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 (72) **Inventeurs/Inventors:**
 AHLIJANIAN, MICHAEL KIRK, US;
 MCNEISH, JOHN DOUGLAS, US
 (73) **Propriétaire/Owner:**
 PFIZER PRODUCTS INC., US
 (74) **Agent:** BERESKIN & PARR

(54) **Titre :** METHODE DE PREPARATION D'UN ANIMAL TRANSGENIQUE NON-HUMAIN EXPRIMANT LE P25
 (54) **Title:** METHOD OF PREPARING A TRANSGENIC NON-HUMAN ANIMAL EXPRESSING P25

(57) **Abrégé/Abstract:**

The invention provides transgenic, non-human animals and transgenic non-human mammalian cells harboring a transgene encoding a p25 (activator of the protein kinase cdk 5) polypeptide. The two neuropathological lesions associated with Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles (NFTs), composed predominantly of amyloid β peptides and hyperphosphorylated tau, respectively. While animal models for plaque formation exist, there is no animal model that recapitulates the formation of NFTs. This invention provides transgenic mice that overexpress human p25, an activator of cdk5, resulting in tau that is hyperphosphorylated at AD-relevant epitopes. Deposition of tau is detected in the amygdala, thalamus and cortex. Increased phosphorylated neurofilament, silver-positive neurons and neuronal death are also observed in these regions. We conclude that the overexpression of p25, an activator of cdk5, is sufficient to produce hyperphosphorylation of tau and neuronal death. The p25 transgenic mouse represents the first model for tau pathology in AD.

ABSTRACT

The invention provides transgenic, non-human animals and transgenic non-human mammalian cells harboring a transgene encoding a p25 (activator of the protein kinase cdk 5) polypeptide.

The two neuropathological lesions associated with Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles (NFTs), composed predominantly of amyloid β peptides and hyperphosphorylated tau, respectively. While animal models for plaque formation exist, there is no animal model that recapitulates the formation of NFTs. This invention provides transgenic mice that overexpress human p25, an activator of cdk5, resulting in tau that is hyperphosphorylated at AD-relevant epitopes. Deposition of tau is detected in the amygdala, thalamus and cortex. Increased phosphorylated neurofilament, silver-positive neurons and neuronal death are also observed in these regions. We conclude that the overexpression of p25, an activator of cdk5, is sufficient to produce hyperphosphorylation of tau and neuronal death. The p25 transgenic mouse represents the first model for tau pathology in AD.

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5 The invention provides transgenic, non-human animals and transgenic non-human mammalian cells harboring a transgene encoding a p25 polypeptide, an activator of the protein kinase cdk5. The invention also provides non-human animals and cells comprising a transgene encoding a p25 polypeptide and further comprising functional overexpression of p25, the p25 transgene and targeting constructs used to produce such transgenic cells and
10 animals, transgenes encoding human p25 polypeptide sequences and methods for using the transgenic animals in pharmaceutical screening and as commercial research animals for modeling neurodegenerative disease such as Alzheimer's disease and p25/cdk5 biochemistry in vivo.

15 Throughout the specification, a number of publications are cited. A complete listing of the publications appears later in the specification.

 Alzheimer's disease (AD) is a progressive, neurodegenerative disorder characterized by loss of cognitive function. The primary neuropathological lesions in AD are amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are composed primarily of
20 amyloid beta (Ab) peptides, varying in length from 39-42 amino acids, which are derived from amyloid precursor protein (APP) (reviewed in 1). NFTs are composed of the microtubule binding protein tau that is hyperphosphorylated at epitopes which exist in a predominantly unphosphorylated state in disease-free brain (2-4). The respective roles that these lesions play in the neuronal loss and dementia observed in patients with AD remain controversial.

25 The precise mechanism of NFT formation is not clear but work from many laboratories suggests that hyperphosphorylation of tau may be an important event. The paired helical filament (PHF) is the fundamental unit of the NFT. Hyperphosphorylation of tau at serine or threonine residues followed by proline (SP or TP) epitopes which are concentrated at the amino and carboxy termini of tau, results in loss of affinity for
30 microtubules, and a presumed concomittant increase in the concentration of cytoplasmic tau (5-8). However, phosphorylation of tau at serine 262, which is not followed by proline, can also reduce the affinity of tau for microtubules (9). *In vitro* experiments with purified tau show that in the presence of endogenous cations (e.g., mRNA, heparin sulfate proteoglycan), tau polymerizes to form structures indistinguishable from the PHF seen in AD brain (10,11). The
35 cation-dependent formation of PHF *in vitro* is independent of the phosphorylation state of tau, suggesting that the key permissive event in initiating PHF formation may be an increase in the cytoplasmic concentration of tau. In support of this hypothesis, recent evidence (12-14) indicates that the mutations in the *tau* gene associated with susceptibility to a form of inherited

5 dementia called frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), reduce the affinity of tau for microtubules (15). Therefore these mutations, like hyperphosphorylation, may result in an increase in cytoplasmic tau concentrations. In the presence of endogenous cations, this increase is presumed to permit PHF formation followed by NFT assembly and, ultimately, neuronal death.

10 As with other phosphoproteins, the phosphorylation state of tau is the sum of protein kinase and protein phosphatase activity. Thus hyperphosphorylation of tau in AD may be due to an increase in kinase activity or a decrease in phosphatase activity. While many protein kinases phosphorylate tau at AD-relevant epitopes in vitro (reviewed in 16,17), only two have been co-purified with microtubules from mammalian brain, GSK3b and cdk5 (18). To our
15 knowledge, only these two kinases will phosphorylate tau when transfected heterologously into mammalian cells (19,24). We chose to focus on cdk5 vs. GSK3b as the latter plays a role in energy metabolism and is expressed in an active form in all cells, while cdk5 is only active in neurons (vide infra).

The kinase cdk5 is a member of the cyclin-dependent protein kinase family and is
20 expressed in nearly all cells (reviewed in 25,26). Unlike other members of the cdk family, there is no known cyclin which activates cdk5. Rather, the positive allosteric regulators of cdk5 are p35 (27), amino terminal proteolytic fragments of p35, e.g, p25, p23 or p21 (28,29) and p39 (30). These proteins share minimal amino acid sequence homology to cyclins (27-29) but computer modeling and biochemical experiments suggests that the mechanism of
25 activation of cdk5 by p25/35 may be similar to that of cyclin A activation of cdk2 (31-33). The protein p25/35 is expressed predominantly in neurons implying that most cdk5 activity is concentrated in neuronal structures (27,28). The protein p35 has a relatively short half life within cells and is rapidly ubiquitinated, suggesting tight regulation of cdk5 activity in neurons (34). The kinase cdk5 plays a pivotal role in neuronal development as evidenced by the
30 abnormal corticogenesis and perinatal lethality of cdk5 knockout mice (35) and the disturbances in neuronal migration and early death in p35 knock-out mice (36). In developmental studies in rodents, the peak catalytic activity of cdk5 occurs at E11 or 12, lending further support for the role of cdk5 in neurogenesis (37,38). Furthermore, in primary cultured neurons, cdk5/p35 is localized to growth cones suggesting a role in neurite outgrowth
35 (39). Recently, evidence of signaling pathways which may modulate cdk5 activity have emerged. For example, it has been demonstrated that cdk5/p35 interacts with Rac and modulates PAK activity (40), and that laminin-enhanced outgrowth of cerebellar neurons is disrupted by suppression of p35 expression (41). A few substrates for cdk5 have been identified and are consistent with the presumed role in neurite outgrowth and plasma
40 membrane dynamics. These include cytoskeletal proteins such as tau (42-45) and

5 neurofilament (46-48), synaptic vesicle proteins such as synapsin and Munc-18 (49,50) and the retinoblastoma protein (51). Nevertheless, neither a clear picture of a signal transduction pathway(s) which regulates cdk5/p35 activity nor the role of both cdk5/p35 in mature brain have been elucidated.

A clear picture of the protein kinases responsible for the hyperphosphorylation of tau
10 in AD is also lacking. However, evidence that cdk5 may play a pathological role is accumulating. For example, in *in vitro* studies, cdk5 will phosphorylate up to eight different epitopes of tau, including those associated with AD and known to decrease the affinity of tau for microtubules (42-45). Additionally, heterologous co-transfection of cdk5/p25 with tau into mammalian cells also results in phosphorylation of several of these epitopes (24). Finally,
15 immunohistochemical evidence suggests that cdk5 is proximal to NFTs in AD brain (52,53).

The development of experimental models of Alzheimer's disease that can be used to define further the underlying biochemical events involved in AD pathogenesis would be highly desirable. Such models could be employed, in one application, to screen for agents that alter the degenerative course of AD. For example, a model system of AD could be used to screen
20 for environmental factors that induce or accelerate the pathogenesis of AD. Alternatively, an experimental model could be used to screen for agents that inhibit, prevent, or reverse the progression of AD. Such models could be employed to develop pharmaceuticals that are effective in preventing, arresting or reversing AD. Only humans and aged non-human primates develop any of the pathological features of AD. The expense and difficulty of using
25 primates and the length of time required for developing the AD pathology makes extensive research on such animals prohibitive. Rodents do not develop AD, even at an extreme age. Despite various reports that certain treatments result in hyperphosphorylation of tau and/or neuronal death associated with the phosphorylation of tau, there is a need in the art for transgenic non-human animals which can produce hyperphosphorylation of tau and
30 associated neuronal death.

Based on the above, it is clear that a need exists for nonhuman cells and nonhuman animals which produce hyperphosphorylation of tau and neuronal cell death. Thus, it is an object of the invention herein to provide methods and compositions for transferring transgenes and homologous recombination constructs into mammalian cells, especially into
35 embryonic stem cells. It is also an object of the invention to provide transgenic non-human cells and transgenic nonhuman animals harboring transgenes resulting in the increased expression of p25, an activator of cdk5. Of further interest to the present invention are the application of such transgenic animals as *in vivo* systems for screening test compounds for the ability to inhibit or prevent the production of hyperphosphorylated tau and associated
40 neuronal death. It is desirable to provide methods and systems for screening test compounds

5 for the ability to inhibit or prevent the phosphorylation of tau and associated neuronal death. In particular, it is be desirable to base such methods and systems on inhibition of cdk5/p25, where the test compound blocks phosphorylation of tau mediated by cdk5/p25, the test compound also blocks neuronal death. Such methods and transgenic animals should provide a rapid, economical and suitable way for screening large numbers of test compounds.

10 We overexpressed human p25 in the brains of mice to determine if an increase in cdk5 activity would result in the hyperphosphorylation of tau at AD-relevant epitopes, and if this hyperphosphorylation would lead to neuronal death. In the brains of p25 transgenic mice, both tau and neurofilament are hyperphosphorylated, and many silver-positive neurons with tangle-like inclusions are present. The silver-positive neurons suggest ongoing neuronal
15 death. These results demonstrate that overexpression of an activator of cdk5 is sufficient to produce tau and neurofilament phosphorylation and silver-positive neurons which are very similar to those seen in AD. The p25 transgenic mouse may serve as a model for the neurofibrillary pathology and neuronal death seen in AD.

Summary of the Invention

20 In one embodiment, the present invention is directed to a purified and isolated recombinant polynucleotide comprising a neuron specific enolase promoter operably linked to a p25 encoding sequence.

In a preferred embodiment, the present invention is directed to recombinant DNA wherein said sequence encoding said p25 protein is genomic DNA.

25 In another preferred embodiment, the present invention is directed to recombinant DNA wherein said sequence encoding said p25 protein is cDNA.

In still another preferred embodiment, the present invention is directed to recombinant DNA wherein said sequence is that of SEQ ID NO: 4

30 In yet another embodiment, the present invention is directed to a vector comprising recombinant DNA according to the present invention.

In another embodiment, the present invention is directed to eukaryotic cell comprising recombinant DNA according to the present invention.

35 In another embodiment, the present invention is directed to a transgenic non-human animal, or progeny thereof, whose germ cells and somatic cells express recombinant DNA according to the present invention

In a preferred embodiment, the present invention is directed to a transgenic non-human animal, or progeny thereof, which is a mouse.

In a further embodiment, the present invention is directed to a method for treating an animal having a disease characterized by the expression of a p25 fragment of a human cdK5

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gene comprising administering a therapeutically effective amount of an inhibitor of said p25 fragment.

In another embodiment, the present invention is directed to a method for determining the ability of a compound to inhibit the expression of a p25 protein comprising the steps of:

- 5 a. creating a transgenic non-human animal by stably incorporating into the embryonic stem cells of said animal a recombinant polynucleotide comprising a neuron-specific enolase promoter operably linked to an encoding sequence for p25;
- b. growing said embryonic stem cells into a mature transgenic non-human animal;
- 10 c. administering to said transgenic non-human animal the compound of interest;
- d. measuring the inhibition of said p25 protein by said compound.

In still another embodiment the present invention is directed to a method for generating data to determining the ability of a compound to inhibit the expression of a p25 protein comprising the steps of:

- a. creating a transgenic non-human animal by stably incorporating into the embryonic stem cells of said animal a recombinant polynucleotide comprising a neuron-specific enolase promoter operably linked to an encoding sequence for p25;
- b. growing said embryonic stem cells into a mature transgenic non-
20 human animal;
- c. administering to said transgenic non-human animal the compound of interest;
- d. measuring the inhibition of said p25 protein by said compound;
- e. using the data derived from said inhibition to synthesize compounds
25 capable of inhibiting said p25 protein.

Figure 1A. Western blots of the amygdala (1), thalamus (2) and cortex (3) from 3 wild type mice were probed with an antibody specific for p25 and p35/39 (generous gift of L.-H. Tsai, Harvard Medical School Cambridge, MA).

30 Figure 1B. Western blots of the amygdala (1), thalamus (2) and cortex (3) from 3 p25 transgenic mice were probed with the same antibody as in FIG 1A.

Expression of the transgene, p25, is apparent in the transgenic but not wild type mice.

35 Figure 2 displays a four month old transgenic mouse brain immunopositive for AT-8 (a commercially available antibody which specifically recognizes phospho-serine 202/205 of tau) specific in the rostral portion of the amygdala. Neuronal cell bodies with accompanying axons (arrows) are positive. Several dark-brown positive cells are seen in this field.

5 Figure 3 shows a four month old wild-type mouse brain minimally positive for AT-8 in the rostral portion of the amygdala. A cell body exhibits non-specific positivity (arrow) due to the secondary antibody.

 Figure 4 shows a four month old transgenic mouse brain demonstrating PHF-13 (an antibody which recognizes phospho-serine 396/404 of tau; (gift from Dr. V. Lee, U. of
10 Pennsylvania) immunopositivity in the rostral portion of the amygdala. Neuronal cell body with a thickened axon (arrow) is positive. Positive neuronal cell body (arrow head) with possible swollen/atrophied axon.

 Figure 5 shows a four month old wild-type mouse brain minimally positive for PHF-13 in the rostral portion of the amygdala. Cell bodies exhibit non-specific positivity (arrows) due
15 to the secondary antibody.

 Figure 6 shows a four month old transgenic mouse brain immunopositive for total tau (an antibody which recognizes total tau commercially available from Accurate Chemical and Scientific Corp Westbury, N.Y.) in several cell bodies of the rostral amygdala (examples marked by arrows).

20 Figure 7 shows a four month old wild-type mouse brain immunopositive for tau in axons (arrow heads) of the rostral amygdala.

 Figure 8 shows a four month old transgenic mouse brain reacted with SMI-34 (a commercially available antibody which recognizes phosphorylated neurofilament H protein). Positive cells (arrows) are seen in the rostral amygdala.

25 Figure 9 shows minimal SMI-34 axonal positivity in the rostral amygdala of a four month old wild-type mouse brain.

 Figure 10 shows silver-positive (an indication of disrupted cytoskeleton and neuronal cell death) cell bodies (arrows) in the rostral amygdala of a four month old transgenic mouse brain.

30 Figure 11 shows minimal silver positivity of axons in the rostral amygdala of a four month old wild type mouse.

 Figure 12 shows several dilated axons (arrows) that exhibit silver positivity in the spinal cord of a five month old transgenic mouse.

 Figure 13 shows several dilated axons (arrows) that exhibit SMI-34 positivity in the
35 spinal cord of a five month old transgenic mouse.

 Figure 14 shows that normal axons in the spinal cord of a six month old wild-type mouse are negative for SMI-34.

 Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, nucleic acid chemistry and hybridization, biochemistry, histology
40 and immunocytochemistry described below are those well known and commonly described in

5 the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, cell culture, transgene incorporation, Western blotting, immunocytochemistry and histological techniques such as silver staining. The techniques and procedures are generally performed according to conventional methods in the art and various general references which are provided throughout this specification. The procedures therein are well known in the art
10 and are provided for the convenience of the reader.

In accordance with the foregoing objects, in one aspect of the invention are provided nonhuman animals harboring at least one copy of a transgene comprising a polynucleotide sequence that encodes a heterologous protein operably linked to regulatory elements that are
15 capable of expressing the heterologous protein in the transgenic nonhuman animal. Said heterologous polypeptide is a truncated portion of the p35 cyclin dependent kinase 5 (cdk5) regulatory protein (27,28). The truncated heterologous polypeptide has the first 98 N-terminal amino acids removed and has a molecular weight of approximately 25,000 daltons. This novel polypeptide will be referred to as p25. Typically, the nonhuman transgenic animal is a mouse
20 and the heterologous gene is the human p25 sequence. Transgenes are typically cDNA sequences that have been operably linked to cis-acting regulatory sequences that direct expression in the host transgenic mammal in cell-type specific manner, typically in neurons. Typically, the transgene will be incorporated into the host chromosomes in random, non-targeted fashion. The invention further provides that the nonhuman, transgenic animal
25 harboring at least one copy of the heterologous p25 sequence transgene or gene targeting vector of the invention either non-homologously or homologously integrated into the chromosomal location express the p25 truncated polypeptide. Transgenic animals are typically produced by introduction of a transgene by microinjection into pronuclei of one-cell embryos or a targeting vector into the host by electroporation, lipofection, or viral transfection
30 of embryonic stem (ES) cells. The transgenic animals that express the p25 polypeptide are suitable for use as models of disease and screening of potential therapeutic compounds including small molecules, proteins and polynucleotides. The invention also provides nonhuman or human cell lines to be derived by transformation of established cell lines or primary cell lines established directly from the nonhuman transgenic animal, for example
35 neurons. It is obvious that the transgenic nonhuman animal can have additional genetic modifications by transgenic art or through traditional matings with other transgenic or naturally occurring animals to produce novel animals that serve as alternative disease models, drug screens or other applications. This includes the inactivation of the murine endogenous p35 gene through gene targeting in ES cells and resultant murine p35-deficient mice that becomes
40 the preferred host for expression of the human p25 heterologous polypeptide. Such

5 heterologous transgenes may be integrated in nonhomologous chromosomal locations in the transgenic animal, typically derived by pronuclear injection or may be integrated by gene targeting in ES cells by methods to inactivate the murine p35 gene and add linked sequences to direct expression of the heterologous human p25 sequences.

10 In general, the invention encompasses methods for the generation and characterization of transgenic animals that express the human p25 polypeptide, a proteolytic fragment of p35, both of which are an allosteric activators of cyclin dependent kinase 5 (cdk5). The transgenic animals express this human protein in the presence of the endogenous homologue. The techniques and procedures are performed according to established protocols that are generally considered to be routine methods in the art and references are provided within this specification. The protein kinase cdk5 when associated with an allosteric activator, 15 e.g., p35 or p25 is reported in the literature to phosphorylate tau *in vitro* and in whole cells (see Background of the Invention). The transgenic mice thus produced establish the role of the human p25 in the formation of hyperphosphorylated tau in neurodegenerative conditions including Alzheimer's disease, Parkinson's disease, amyelolateral sclerosis, Huntington's chorea, stroke, traumatic brain injury, Pick's disease, neuraxonal dystrophy, multiple 20 sclerosis, motor neuron disease, and spinocerebellar degeneration and other neurodegenerative diseases. It is apparent that the preparation of other transgenic animals that express the human p25 protein is easily accomplished including rats, hamsters, guinea pigs and rabbits. The transgenic animals that express the human p25 protein can be monitored for the level of expression and tau phosphorylation. It will be appreciated that under different conditions the level of expression and degree of tau phosphorylation will be inhibited in such an animal model. In particular, the screening of therapeutic agents that inhibit the activity of the human p25 protein will be greatly facilitated in animal models. It is apparent that the development of cell lines from the affected transgenic animals, (e.g., neurons) will improve 25 the throughput in which biochemical and pharmacological analysis of the human p25 protein can be assessed.

Particularly preferred animal models for p25 overexpression are transgenic animals which express p25 as described above. Such transgenic animals, particularly transgenic mice according to this invention, produce high quantities of p25 and hyperphosphorylated tau 35 and neuronal death which may be detected according to the methods of the present invention. In accordance to this invention particular, the overexpression of p25 will be equal to or greater than endogenous p25 expression in such animals. Further, this level of p25 overexpression results in tau hyperphosphorylation which is minimal in wild type animals. With such elevated levels of p25, monitoring of hyperphosphorylation of tau and neuronal death is greatly 40 facilitated. In particular, screening for compounds and other therapies for inhibiting tau

5 phosphorylation are greatly simplified in animals overexpressing p25 according to this invention.

Agents are administered to test animals, such as test mice, which are transgenic and which overexpress p25. Particular techniques for producing transgenic mice which overexpress p25 are described below. It will be appreciated that the preparation of other
10 transgenic animals overexpressing p25 may easily be accomplished, including rats, hamsters, guinea pigs, rabbits, and the like. In light of this disclosure, the effect of test compounds on the hyperphosphorylation of tau in the test animals may be measured in various specimens from the test animals.

The effect of test agents on hyperphosphorylation of tau may be measured in various
15 specimens from the test animals. In all cases, it will be necessary to obtain a control value which is characteristic of the level of tau phosphorylation and neuronal death in the test animal in the absence of the test compound(s). In cases where the animal is sacrificed, it will be necessary to base such control values on an average or a typical value from other test animals which have been transgenically modified to overexpress p25 but which have not
20 received the administration of any test compounds or any other substances expected to affect the level of tau phosphorylation or neuronal death. Once such control level is determined, test compounds can be administered to additional test animals, where deviation from the average control value indicates that the test compound had an effect on the tau phosphorylation or neuronal death in the animal. Test substances considered positive, i.e., likely to be beneficial
25 in the treatment of AD or other neurodegenerative diseases, will be those which are able to reduce the level of tau phosphorylation or neuronal death preferably by at least 20% and most preferably by 80%. In addition there may be paired helical or straight filament formation in transgenic animals which overexpress p25 and display tau hyperphosphorylation. In these cases, test compounds can be administered to test animals and the reduction of filament
30 formation monitored as a result of exposure to the compound. In addition, there may be behavioral alterations in the transgenic animals which overexpress p25 and display tau hyperphosphorylation. In these cases it will be necessary to obtain a control value from live animals performing a behavioral task (e.g., the measurement of locomotor activity) in the test animal in the absence of test compound(s). Such a control will also be determined in non-
35 transgenic, wild type mice. The difference between the wild type and transgenic mice will serve as the outcome measure for the effects of compounds. Once such control levels are determined, test compounds can be administered to additional test animals, where reduction in or reversal of the difference between the wild type and transgenic mice indicates that test compound has an effect on the behavioral test being measured. Test substances considered
40 positive, i.e., likely to be beneficial in the treatment of AD or other neurodegenerative diseases,

5 preferably will be those which are able to reverse or, substantially reverse, or favorably modify the behavioral abnormality in the transgenic animal to the level found in wild type mice.

Test agents will be defined as any small molecule, protein, polysaccharides, deoxy or ribonucleotides, or any combination thereof that when added to the cell culture or animal will not adversely interfere with the cell or animal viability. Agents that alter the level of human p25
10 expression and tau phosphorylation will be considered as candidates for further evaluation as potential therapeutics. The test compound will typically be administered to transgenic animals at a dosage of from 1ng/kg to 100mg/kg, usually from 10ug/kg to 32 mg/kg.

Test compounds which are able to inhibit phosphorylation of tau are considered as candidates for further determinations of the ability to block tau phosphorylation in animals and
15 humans. Inhibition of tau phosphorylation indicates that cdk5/p25 activity has been at least partly blocked, reducing the amount of cdk5/p25 available to phosphorylate tau.

The present invention further comprises pharmaceutical compositions incorporating a compound selected by the above-described method and including a pharmaceutically acceptable carrier. Such pharmaceutical compositions should contain a therapeutic or
20 prophylactic amount of at least one compound identified by the method of the present invention. The pharmaceutically acceptable carrier can be any compatible, non-toxic substance suitable to deliver the compound or compounds to an intended host. Sterile water, alcohol, fats, waxes and inert solids may be used as the carrier. Pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like may also be incorporated into the
25 pharmaceutical compositions. Preparation of pharmaceutical compositions incorporating active agents is well described in the medical and scientific literature. See, for example, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, 16th Ed., 1982.

The pharmaceutical compositions just described are suitable for systemic
30 administration to the host, including both parenteral, topical, and oral administration. The pharmaceutical compositions may be administered parenterally, i.e., subcutaneously, intramuscularly or intravenously. Thus, the present invention provides compositions for administration to a host where the compositions comprise a pharmaceutically acceptable solution of the identified compound in an acceptable carrier, as described above.

35 Commercial Research and Screening Uses

Non-human animals comprising transgenes which encode p25 can be used commercially to screen for agents having the effect of preventing or reducing the phosphorylation of tau and neuronal death. Such agents can be developed as pharmaceuticals for treating hyperphosphorylation of tau and AD amongst other
40 neurodegenerative diseases. For example, the p53 knockout mice of Donehower et al. (54)

5 have found wide acceptance as commercial products for carcinogen screening and the like. The transgenic animals of the present invention exhibit abnormal tau phosphorylation and can be used for pharmaceutical screening and as disease models for neurodegenerative diseases and cdk5/p25 and tau biochemistry. Such animals have many uses including but not limited to identifying compounds that affect tau hyperphosphorylation; in one variation, the agents are
10 thereby identified as candidate pharmaceutical agents. The transgenic animals can also be used to develop agents that modulate cdk5 or p25 expression and or stability; such agents can serve as therapeutic agents to treat neurodegenerative diseases. The p25 overexpressing mice of the invention can also serve as disease models for investigating tau-related pathologies (e.g., AD, Pick's disease, Parkinson's disease, frontal temporal lobe dementia
15 associated with chromosome 17, stroke, traumatic brain injury, mild cognitive impairment and the like). Such transgenic animals can be commercially marketed to researchers, among other uses.

Having described the invention in general terms, reference is now made to specific examples. It is to be understood that these examples are not meant to limit the present
20 invention, the scope of which is to be determined by the appended claims.

The plasmid pNSE-p25 (rat neuron specific enolase promoter, human p25 transgene) contains DNA from 4 different sources: the rat neuron specific enolase promoter; the human cDNA for the p25 catalytic fragment of the p35 protein; the SV40 polyadenylation signal sequence and a commercially available plasmid vector. The transgene is isolated by BamH1
25 restriction enzyme digestion of pNSE-p25. This restriction digest releases the NSE-p25 transgene (3212 bp) from the plasmid cloning vector pSP72 (2462 bp) (Promega, Madison, WI). The rat Neuron Specific Enolase (NSE) promoter sequence is 1826 bp (SEQ ID NO: 1) and has been demonstrated to efficiently express DNA coding sequences in neurons of transgenic mice (55). The coding region for the human p25 sequence is 633 bp. (SEQ ID NO:
30 2). The 3-prime untranslated and SV40 polyadenylation sequence are 688 bp (SEQ ID NO: 3). The microinjected NSE-p25 transgene is 3212 bp (SEQ ID NO: 4). Enzymatic reactions of recombinant DNA, including ligations, restriction endonuclease digestions, DNA synthesis reactions, single strand fill-in reactions, and bacterial transformations performed are well established procedures as described by Sambrook, J., Fritsch, E.F. and Maniatis, T.
35 **Molecular Cloning: A Laboratory Manual.** 2nd edition, Cold Spring Harbor Press, New York, 1989.

The SV 40 polyadenylation sequence (SEQ ID NO: 3) was cloned into the commercially available plasmid pSP72 (SEQ ID NO: 5) at Xba1 and BstX1 sites. This plasmid, designated pSP-SVpA, served as the backbone vector for the construction of the
40 NSE-p25 transgene plasmid. The cDNA for the human p25 catalytic fragment was derived

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5 using polymerase chain reaction (PCR). The human p35 full length cDNA was used as the template for amplification of the p25 insert. We received the p35 template DNA from Dr. David Auperin of Pfizer; the human p35 sequence was cloned into the EcoR1 site of the pFastBac1, baculoviral expression vector (Gibco-BRL, Gaithersburg, MD). The PCR primers were designed to have Not1 restriction sites outside the coding sequence to facilitate transgene

10 construction. The forward primer also introduced a new ATG start codon adjacent to the GCC (Ala codon at position 99 of the human p35 sequence). The primers (submitted in 5-prime to 3-prime orientation) were p25-forward:

GCGGCCGCATGGCCCAGCCCCACCGGCCAGCCGCCTGCA

(SEQ ID NO: 6) and p25-reverse:

15 CTCCTCCTAGGCCTGGAATCGGTGAGGCGGCCGC (SEQ ID NO: 7). The ATG is introduced start codon, the TGA is the stop codon and the GCGGCCGC are the Not1 restriction sites added to the PCR primers. The resulting PCR product is 648 bp containing the entire 633 bp human p25 cDNA sequence with engineered ATG start site (SEQ ID NO: 2) and an additional 15 bp from addition of the Not 1 cloning sites. The 648 bp fragment was

20 subcloned into the pCR2.1 (Invitrogen, Carlsbad, CA) vector for verification of sequence prior to use as insert in the final transgene. The confirmed 648 bp Not 1 fragment was ligated into the Not 1 restriction site of pSP-SVpA producing the plasmid pSP-SVpA:p25. The rat Neuron Specific Enolase (NSE) promoter was isolated from the plasmid pNSE-lacZ obtained from Dr. J.G. Sutcliffe of the Research Institute of Scripps Clinic (LaJolla, CA). The rat NSE promoter

25 sequence (SEQ ID NO: 1) was isolated by EcoR1 and Hind III restriction digestion of the pNSE-lacZ plasmid. The 1.8 kb fragment was extracted by agarose gel electrophoresis and purified. The 5-prime single strand overhangs generated by the restriction enzymes were filled by Klenow reaction to produce the rat NSE promoter fragment with double stranded blunt ends. The 1826 bp rat NSE blunt end promoter fragment was blunt end ligated into the Sma I

30 restriction site of pSP-SVpA:p25. The correct orientation of the rat NSE promoter within the pSP-SVpA:p25 was verified by double restriction digests of BamH1 and Xho1. Plasmids with the correct orientation were assigned the designation as pNSE-p25. The entire sequence of the 3212 bp NSE-p25 transgene (SEQ ID NO: 4) was analyzed and verified by automated sequencing on ABI 373 sequencer (Foster City, CA) using the standard dye-terminator

35 chemistry protocol outlined by ABI. All plasmids were grown in DH5alpha bacterial cells (Gibco BRL Gaithersburg MD).

Production of transgenic mice overexpressing human p25.

The 3212 bp NSE-p25 transgene DNA fragment was excised from the pNSE-p25 plasmid by BamH1 restriction endonuclease reaction. The 3.2 kbp fragment was isolated by

40 electroelution (50V, 3 hrs) after electrophoresis in 1% agarose gel (FMC Bioproducts,

5 Rockland ME). The fragment was further purified by on a Schleicher and Schuell (Keene, NH) Elutip-d column following protocols established by the manufacturer for DNA purification prior to murine embryo microinjection.

Production of transgenic mice by pronuclear microinjection was carried out by published procedures as outlined in Hogan, B. et al **Manipulating the Mouse Embryo: A**
10 **Laboratory Manual** 2nd edition, Cold Spring Harbor Laboratories, New York, 1994. Pronuclear stage embryos from F1 females mice of the strain FVB/N (Charles River Labs, Wilmington, MA) were obtained after superovulation with 5 international units (IU) of follicle stimulating hormone from pregnant mare serum (Sigma St Louis, Mo.) and 2.5 I.U. human chorionic gonadotropin (Sigma). The actual microinjection procedure was performed as
15 described by Wagner, T. et al. (56) except that the embryos were transferred immediately to pseudopregnant CD-1 recipient females (Charles River Laboratories, Wilmington, MA) for development of embryos to term. Mice resulting from the reimplantation events were tested for the presence of the NSE-p25 transgene by PCR analysis of genomic DNA isolated from tail biopsies at 3 weeks of age. The mice that demonstrated positive for the presence of the NSE-
20 p25 transgene were mated with wild type FVB/N mice (Charles River Laboratories, Wilmington, MA) of the opposite sex. Offspring of these matings were tested for germline transmission by PCR analysis or Southern blot analysis of genomic DNA isolated from tail biopsies at 3 weeks of age. Transgenic lines were produced from 7 founder transgenic mice and were maintained by breeding to wild type FVB/N mice and PCR genotyping for the
25 presence of the NSE-p25 transgene.

The experiments described below were done in mice heterozygous for the inserted transgene. These mice were derived by breeding mice positive for the transgene insert with FVB/N wild-type animals and screening offspring by PCR for presence of the NSE-p25 transgene sequences.

30 **Detection of overexpression of p25 protein.**

Western Blot

Whole brains from 1 or 4 month old transgenic (Tg) and wild type (wt) mice were removed and snap frozen in liquid nitrogen. Amygdala, thalamus and cortex were dissected and homogenized in 1 ml of lysis buffer (as described in 37). The samples were boiled for 10
35 minutes and then centrifuged at 13,000 rpm. Protein concentrations of the resulting supernatants were determined by the Pierce BCA method (BCA micro protein assay, catalog #23225, Pierce, Rockford ILL). Ten micrograms of each sample were electrophoresed on an SDS polyacrylamide gel (57) and transferred to ProBlott protein paper for western blot analysis [Western blotting protocols, ECL detection, Amersham Life Science Arlington
40 Heights, ILL (1995)]. Non specific sites were blocked by incubating the blots in 5% nonfat

5 milk in tris-buffered saline (20mM Tris Base, 127 mM NaCl, 3.8 mM HCl pH 7.6, Sigma) included 0.1% tween-20 (TBS-T). Primary antibodies were diluted in 5% nonfat milk\TBS-T. The blots were placed in the diluted antibody solutions and rotated for 1 hr at 23°C. The western blots were then washed for 30 min in TBS-T. Secondary horseradish peroxidase linked antibodies were diluted in 5% nonfat milk\TBS-T. The blots were incubated with the
10 secondary antibody solution and rotated for 45 min at 23°C. The blots were then washed for 30 min in TBS-T. Equal volumes of Amersham's ECL developing solutions A and B were mixed together. The Western blots were incubated for 1 min in the developing solution. The blots were wrapped in Saran ® wrap and then exposed to imaging film (Kodak Rochester N.Y. X-OMAT AR™, catalog #165 1454).

15 Western blots of the amygdala (1), thalamus (2) and cortex (3) from 3 mice were probed with an antibody specific for p25 and p35/39 (gift of L.-H. Tsai, Harvard Medical School, Figure 1A). While detection of p35/39 is apparent, no p25 is detected in wild type mice. When the same analysis is performed in the transgenic mice (Figure 1B), robust expression of p25 in addition to the constitutive expression of p35 is detected. These results
20 confirm that expression of the transgene is robust in the p25 transgenic animals.

Silver Stain & SMI 34 Immunohistochemistry

Tissues collected from scheduled sacrifice mice were perfusion-fixed in situ with 10% neutral-buffered formalin or 4% paraformaldehyde, whereas tissues collected from mice found dead were immersion fixed in formalin. Following fixation, trimmed tissues were dehydrated
25 through graded alcohols and embedded in paraffin. Standard cross sections of brain were identified by analogy to figures in The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 1997). Paraffin sections (8µm thick) were stained with modified Bielschowsky silver stain and, immunohistochemically, with an antibody directed against phosphorylated neurofilament (SMI 34, commercially available from Sternberger Monoclonals, Inc Lutherville,
30 MD).

Immunohistochemistry

For immunohistochemical studies, transgenic and wild type mice were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the ascending aorta with 20 ml of 0.1M NaPO₄ containing 0.9% NaCl (PBS, 7.4) followed by 30 ml of fixative
35 containing 4% paraformaldehyde in 0.1M NaPO₄ (PB, 7.4) buffer. The brains were removed, cryoprotected for 48 hr in PBS containing 20% sucrose, frozen in a bed of pulverized dry ice, and then cut into 35 micron sections on a sliding microtome. Consecutive 1 in 18 series of sections were collected in 0.1M PB and processed for immunohistochemistry as described below.

5 For immunohistochemistry, sections were incubated for 1 hr in 50 mM Tris buffer, pH 7.4 containing 0.9% NaCl (TBS) and 0.1% Triton-X-100™. The sections were then washed in TBS and incubated for 1 hr in antibody vehicle (4% goat or horse serum, 0.1% Triton-X-100™ in TBS). After additional washes in TBS, sections were incubated overnight at 4°C in vehicle containing the primary antibody.

10 Following overnight incubation in the primary antibody, sections were washed in TBS, incubated for 1 hr in vehicle containing biotinylated horse anti-mouse or goat anti-rabbit antibody (as per manufacturer, Vector Labs, Burlingame, CA.), washed in TBS, and then incubated for 1 hr in TBS containing ABC/horseradish peroxidase reagent (Vector Labs, Burlingame, CA).

15 After additional washes in TBS, immunolocalization products were visualized by developing sections for 3 - 5 min in 50mM Tris buffer (pH 7.6) containing 0.04% diaminobenzidine (DAB) and 0.003% H₂O₂. Sections were subsequently washed in the Tris buffer, mounted onto slides and dehydrated and coverslipped using DPX (Fluka, Ronkokoma, N.Y.).

20 Antibodies

AT-8:

Used at 1:1,000 (200ng/ml), mouse monoclonal which recognizes ser 202 and thr 205 of human phosphorylated tau. (Innogenetics, Inc. Zwijndrecht (Belgium))

PHF-13:

25 Used at 1: 20,000, mouse monoclonal which recognizes ser 396 and thr 404 of human phosphorylated tau. (Gift from V. Lee, University of Pennsylvania)

anti-TAU:

Used at 1:7,000, rabbit polyclonal which recognizes total tau. (Accurate Chemical and Scientific Corp Westbury, N.Y.).

30 SMI 34:

Used at 1:1000, mouse monoclonal which recognizes phosphorylated neurofilament.H (Sternberger Monoclonals, Inc., Lutherville MD)

35 Brains from four month old transgenic and wild type mice were compared for the presence of tau and neurofilament phosphoepitopes by immunohistochemistry. AT-8 and PHF-13, monoclonal antibodies which recognize phosphorylated Ser-202 and Thr-205, and Ser-396,404, respectively, labeled neurons in amygdala (Figs. 2 and 4, respectively), thalamus/hypothalamus and cortex adjacent to external capsule (not shown) of transgenic animals but not in corresponding regions of wild-type animals (Figs. 3 and 5). In addition, staining for total tau revealed numerous cell bodies in p25 transgenic animals (Fig. 6) while
40 mostly axons and very few cell bodies were labeled in wild type mice (Fig. 7). A monoclonal

5 antibody (SMI34) recognizing phosphorylated neurofilament H also showed increased immunostaining in these three brain regions and spinal cord of transgenic mice (amygdala is depicted in Fig. 8), but not in wild type (Fig. 9). All three of these markers are known to be increased in Alzheimer brain relative to age-matched control.

In Alzheimer's brain, pathological changes in neurons containing altered tau proteins
10 have classically been identified using silver-based staining methods. We found that, in the transgenic mice of this invention, neurons in the same three brain regions described above and spinal cord show positive labelling using the modified Bielschowsky silver stain (amygdala, Fig. 10). This staining was once again absent in wild-type control animals (Fig. 11). We also detected increases in silver staining and phosphorylated neurofilament H in
15 obviously enlarged axons in the spinal cord of transgenic mice (Figs. 12 and 13, respectively), while in wild type mice, axons were of the expected diameter and neurofilament staining was normal (Fig. 14).

With regard to AT-8 immunoreactivity, most labelled neurons in the amygdala exhibited well-defined, densely staining soma, often with a swollen, dystrophic hillock and
20 contorted axon (Fig. 2). Less commonly, diffuse labelling of unidentified cells was seen, and occasionally cells having astroglial or microglial morphology were labelled. In cortex adjacent to external capsule, the majority of AT-8 staining was found in intensely-labelled neuronal cell bodies, often accompanied by axons when present in the plane of the section. Thalamic/hypothalamic staining was more diverse, including both neuronal staining as seen in
25 other sections.

Histologic examination of brain and spinal cord from p25 Tg mice revealed changes in neurons and axons of brain or spinal cord (n = 19) but not in these tissues from 10 age-matched, wild-type controls. Axonal changes were most pronounced in the spinal cord (cervical and thoracic) and consisted of marked dilation of axoplasm to diameters of 20 to 50
30 μm . Dilated axons were filled with both silver staining and phosphorylated neurofilament H (Figs. 12, 13). A given cross section of affected cord generally contained between 10 and 100 overtly dilated axons distributed among all funiculi of white matter. Luxol fast blue staining illustrated dissolution of the internal portion of myelin sheath of affected axons (not shown).

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- 22 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Pfizer Products Inc.
- (ii) TITLE OF INVENTION: Transgenic Animals Expressing Human P25
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: BERESKIN & PARR
 - (B) STREET: 40 King Street West
 - (C) CITY: Toronto
 - (D) STATE: Ontario
 - (E) COUNTRY: Canada
 - (F) ZIP: M5H 3Y2
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC™ compatible
 - (C) OPERATING SYSTEM: PC-DOS™/MS-DOS™
 - (D) SOFTWARE: PatentIn™ Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: CA 2,295,861
 - (B) FILING DATE: 03-FEB-2000
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/118,478
 - (B) FILING DATE: 03-FEB-1999
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Rudolph, John R.
 - (B) REGISTRATION NUMBER: 4213
 - (C) REFERENCE/DOCKET NUMBER: 10986-12
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (416) 364-7311
 - (B) TELEFAX: (416) 361-1398

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1867 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: other nucleic acid
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
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- 23 -

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| CGGCCGC    |            |            |            |             |             |            | 1867 |

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 650 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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| CGGGTTCCTA | GACCGGGGGC | TCCTCCTCAG | TCAAGAAAGC | CCCTCACCT  | GCCGTCACCT | 120 |
| CCGCAGGGAC | GCCCAAACGG | GTCATCGTCC | AGGCGTCCAC | CAGTGAGCTG | CTTCGCTGCC | 180 |
| TGGGTGAGTT | TCTCTGCCGC | CGGTGCTACC | GCCTGAAGCA | CCTGTCCCC  | ACGGACCCCG | 240 |
| TGCTCTGGCT | GCGCAGCGTG | GACCGCTCGC | TGCTTCTGCA | GGGCTGGCAG | GACCAGGGCT | 300 |
| TCATCACGCC | GGCCAACGTG | GTCTTCCTCT | ACATGCTCTG | CAGGGATGTT | ATCTCCTCCG | 360 |
| AGGTGGGCTC | GGATCACGAG | CTCCAGGCCG | TCCTGCTGAC | ATGCCTGTAC | CTCTCCTACT | 420 |
| CCTACATGGG | CAACGAGATC | TCCTACCCGC | TCAAGCCCTT | CCTGGTGGAG | AGCTGCAAGG | 480 |
| AGGCCTTTTG | GGACCGTTGC | CTCTCTGTCA | TCAACCTCAT | GAGCTCAAAG | ATGCTGCAGA | 540 |
| TAAATGCCGA | CCCACACTAC | TPCACACAGG | TCTTCTCCGA | CCTGAAGAAC | GAGAGCGGCC | 600 |
| AGGAGGACAA | GAAGCGGCTC | CTCCTAGGCC | TGGATCGGTG | AGGCGGCCGC |            | 650 |

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 717 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

|            |            |            |            |            |             |     |
|------------|------------|------------|------------|------------|-------------|-----|
| GCGGCCGCGA | CTCTAGAGGA | TCTTTGTGAA | GGAACCTTAC | TTCTGTGGTG | TGACATAATT  | 60  |
| GGACAAACTA | CCTACAGAGA | TTTAAAGCTC | TAAGGTAAAT | ATAAAATTTT | TAAGTGTATA  | 120 |
| ATGTGTTAAA | CTACTGATTC | TAATTGTTTG | TGTATTTTAG | ATTCCAACCT | ATGGAACCTGA | 180 |
| TGAATGGGAG | CAGTGGTGGG | ATGCCTTTAA | TGAGGAAAAC | CTGTTTTGCT | CAGAAGAAAT  | 240 |
| GCCATCTAGT | GATGATGAGG | CTACTGCTGA | CTCTCAACAT | TCTACTCCTC | CAAAAAGAA   | 300 |

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|            |            |            |            |            |             |     |
|------------|------------|------------|------------|------------|-------------|-----|
| GAGAAAGGTA | GAAGACCCCA | AGGACTTTCC | TTCAGAATTG | CTAAGTTTTT | TGAGTCATGC  | 360 |
| TGTGTTTAGT | AATAGAACTC | TTGCTTGCTT | TGCTATTTAC | ACCACAAAGG | AAAAAGCTGC  | 420 |
| ACTGCTATAC | AAGAAAATTA | TGGAAAAATA | TTTGATGTAT | AGTGCCTTGA | CTAGAGATCA  | 480 |
| TAATCAGCCA | TACCACATTT | GTAGAGGTTT | TACTTGCTTT | AAAAAACCTC | CCACACCTCC  | 540 |
| CCCTGAACCT | GAAACATAAA | ATGAATGCAA | TTGTTGTTGT | TAACCTGTTT | ATTGCAGCTT  | 600 |
| ATAATGGTTA | CAAATAAAGC | AATAGCATCA | CAAATTTTAC | AAATAAAGCA | TTTTTTTTCAC | 660 |
| TGCATTCTAG | TTGTGGTTTG | TCCAAACTCA | TCAATGTATC | TTATCATGTC | TGGATCC     | 717 |

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3218 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

|             |            |            |            |            |             |      |
|-------------|------------|------------|------------|------------|-------------|------|
| GGATCCCCAA  | TTCGAGCTCC | TCCTCTGCTC | GCCCAATCCT | TCCAACCCCC | TATGGTGGTA  | 60   |
| TGGCTGACAC  | AGAAAATGTC | TGCTCCTGTA | TGGGACATTT | GCCCCTCTTC | TCCAAATATA  | 120  |
| AGACAGGATG  | AGGCCTAGCT | TTTGCTGCTC | CAAAGTTTTA | AAAGAACACA | TTGCACGGCA  | 180  |
| TTTAGGGACT  | CTAAAGGGTG | GAGGAGGAAT | GAGGGAATTG | CATCATGCCA | AGGCTGGTCC  | 240  |
| TCATCCATCA  | CTGCTTCCAG | GGCCCAGAGT | GGCTTCCAGG | AGGTATTCTT | ACAAAGGAAG  | 300  |
| CCCGATCTGT  | AGCTAACACT | CAGAGCCCAT | TTTCCTGCGT | TAACCCCTCC | CGACCTCATA  | 360  |
| TACAGGAGTA  | ACATGATCAG | TGACCTGGGG | GAGCTGGCCA | AACTGCGGGA | CCTGCCCAAG  | 420  |
| CTGAGGGCCT  | TGGTGCTGCT | GGACAACCCC | TGTGCCGATG | AGACTGACTA | CCGCCAGGAG  | 480  |
| GCCCTGGTGC  | AGATGGCACA | CCTAGAGCGC | CTAGACAAAG | AGTACTATGA | GGACGAGGAC  | 540  |
| CGGGCAGAAG  | CTGAGGAGAT | CCGACAGAGG | CTGAAGGAGG | AACAGGAGCA | AGAACTCGAC  | 600  |
| CCGGACCAAG  | ACATGGAACC | GTACCTCCCG | CCAACCTAGT | GGCTCCTCTA | GCCTGCAGGG  | 660  |
| ACAGTAAAGG  | TGATGGCAGG | AAGGCAGCCC | CCGGAGGTCA | AAGGCTGGGC | ACGCGGGAGG  | 720  |
| AGAGGCCAGA  | GTCAGAGGCT | GCGGGTATCT | CAGATATGAA | GGAAAGATGA | GAGAGGCTCA  | 780  |
| GGAAGAGGTA  | AGAAAAGACA | CAAGAGACCA | GAGAAGGGAG | AAGAATTAGA | GAGGGAGGCA  | 840  |
| GAGGACCGCT  | GTCTCTACAG | ACATAGCTGG | TAGAGACTGG | GAGGAAGGGA | TGAACCCTGA  | 900  |
| GCGCATGAAG  | GGAAGGAGGT | GGCTGGTGGT | ATATGGAGGA | TGTAGCTGGG | CCAGGGAAAA  | 960  |
| GATCCTGCAC  | TAAAAATCTG | AAGCTAAAAA | TAACAGGACA | CGGGGTGGAG | AGGCGAAAGG  | 1020 |
| AGGGCAGATT  | GAGGCAGAGA | GACTGAGAGG | CCTGGGGATG | TGGGCATTCC | GGTAGGGCAC  | 1080 |
| ACAGTTCACT  | TGTCTTCTCT | TTTTCCAGGA | GGCCAAAGAT | GCTGACCTCA | AGAACTCATA  | 1140 |
| ATACCCCAGT  | GGGGACCACC | GCATTCATAG | CCCTGTTACA | AGAAGTGGGA | GATGTTCCCTT | 1200 |
| TTTGTCACCAG | ACTGGAAATC | CATTACATCC | CGAGGCTCAG | GTTCTGTGGT | GGTCATCTCT  | 1260 |
| GTGTGGCTTG  | TTCTGTGGGC | CTACCTAAAG | TCCTAAGCAC | AGCTCTCAAG | CAGATCCGAG  | 1320 |
| GCGACTAAGA  | TGCTAGTAGG | GGTTGTCTGG | AGAGAAGAGC | CGAGGAGGTG | GGCTGTGATG  | 1380 |
| GATCAGTTCA  | GCTTTCAAAT | AAAAAGGCGT | TTTTATATTC | TGTGTCGAGT | TCGTGAACCC  | 1440 |
| CTGTGGTGGG  | CTTCTCCATC | TGTCTGGGTT | AGTACCTGCC | ACTATACTGG | AATAAGGGGA  | 1500 |
| CGCCTGCTTC  | CCTCGAGTTG | GCTGGACAAG | GTTATGAGCA | TCCGTGTACT | TATGGGGTTG  | 1560 |
| CCAGCTTGGT  | CCTGGATCGC | CCGGGCCCTT | CCCCACCCG  | TTCGGTTCCC | CACCACCACC  | 1620 |
| CGCGCTCGTA  | CGTGCGTCTC | CGCCTGCAGC | TCTTACTCA  | TCGGGGCCCC | CGGGTCACAT  | 1680 |
| GCGCTCGCTC  | GGCTCTATAG | GCGCCGCCCC | CTGCCACCC  | CCCGCCCGCG | CTGGGAGCCG  | 1740 |
| CAGCCGCCGC  | CACTCCTGCT | CTCTCTGCGC | CGCCGCCGTC | ACCACCGCCA | CCGCCACCGG  | 1800 |
| CTGAGTCTGC  | AGTCCTCGAC | CTGCAGGCAT | GCAAGCTGGG | TACCGAGCTC | GAATTGGTCG  | 1860 |

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|            |            |            |             |             |            |      |
|------------|------------|------------|-------------|-------------|------------|------|
| CGGCCGCATG | GCCCAGCCCC | CACCGGCCCA | GCCGCCTGCA  | CCCCCGGCCA  | GCCAGCTCTC | 1920 |
| GGGTTCCCAG | ACCGGGGGCT | CCTCCTCAGT | CAAGAAAGCC  | CCTCACCCCTG | CCGTCACCTC | 1980 |
| CGCAGGGACG | CCCAAACGGG | TCATCGTCCA | GGCGTCCACC  | AGTGAGCTGC  | TTCGCTGCCT | 2040 |
| GGGTGAGTTT | CTCTGCCGCC | GGTGCTACCG | CCTGAAGCAC  | CTGTCCCCCA  | CGGACCCCGT | 2100 |
| GCTCTGGCTG | CGCAGCGTGG | ACCGCTCGCT | GCTTCTGCAG  | GGCTGGCAGG  | ACCAGGGCTT | 2160 |
| CATCACGCCG | GCCAACGTGG | TCTTCCTCTA | CATGCTCTGC  | AGGGATGTTA  | TCTCCTCCGA | 2220 |
| GGTGGGCTCG | GATCACGAGC | TCCAGGCCGT | CCTGCTGACA  | TGCCTGTACC  | TCTCCTACTC | 2280 |
| CTACATGGGC | AACGAGATCT | CCTACCCGCT | CAAGCCCTTC  | CTGGTGGAGA  | GCTGCAAGGA | 2340 |
| GGCCTTTTGG | GACCGTTGCC | TCTCTGTCA  | CAACCTCATG  | AGCTCAAAGA  | TGCTGCAGAT | 2400 |
| AAATGCCGAC | CCACACTACT | TCACACAGGT | CTTCTCCGAC  | CTGAAGAACG  | AGAGCGGCCA | 2460 |
| GGAGGACAAG | AAGCGGCTCC | TCCTAGGCC  | GGATCGGTGA  | GGCGGCCGCG  | ACTCTAGAGG | 2520 |
| ATCTTTGTGA | AGGAACCTTA | CTTCTGTGGT | GTGACATAAT  | TGGACAAACT  | ACCTACAGAG | 2580 |
| ATTTAAAGCT | CTAAGGTAAA | TATAAAATTT | TTAAGTGTAT  | AATGTGTTAA  | ACTACTGATT | 2640 |
| CTAATTGTTT | GTGTATTTTA | GATTCCAACC | TATGGAAGCTG | ATGAATGGGA  | GCAGTGGTGG | 2700 |
| AATGCCTTTA | ATGAGGAAAA | CCTGTTTTGC | TCAGAAGAAA  | TGCCATCTAG  | TGATGATGAG | 2760 |
| GCTACTGCTG | ACTCTCAACA | TTCTACTCCT | CCAAAAAAGA  | AGAGAAAGGT  | AGAAGACCCC | 2820 |
| AAGGACTTTC | CTTCAGAATT | GCTAAGTTTT | TTGAGTCATG  | CTGTGTTTAG  | TAATAGAACT | 2880 |
| CTTGCTTGCT | TTGCTATTTA | CACCACAAAG | GAAAAAGCTG  | CACTGCTATA  | CAAGAAAATT | 2940 |
| ATGGAAAAAT | ATTTGATGTA | TAGTGCCTTG | ACTAGAGATC  | ATAATCAGCC  | ATACCACATT | 3000 |
| TGTAGAGGTT | TTACTTGCTT | TAAAAACCT  | CCCACACCTC  | CCCCTGAACC  | TGAAACATAA | 3060 |
| AATGAATGCA | ATTGTTGTTG | TTAACTTGTT | TATTGCAGCT  | TATAATGGTT  | ACAAATAAAG | 3120 |
| CAATAGCATC | ACAAATTTCA | CAAATAAAGC | ATTTTTTTCA  | CTGCATTCTA  | GTTGTGGTTT | 3180 |
| GTCCAAACTC | ATCAATGTAT | CTTATCATGT | CTGGATCC    |             |            | 3218 |

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2462 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

|            |            |            |            |            |            |     |
|------------|------------|------------|------------|------------|------------|-----|
| GAACTCGAGC | AGCTGAAGCT | TGCATGCCTG | CAGGTCGACT | CTAGAGGATC | CCCGGGTACC | 60  |
| GAGCTCGAAT | TCATCGATGA | TATCAGATCT | GCCGGTCTCC | CTATAGTGAG | TCGTATTAAT | 120 |
| TTCGATAAGC | CAGGTTAACC | TGCATTAATG | AATCGGCCAA | CGCGCGGGGA | GAGGCGGTTT | 180 |
| GCGTATTGGG | CGCTCTTCCG | CTTCCTCGCT | CACTGACTCG | CTGCGCTCGG | TCGTTCGGCT | 240 |
| GCGGCGAGCG | GTATCAGCTC | ACTCAAAGGC | GGTAATACGG | TTATCCACAG | AATCAGGGGA | 300 |
| TAACGCAGGA | AAGAACATGT | GAGCAAAAGG | CCAGCAAAAG | GCCAGGAACC | GTAAAAAGGC | 360 |
| CGCGTTGCTG | GCGTTTTTCC | ATAGGCTCCG | CCCCCTGAC  | GAGCATCACA | AAAATCGACG | 420 |
| CTCAAGTCAG | AGGTGGCGAA | ACCCGACAGG | ACTATAAAGA | TACCAGGCGT | TTCCCCCTGG | 480 |
| AAGCTCCCTC | GTGCGCTCTC | CTGTTCCGAC | CCTGCCGCTT | ACCGGATACC | TGTCCGCCTT | 540 |
| TCTCCCTTCG | GGAAGCGTGG | CGCTTCTCA  | ATGCTCACGC | TGTAGGTATC | TCAGTTCGGT | 600 |
| GTAGGTCGTT | CGCTCCAAGC | TGGGCTGTGT | GCACGAACCC | CCCGTTCAGC | CCGACCGCTG | 660 |
| CGCCTTATCC | GGTAACTATC | GTCTTGAGTC | CAACCCGGTA | AGACACGACT | TATCGCCACT | 720 |
| GGCAGCAGCC | ACTGGTAACA | GGATTAGCAG | AGCGAGGTAT | GTAGGCGGTG | CTACAGAGTT | 780 |
| CTTGAAGTGG | TGGCCTAACT | ACGGCTACAC | TAGAAGGACA | GTATTTGGTA | TCTGCGCTCT | 840 |
| GCTGAAGCCA | GTTACCTTCG | GAAAAAGAGT | TGGTAGCTCT | TGATCCGGCA | AACAAACCAC | 900 |

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|            |            |              |            |             |             |      |
|------------|------------|--------------|------------|-------------|-------------|------|
| CGCTGGTAGC | GGTGGTTTTT | TTGTTTGCAA   | GCAGCAGATT | ACGCGCAGAA  | AAAAAGGATC  | 960  |
| TCAAGAAGAT | CCTTTGATCT | TTTCTACGGG   | GTCTGACGCT | CAGTGGAACG  | AAAAC TCACG | 1020 |
| TTAAGGGATT | TTGGTCATGA | GATTATCAAA   | AAGGATCTTC | ACCTAGATCC  | TTTTAAATTA  | 1080 |
| AAAATGAAGT | TTTAAATCAA | TCTAAAGTAT   | ATATGAGTAA | ACTTGGTCTG  | ACAGTTACCA  | 1140 |
| ATGCTTAATC | AGTGAGGCAC | CTATCTCAGC   | GATCTGTCTA | TTTCGTTTCAT | CCATAGTTGC  | 1200 |
| CTGACTCCCC | GTCGTGTAGA | TAACTACGAT   | ACGGGAGGGC | TTACCATCTG  | GCCCCAGTGC  | 1260 |
| TGCAATGATA | CCGCGAGACC | CACGCTCACC   | GGCTCCAGAT | TTATCAGCAA  | TAAACCAGCC  | 1320 |
| AGCCGGAAGG | GCCGAGCGCA | GAAGTGGTCC   | TGCAACTTTA | TCCGCCTCCA  | TCCAGTCTAT  | 1380 |
| TAATTGTTGC | CGGGAAGCTA | GAGTAAGTAG   | TTCGCCAGTT | AATAGTTTGC  | GCAACGTTGT  | 1440 |
| TGCCATTGCT | ACAGGCATCG | TGGTGTCACG   | CTCGTCGTTT | GGTATGGCTT  | CATTCAGCTC  | 1500 |
| CGGTTCCCAA | CGATCAAGGC | GAGTTACATG   | ATCCCCCATG | TTGTGCAAAA  | AAGCGGTTAG  | 1560 |
| CTCCTTCGGT | CCTCCGATCG | TTGTCAGAAG   | TAAGTTGGCC | GCAGTGTAT   | CACTCATGGT  | 1620 |
| TATGGCAGCA | CTGCATAATT | CTCTTACTGT   | CATGCCATCC | GTAAGATGCT  | TTTCTGTGAC  | 1680 |
| TGGTGAGTAC | TCAACCAAGT | CATTCTGAGA   | ATAGTGTATG | CGGCGACCGA  | GTTGCTCTTG  | 1740 |
| CCCGGCGTCA | ATACGGGATA | ATACCGCGCC   | ACATAGCAGA | ACTTTAAAAG  | TGCTCATCAT  | 1800 |
| TGGAAAACGT | TCTTCGGGGC | GAAAAC TCTC  | AAGGATCTTA | CCGCTGTTGA  | GATCCAGTTC  | 1860 |
| GATGTAACCC | ACTCGTGAC  | CCAAC T GATC | TTCAGCATCT | TTTACTTTCA  | CCAGCGTTTC  | 1920 |
| TGGGTGAGCA | AAAACAGGAA | GGCAAAAATGC  | CGCAAAAAG  | GGAATAAGGG  | CGACACGGAA  | 1980 |
| ATGTTGAATA | CTCATACTCT | TCCTTTTTTCA  | ATATTATTGA | AGCATTTATC  | AGGGTTATTG  | 2040 |
| TCTCATGAGC | GGATACATAT | TTGAATGTAT   | TTAGAAAAAT | AAACAAATAG  | GGGTTCCGCG  | 2100 |
| CACATTTCCC | CGAAAAGTGC | CACCTGACGT   | CTAAGAAACC | ATTATTATCA  | TGACATTAAC  | 2160 |
| CTATAAAAAT | AGGCGTATCA | CGAGGCCCTT   | TCGTCTCGCG | CGTTTCGGTG  | ATGACGGTGA  | 2220 |
| AAACCTCTGA | CACATGCAGC | TCCCGGAGAC   | GGTCACAGCT | TGTCTGTAAG  | CGGATGCCGG  | 2280 |
| GAGCAGACAA | GCCCGTCAGG | GCGCGTCAGC   | GGGTGTTGGC | GGGTGTCGGG  | GCTGGCTTAA  | 2340 |
| CTATGCGGCA | TCAGAGCAGA | TTGTACTGAG   | AGTGCACCAT | ATGGACATAT  | TGTCGTTAGA  | 2400 |
| ACGCGGCTAC | AATTAATACA | TAACCTTATG   | TATCATAAC  | ATACGATTTA  | GGTGACACTA  | 2460 |
| TA         |            |              |            |             |             | 2462 |

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 41 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

|            |           |           |            |   |    |
|------------|-----------|-----------|------------|---|----|
| GCGGCCGCAT | GGCCAGCCC | CCACCGGCC | AGCCGCCTGC | A | 41 |
|------------|-----------|-----------|------------|---|----|

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

|            |            |            |      |    |
|------------|------------|------------|------|----|
| CTCCTCCTAG | GCCTGGAATC | GGTGAGGCGG | CCGC | 34 |
|------------|------------|------------|------|----|

**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A purified and isolated recombinant polynucleotide comprising a neuron-specific enolase promoter operably linked to an encoding sequence for p25.  
5
2. The polynucleotide according to claim 1 wherein said sequence encoding said p25 protein has the characteristics of genomic DNA.
- 10 3. The polynucleotide according to claim 1 wherein said sequence encoding said p25 protein has the characteristics of cDNA.
4. The polynucleotide according to claim 1 wherein said sequence is that of SEQ ID NO: 4.  
15
5. A vector comprising the polynucleotide according to claim 1.
6. A vector comprising the polynucleotide according to claim 2.
- 20 7. A vector comprising the polynucleotide according to claim 3 or 4.
8. An isolated eukaryotic cell comprising the polynucleotide according to claim 1.
- 25 9. An isolated eukaryotic cell comprising the polynucleotide according to claim 2.
10. An isolated eukaryotic cell comprising the polynucleotide according to claim 3.  
30
11. An isolated eukaryotic cell according to any one of claims 8-10 which is a non-human embryonic stem cell.
12. An isolated eukaryotic cell according to any one of claims 8-11 which is a mouse cell.  
35

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13. A method for determining the ability of a compound to inhibit p25 protein comprising the steps of:
- 5
- a. providing a transgenic non-human animal by stably incorporating into the embryonic stem cells of said animal the polynucleotide of any one of claims 1 to 4;
  - b. growing said embryonic stem cells into a mature transgenic non-human animal;
  - 10
  - c. administering to said transgenic non-human animal the compound of interest; and
  - d. measuring the inhibition of said p25 protein by said compound.
  - 15
14. A method for generating data to determine the ability of a compound to inhibit p25 comprising the steps of:
- 20
- a. creating a transgenic non-human animal by stably incorporating into the embryonic stem cells of said animal the polynucleotide of any one of claims 1 to 4;
  - b. growing said embryonic stems cells into a mature transgenic non-human animal;
  - 25
  - c. administering to said transgenic non-human animal the compound of interest;
  - d. measuring the inhibition of said p25 protein by said compound; and
  - 30
  - e. using the data derived from said inhibition of synthesize compounds capable of inhibiting said p25 protein.

15. A method of preparing a transgenic non-human animal whose germ cells and somatic cells express a polynucleotide according to any one of claims 1 to 4 comprising the steps of:
- 5 a. incorporating the polynucleotide of any one of claims 1 to 4 into non-human embryonic stem cells;
- b. transferring the embryonic stem cells to a recipient female non-human animal; and
- 10 c. growing the embryonic stem cells into a mature transgenic non-human animal.
16. The method according to claim 15 wherein the transgenic non-human animal is a mouse.
- 15

Application number / numéro de demande: 2295861

Figures: 1-14

Pages: \_\_\_\_\_

Unscannable items  
received with this application  
(Request original documents in File Prep. Section on the 10<sup>th</sup> floor)

Documents reçu avec cette demande ne pouvant être balayés  
(Commander les documents originaux dans la section de préparation des dossiers au  
10<sup>ème</sup> étage)