas Offers Challenges and Promises from Bench to Bed", PHARMACOLOGICAL REVIEWS, vol. 65, no. 1, 8 January 2013 (2013-01-08), pages 90-104, XP055173064, DOI: 10.1124/pr.112.006577 page 99 - page 101 -----	1-7
X	A. PROCHIANTZ ET AL: "Postnatal signalling with homeoprotein transcription factors", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 282, no. 12, 18 August 2014 (2014-08-18), pages 8994-20130518, XP055173065, ISSN: 0021-9258, DOI: 10.1074/jbc.M609246200 page 8994 -----	1-7
X,P	HOCINE REKAIK ET AL: "Engrailed Homeoprotein Protects Mesencephalic Dopaminergic Neurons from Oxidative Stress", CELL REPORTS, vol. 13, no. 2, 24 September 2015 (2015-09-24), pages 242-250, XP055273273, US ISSN: 2211-1247, DOI: 10.1016/j.celrep.2015.08.076 page 243 ----- -/--	1-7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/072675

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MATIAS ALVAREZ-SAAVEDRA ET AL: "Coordinated epigenetic regulation of Engrailed-1 by the chromatin remodelers Smarca1 and Smarca5 mediates cerebellar morphogenesis", EPIGENETICS & CHROMATIN, BIOMED CENTRAL LTD, LONDON, UK, vol. 6, no. Suppl 1, 8 April 2013 (2013-04-08), page P105, XP021147529, ISSN: 1756-8935, DOI: 10.1186/1756-8935-6-S1-P105 page P105	1-7
X	----- WO 2004/104030 A2 (CENTRE NAT RECH SCIENT [FR]; ECOLE NORMALE SUPERIEURE [FR]; UNIV PARIS) 2 December 2004 (2004-12-02) page 5	1-7
X	----- WO 2013/128239 A1 (CENTRE NAT RECH SCIENT [FR]; COLLEGE DE FRANCE [FR]; PROCHIANTZ ALAIN) 6 September 2013 (2013-09-06) cited in the application page 1, line 23 - line 33	1-7
A	----- FR 2 662 698 A1 (CENTRE NAT RECH SCIENT [FR]) 6 December 1991 (1991-12-06) claim 4	1-7
X,P	----- FRANÇOIS-XAVIER BLAUDIN DE THÉ ET AL: "Neuroprotective Transcription Factors in Animal Models of Parkinson Disease", NEURAL PLASTICITY, vol. 20, no. 4, 31 December 2015 (2015-12-31), pages 1275-11, XP055273348, ISSN: 2090-5904, DOI: 10.1016/j.neuron.2015.01.019 page 1275 -----	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/072675

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7(partially)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7(partially)

A homeoprotein, or peptide derivatives thereof, for use in the treatment and/or prevention of a neurodegenerative disorder, wherein the homeoprotein is Engrailed protein and the disorder is Parkinson disease (PD).

2-18. claims: 8-10(completely); 1-7(partially)

As invention 1, but with the following features of inventions 2-24 (invention number in brackets):

- homeoprotein: Engrailed, disorder: Alzheimer disease (AD) (2), amyotrophic lateral sclerosis (ALS) (3), Huntington disease (HD) (4), neurodegenerative diseases affecting the eyes (5), neurodegenerative diseases affecting the ears (6);
- homeoprotein: Gbx2, disorder: PD (7), AD (8), ALS (9), HD (10), neurodegenerative diseases affecting the eyes (11), neurodegenerative diseases affecting the ears (12);
- homeoprotein: Lhx9, disorder: PD (13), AD (14), ALS (15), HD (16), neurodegenerative diseases affecting the eyes (17), neurodegenerative diseases affecting the ears (18).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/072675

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004104030	A2	02-12-2004	AT 339441 T 15-10-2006
		DE 602004002400 T2	20-09-2007
		DK 1625152 T3	22-01-2007
		EP 1625152 A2	15-02-2006
		ES 2273277 T3	01-05-2007
		FR 2855178 A1	26-11-2004
		JP 2007532093 A	15-11-2007
		PT 1625152 E	31-01-2007
		US 2007155668 A1	05-07-2007
		US 2009131320 A1	21-05-2009
		WO 2004104030 A2	02-12-2004

WO 2013128239	A1	06-09-2013	US 2015057231 A1 26-02-2015
			WO 2013128239 A1 06-09-2013

FR 2662698	A1	06-12-1991	AT 143691 T 15-10-1996
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