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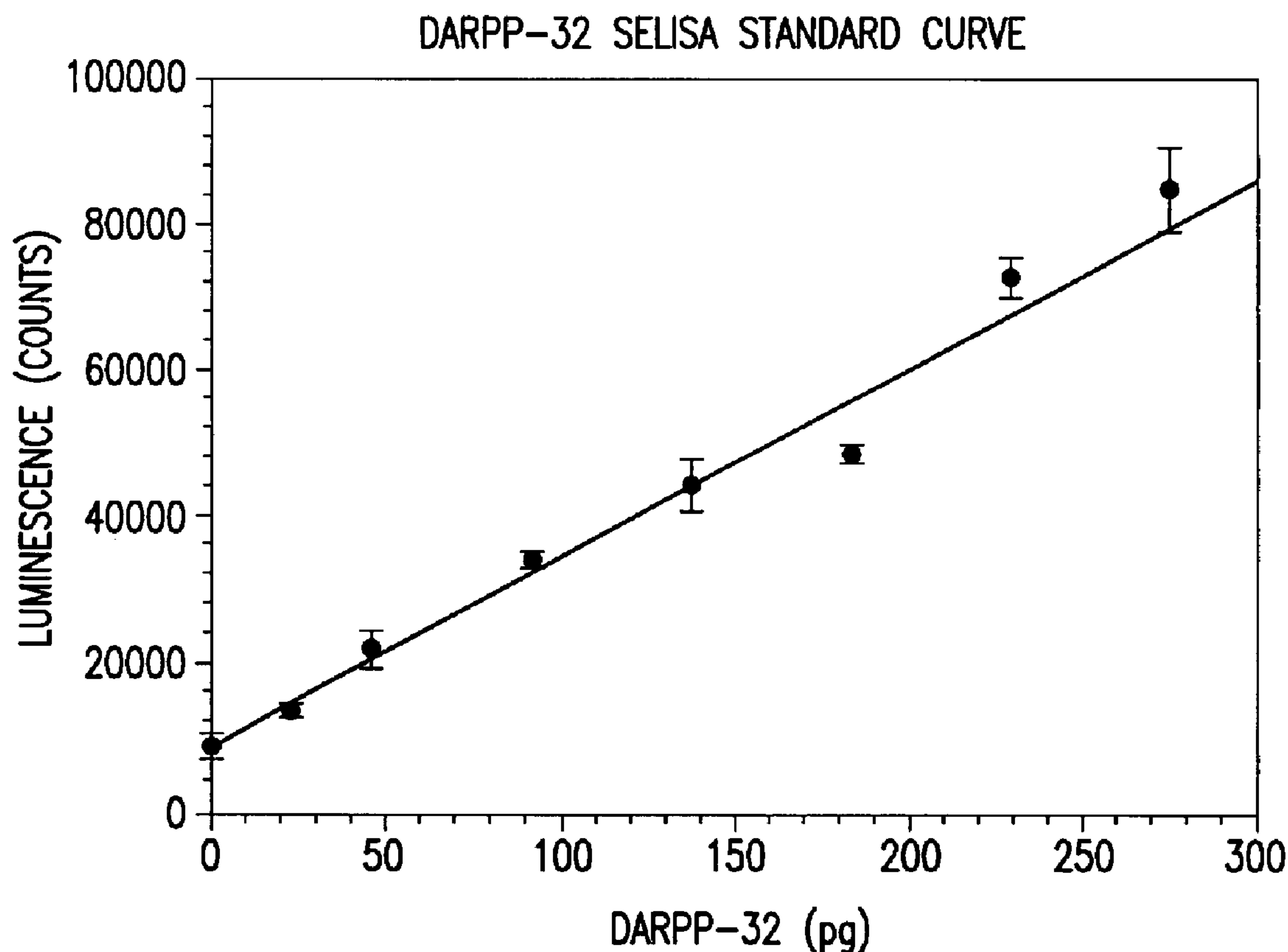
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(54) Titre : MOLECULES D'ACIDES NUCLEIQUES ISOLEES CODANT UNE NOUVELLE PHOSPHOPROTEINE-DARPP-32, PROTEINE CODEE ET UTILISATIONS ASSOCIEES

(54) Title: ISOLATED NUCLEIC ACID MOLECULES ENCODING A NOVEL PHOSPHOPROTEIN-DARPP-32, ENCODED PROTEIN AND USES THEREOF



(57) **Abrégé/Abstract:**

Provided herein are novel nucleic acid and amino acids sequences of a phosphoprotein derived from a Guinea pig. Methods and compositions for modulating the phosphorylation of the novel protein in a dopaminergic signaling pathway are also provided.

**(57) Abrégé(suite)/Abstract(continued):**

Compounds identified by the disclosed methods may be used to treat disorders characterized by a aberrant or dysfunctional intracellular signaling pathway regulated by DARPP-32.

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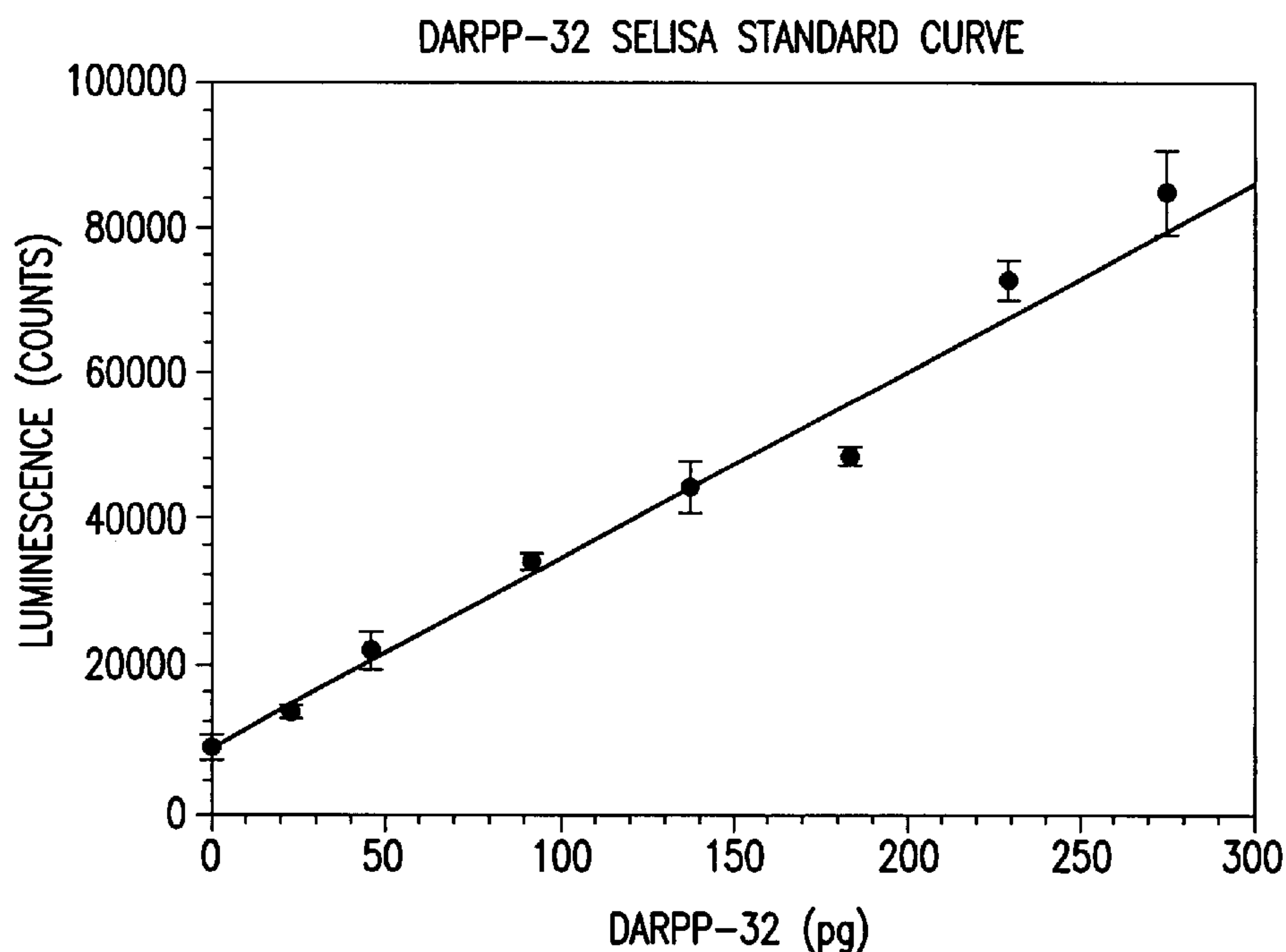
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## TITLE OF THE INVENTION

ISOLATED NUCLEIC ACID MOLECULES ENCODING A NOVEL PHOSPHOPROTEIN- DARPP-32, ENCODED PROTEIN AND USES THEREOF

## 5 CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/572,634 filed May 19, 2004, the contents of which are incorporated herein by reference in their entirety.

## FIELD OF THE INVENTION

10 The invention relates to the field of neurobiology. The invention provides novel nucleic acid molecules, encoded proteins and methods of using either in identifying therapeutic moieties for use in treating psychotic disorders. Specifically, the invention provides nucleic acid molecules encoding a phosphoprotein designated herein as DARPP-32 derived from a non-human source, e.g., guinea pig, encoded proteins and use of the sequences in the study, diagnosis, and treatment of diseases affecting  
15 brain function.

## BACKGROUND OF THE INVENTION

Protein phosphorylation appears to be an important mechanism in neuronal signal transduction, whereby extracellular stimuli are relayed to the interior of cells and subsequently these  
20 stimuli regulate diverse cellular processes. The triggering mechanisms for activation of protein phosphorylation include many established second messengers (cAMP, cGMP, calcium), which are generated by interaction of neurotransmitters with their receptors and which, in turn, activate protein kinases (protein-phosphorylating enzymes) that transfer phosphate from adenosine triphosphate (ATP) to substrate proteins. These substrate proteins, in turn, mediate many of the physiological effects attributed  
25 to the transmitter-receptor interaction.

Studies of signal transduction mediated by protein phosphorylation have demonstrated a central role for one particular phosphoprotein designated - DARPP-32 (dopamine- and cyclic AMP (cAMP)-regulated phosphoprotein having a molecular weight of 32 kilo daltons). This protein is a  
30 cystolic protein that is selectively enriched in medium-sized spiny neurons in neostriatum, which is a major target for midbrain dopaminergic neurons. Ouimet et al., 1984. Since, medium spiny neurons are the only known projection neurons in the neostriatum, these function to integrate all input to this brain region. The spiny neurons contain both D1 class (D1, D5) and D2 class (D2, D3, D4) dopamine receptors (Sibley, D.R. and Monsma, F.J.Jr. (1992). Molecular biology of dopamine receptors. Trends Pharmacol. Sci 13, 61-69.). (Surmeier, D.J., Song, W.J., and Yan, Z. (1996).

Dopamine is a neurotransmitter in the brain. Since its discovery in the 1950s, its function in the brain has been intensely explored. It is well settled that dopamine is essential in several aspects of brain function.

Dopamine produces its biological effects on the neurons via activation of a biochemical cascade involving stimulation of D1 receptors, activation of adenylyl cyclase, increased cAMP formation and increased activity of PKA (Walaas and Greengard, 1984). Briefly, dopamine released from nigrostriatal nerve terminals acts on D1 dopamine receptors to increase the activity of adenylyl cyclase, increase cAMP formation, and stimulate cAMP-dependent protein kinase (PKA). In turn, PKA phosphorylates DARPP-32 at a single threonine residue (Thr34), converting the protein into a potent inhibitor of PP-1, which is a major multifunctional serine/threonine protein phosphatase in the brain. PP-1, in turn, regulates the phosphorylation state and activity of many downstream physiological effectors, including various neurotransmitter receptors and voltage-gated ion channels. To date several classes of ion channels, including Na<sup>+</sup> channels and the L-, N- and P-classes of Ca<sup>2+</sup> channels, the NMDA-R1 class of glutamate receptor, and the electrogenic ion pump, Na<sup>+</sup>,K<sup>+</sup>-ATPase, have been shown to be regulated by PP-1. Since protein phosphatase-1 is a major protein phosphatase in the brain, this inhibitory role of DARPP-32 has considerable physiological significance. Conversely, dopamine, acting on D2-like receptors, through both inhibition of PKA and activation of calcium/calmodulin-dependent protein phosphatase (protein phosphatase 2B – PP-2B/calcineurin), causes the dephosphorylation of DARPP-32. PP-2B also acts synergistically with PP-2A to regulate the dephosphorylation of DARPP-32 (Nishi et al. 1999.). Various studies have confirmed that the DARPP-32/PP-1 cascade is a major target for psycho stimulants and anti schizophrenic drugs. Consequently, modulators of dopaminergic function will find use in the treatment of a wide range of disorders affecting brain functions.

The role of DARPP-32 in the DARPP-32/protein phosphatase-1 (PP-1) cascade in integrating the neurotransmitter pathways in medium spiny neurons of the neostriatum is well documented. For example, Forskolin, a drug which directly activates adenylyl cyclase to increase cAMP formation, mimics the effects of D1 receptor stimulation, resulting in increased phosphorylation of DARPP-32 in neostriatal slices. N-methyl-D-aspartate (NMDA) receptors, which are present on all medium spiny neurons, have been shown to participate in glutamate-mediated dephosphorylation of DARPP-32, probably through Ca<sup>2+</sup>-dependent activation of PP-2B. The dephosphorylation of DARPP-32 in response to NMDA has been shown to be blocked by cyclosporin A, a highly specific PP-2B inhibitor, confirming the involvement of PP-2B as an enzyme that dephosphorylates Thr34 in intact cells. Thus, dopamine and glutamate have opposing actions on the phosphorylation state and activity of DARPP-32, which may contribute to certain of the antagonistic effects of these two neurotransmitters on neostriatal neuron excitability. In addition to PP2B, PP2C and CK1, cyclin-dependent kinase 5 (cdk5) and PP2A are also involved in regulating the state of phosphorylation of DARPP-32.

Likewise, studies in mice lacking the DARPP-32 gene, have revealed that inactivation of the DARPP-32 gene markedly reduced, and in some cases abolished, various responses to dopaminergic agonists and antagonists. In some instances, the impairment of responses could be overcome by increasing the concentration of the test substance used. These studies thus confirm that this protein plays an essential role in mediating the actions and interactions of dopamine and other neurotransmitters that act on dopaminergic neurons. See Greengard et al., *Neuron*, 23: 435-447 (1999).

Other studies have also confirmed that a cascade involving dopamine-mediated receptor activation of DARPP-32, inhibition of PP-1, and potentiation of phosphorylation of neuronal substrates plays a major role in regulating the efficacy of dopaminergic neurotransmission under physiological conditions. See Hemmings, Jr. et al. *Nature* 310 (1984), pp. 503-505 which shows that dopamine-dependent changes in either the phosphorylation or regulation of NR1 NMDA receptors, Na<sup>+</sup>,K<sup>+</sup>-ATPase, and N- and P-type Ca<sup>2+</sup> channels are attenuated in the DARPP-32 knockout mice.

Depression is among the most debilitating psychiatric disorders and is generally associated with heterogeneous dysregulation of the biogenic amines. While few pharmacological approaches exist for treating depression, most of the current treatment paradigms for depression involve modulation of serotonergic neurotransmission through the usage of compounds/agents that alter the level of serotonin in the synaptic cleft. Recently, dopamine has been implicated in the pathophysiology of depression. The suggestion has been made that dopamine may be reduced in depression and increased in mania. See Guitart, X and E.J. Nestler, *J. Neurochem.* 59: 1164-1167 (1992), who show a nexus between the serotonergic pathways in the brain and DARPP-32. Two prevalent theories regarding dopamine and depression are that the mesolimbic dopamine pathway may be dysfunctional in depression and that the dopamine type 1 (D1) receptor may be hypoactive in depression (Ch.9. Mood Disorders, in: *CONCISE TEXTBOOK OF CLINICAL PSYCHIATRY*. Ed. by H I Kaplan and B J Sadock. Williams & Wilkins, Baltimore, Md., 1996, pp. 159-188). For example, drugs that reduce dopamine concentrations (e.g., reserpine) and diseases that reduce dopamine concentrations (e.g., Parkinson's disease) are associated with depressive symptoms. As well, drugs that increase dopamine concentrations (e.g., tyrosine, amphetamine and bupropion) reduce the symptoms of depression. Consequently, in view of the high expression levels of DARPP-32 in prefrontal cortex and the striatum, and the nexus between DARPP-32 and depression, it is believed that the novel sequences disclosed herein will provide means to develop novel assays that can be used to develop novel therapeutic moieties that can be used to treat disorders related to a dysfunctional serotonergic intracellular signaling pathways, more particularly depression.

Dopamine has also been implicated in the pathophysiology of schizophrenia, which is a multi-factorial disease characterized by multiple genetic susceptibility elements, each likely contributing a modest increase in risk (Karaylorgou, M. & Gogos, J. A. (1997) *Neuron* 19, 967-79). The dopamine hypothesis for the pathophysiology of schizophrenia maintains that dysfunction of the dopamine

neurotransmitter system plays a key role in the abnormalities that occur in schizophrenia. (Seeman, P. (1987) Synapse 1, 133-52; Carlsson, A., et al., (2001) Annu Rev Pharmacol Toxicol 41, 237-60).

Schizophrenia is characterized as having both "positive symptoms" (hallucinations, delusions, and conceptual disorganization) and "negative symptoms" (apathy, social withdrawal, affect, and poverty of speech). While many drugs effective in treating schizophrenia share the common property of blocking dopamine receptors, such neuroleptics are only effective for treating the positive symptoms of schizophrenia, but have little or no effect on the negative symptoms. As well, neuroleptics-resistant negative symptoms account for most of the social and vocational disability caused by schizophrenia. Further, neuroleptics cause extrapyramidal symptoms, including rigidity, tremor, bradykinesia (slow movement), and bradyphrenia (slow thought), as well as tardive dyskinesias and dystonias. It is believed that the selection of a preferred therapy for a particular subject may prevent the subject from having to endure possibly irreversible side effects from therapies that are ineffective for that subject. Reduced side effects of the preferred therapy compared to other therapies may result in greater patient compliance, further increasing the likelihood of therapeutic benefit from the therapy. Consequently, the dysfunctional dopamine hypothesis strongly argues for the use of the herein disclosed sequences in identifying therapeutic moieties that may play a role in the DARPP-32/protein phosphatase-1 (PP-1) cascade as a means of treating Schizophrenia.

Abnormalities in dopaminergic neurotransmission have also been implicated in supranuclear palsy, Tourette's syndrome and obsessive-compulsive disorder. Finally, drugs of abuse such as cocaine, amphetamine, and opiate classes, as well as nicotine and alcohol, achieve some of their addictive actions by modifying dopaminergic transmission.

However, currently available dopaminergic pharmaceuticals have severe side effects, such as extra pyramidal side effects and tardive dyskinesia in dopaminergic antagonists used as antipsychotic moieties, and dyskinesia and psychosis in dopaminergic agonists used as anti-Parkinson's agents. In addition, the therapeutic effects are unsatisfactory as a whole.

Collectively, the data favor the suggestion that regulation of DARPP-32 via phosphorylation or dephosphorylation is probably the major molecular mechanism by which information received through dopaminergic and other signaling pathways is integrated in these neurons, which constitute the principal efferent pathway from the striatum. For example, in neostriatum, dopamine-mediated effects on the function of calcium channels (Surmeier et al., 1994), voltage-dependent sodium channels (Surmeier et al., 1992; Schiffman et al., 1994) and Na.sup.+ ,K.sup.+ -ATPase (Aperia et al., 1991) are regulated directly or indirectly by protein phosphatase-1.

Therefore, there is a need in the art to provide new methods of screening that can be used to develop novel compositions or drugs that can be used to treat psychotic diseases or disorders. In addition, there is a need for simple tests of intracellular consequences of antipsychotic action. Since all

anti- psychotics act upon multiple receptors, with widely varying downstream effects in terms of both effective relief of symptoms and unwanted side effects, analysis of the intracellular integration of these signals provides a straightforward, cost-effective, and mechanism-based comparison useful for development of the next generation of therapeutic drugs. As well, there is also a need to develop  
5 treatments for such diseases or disorders that are due, at least in part, to an aberration or dysregulation of an intracellular signaling pathway regulated by DARPP-32.

Consequently, therapeutic moieties that mimic or block the inhibitory effects of DARPP-32 on PP-1 should find use in the treatment of Parkinson's disease, schizophrenia, drug addiction, and other neuropsychiatric disorders involving abnormal dopaminergic function. Such moieties would have  
10 their use in the activation of downstream components of the dopamine signaling cascade. Significantly, the selective enrichment of DARPP-32 in dopaminoceptive neurons and its regulation by dopamine strongly indicate that DARPP-32, by regulating protein phosphatase-1 activity, plays a key role in mediating the effects of dopamine on these cells. As well, the control of protein phosphatase- I activity by DARPP-32 is likely to have a significant role in the regulation of neuronal excitability. Consequently,  
15 the sequences of the present invention aim to remedy the void attending the current treatment paradigms for treating various psychotic disorders by enabling the identification of compounds that modulate the dopaminergic signaling pathway as an effective means for treating various disorders of the brain.

#### SUMMARY OF THE INVENTION

20 In its broadest aspect, invention features isolated and substantially purified polynucleotides that encode a mammalian phosphoprotein. An illustrative nucleic acid molecule has the nucleotide sequence of SEQ ID NO:1 of 896 nucleotides, of which the coding sequence encompasses nucleotides 1 to 567 nucleotides. The encoded polypeptide has the amino acid sequence as set forth in SEQ ID NO:2. The polypeptide is designated herein as nhDARPP-32. Preferably, the polynucleotide  
25 sequences of the invention is/are derived from a guinea pig.

The invention additionally features fragments, portions or antisense molecules of the disclosed sequences thereof, and expression vectors and host cells comprising polynucleotides that encode nhDARPP-32. Variants of the polynucleotide as well as a polynucleotide sequence comprising the complement of SEQ ID NO:1 are also included within the scope of the invention.

30 In addition, the invention features polynucleotide sequences which hybridize under stringent conditions to SEQ ID NO:1.

The present invention also features antibodies which bind specifically to DARPP-32 and/or various phosphorylated fragments thereof, i.e., Thr34phosphorylated DARPP-32, and preferably the amino acids sequence(s) of the invention. Pharmaceutical compositions comprising substantially  
35 purified nhDARPP-32 are also contemplated.

The use of the herein disclosed nhDARPP-32 polypeptide, and of the nucleic acid sequences which encode it, is also based on the amino acid and structural homologies between the herein disclosed nhDARPP-32 and the other known DARPP-32 polypeptides as well as on the known associations and functions of such types of phosphoproteins. The disclosed polynucleotides of the invention share 90 percent of sequence identity with a mammalian counterpart e.g., human DARPP-32, which has been sequenced. See Brene et al., J. Neuroscience, 14: 985-998 (1994). Consequently, the nhDARPP-32 of the invention may be used to treat disorders or diseases based, in part, on an aberrant or dysregulated intracellular signaling pathway regulated by DARPP-32. Thus, sequences of the invention will find use in *in vitro* or *in vivo* assays to identify modulators of a dysfunctional dopaminergic signaling pathway associated disorder, particularly those that are regulated by DARPP-32. In this respect, it is understood that the proteins of the inventions for use in any *in vivo* and *in vitro* assays proposed herein., will comprise the amino acid sequences of SEQ ID NO: 2 or encoded by the nucleotide sequence comprising SEQ ID NO:1 or biologically equivalent fragments thereof. For example, *in vivo* assays of the invention propose transforming cells with the cDNA of SEQ ID NO: 1 such that the cell expresses a functional protein of SEQ ID NO: 2 or a biologically equivalent fragment thereof.

As would be appreciated by those skilled in the relevant art, and as mentioned above, the dopaminergic signaling pathways generally involve checks and balances, e.g., a cascade of phosphorylations and dephosphorylations. Hence, one way to address the current voids in anti-psychotics is to identify therapeutic moieties that either mimic or facilitate the effects of D1 receptor stimulation or mimic or block the inhibitory effects of DARPP-32 on PP-1. For example therapeutic moieties that mimic or block the inhibitory effects of DARPP-32 on PP-1 should be useful in treating Parkinson's disease, schizophrenia, drug addiction, and other neuropsychiatric disorders involving aberrant or abnormal dopaminergic function mediated by DARPP-32. Such moieties would have their use in the activation of downstream components of the dopamine signaling cascade.

A broad aspect of the invention provides a method for identifying a compound to be tested for its ability to modulate the activity of a dopaminergic intracellular signaling pathway in a cell comprising: (a) determining a first level of dopamine activity in said cell; (b) contacting said cell with a test compound; and (c) determining a second level of dopamine activity, respectively, in said cell, wherein a difference in said first level and said second level of dopamine activity is indicative of the ability of said test compound to modulate the dopaminergic intracellular signaling pathway. In preferred embodiments, dopamine activity entails determining the level of phosphorylation of Thr34-phosphorylated DARPP-32. The same assays can be used to determine the ability of other kinases and phosphatases to phosphorylate DARPP-32 (SEQ ID NO:2) at distinct sites well known to a skilled artisan. Such kinases and phosphatases include Cdk5, PP-1, PP2C, PP2B and PP2A.

The proposed assay further comprises the additional step of determining whether the dopaminergic intracellular signaling pathway is modulated.

A representative embodiment proposes a method of identifying a therapeutic moiety to be tested for its ability to modulate the activity of a dopaminergic intracellular signaling pathway regulated by DARPP-32 in a cell comprising: (a) determining a first level of phosphorylated Thr34-DARPP-32 in said cell; (b) contacting said cell with the therapeutic moiety under investigation, and (c) determining a second level of phosphorylated Thr34-DARPP-32, respectively, in said cell, wherein a difference in said first level and said second level of phosphorylated Thr34-DARPP-32 is indicative of the ability of said test compound to modulate the dopaminergic intracellular signaling pathway. The amount of dephosphorylated DARPP-32 versus phosphorylated DARPP-32 can also be used as the end point according to the above assays. As well, the level of phosphorylated DARPP-32 at other residues such as Thr75, Ser137 etc. can also be used. It is noted that DARPP-32 can also be phosphorylated by casein 2 (CK2) at serine 102.

In another aspect, the present invention provides a method for identifying therapeutic moieties that can modulate the activity of a dopaminergic intracellular signaling pathway via modulation of PKA, CK1, CK2, Cdk5, protein phosphatase 1 ("PP-1"), protein phosphatase-2C ("PP2C"), protein phosphatase-2B ("PP2B") or protein phosphatase-2A ("PP2A") activity.

An illustrative method for modulating one of the above comprises contacting the a transformed cell with an effective amount of a compound that alters the activity of a dopaminergic receptor intracellular signaling molecule, wherein contact of the cell with the compound results in a modulation of the activity of PKA, CK1, CK2, Cdk5, PP-1, PP2C, PP2B and/or PP2A, whose modulation may be quantified via the determination of the rate of phosphorylation or dephosphorylation of DARPP-32 of SEQ ID NO:2 at distinct residues known to one skilled in the art.

A representative method contemplates detecting the increase (or decrease) in the amount of phosphorylation (or dephosphorylation) of Thr34-phosphorylated DARPP-32, Ser137-phosphorylated DARPP-32, or Thr75-phosphorylated DARPP-32. Preferably, the DARPP-32 polypeptide comprises the amino acid sequence of SEQ ID NO:2 or a functionally effective fragment thereof. Detecting an increase or decrease in the phosphorylation of other residues mediated by the modulation of any one of PKA, CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A are well known to one skilled in the art.

The invention also provides methods of screening potential therapeutic moieties (or drugs or compounds) that are capable of potentially ameliorating and/or being used in the treatment of a dysfunctional dopaminergic signaling pathway preferably mediated by DARPP-32.

Methods for identifying therapeutic moieties (or drugs or compounds), e.g., drug screening assays, to identify those moieties that may be used in therapeutic methods for the treatment of a

dysfunctional dopaminergic intracellular signaling pathway preferably mediated or regulated by DARPP-32 are also provided.

Methods of treating a disorder or disease due in part by the aberration or dysregulation of an intracellular pathway regulated or mediated by DARPP-32 are also provided. The proposed method  
5 proposes administering to a patient in need thereof a therapeutic moiety that alters the phosphorylation of phosphorylated DARPP-32, wherein the therapeutic moiety modulates the activity of PKA, CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A.

An alternative embodiment provides a method for regulating phosphorylation-dependent activation of one or more dopamine receptors, such as the D1 receptors in a cell. The method proposes  
10 administering an effective amount of a compound that modulates activity of PKA, CK1, CK2, Cdk5, PP-1, PP2C, PP2B and/or PP2A, wherein modulation of the activity of one of PKA, CK1, CK2, Cdk5, PP-1, PP2C, PP2B and/or PP2A results in an alteration in the phosphorylation-dependent activation of the D1 receptors in the cell, e.g., DARPP-32.

A representative embodiment provides a method for treating a disorder characterized by  
15 dysfunctional dopaminergic intracellular signaling pathway mediated by DARPP-32 in a patient in need thereof comprising administering to the patient therapeutic moiety that inhibits the dephosphorylation of Thr34-phosphorylated DARPP-32, wherein the therapeutic moiety decreases PP2B activity.

In accordance with the above, the invention provides a method for identifying a therapeutic moiety for use in the treatment of dopamine mediated disorder regulated by DARPP-32 in a  
20 patient in need of such treatment comprising: (a) contacting the potential therapeutic moiety with PP2B and Thr34-phosphorylated DARPP-32; and (b) detecting the amount of dephosphorylation of Thr34-phosphorylated DARPP-32; wherein a decrease in the dephosphorylation of Thr34-phosphorylated DARPP-32 in the presence of the therapeutic moiety is indicative that the therapeutic moiety has therapeutic utility in the treatment of a dopamine mediated disorder.

In yet another embodiment, the invention provides for a method treating a dysfunctional  
25 dopaminergic signaling pathway related disorder in a patient in need thereof comprising administering to the patient a therapeutic moiety that decreases the dephosphorylation of Thr34-phosphorylated DARPP-32, wherein the therapeutic moiety decreases PP-1 activity.

In accordance with the above, the invention provides a method for identifying therapeutic  
30 moiety for use in the treatment a patient presenting a disease or disorder characterized by dysfunctional dopaminergic intracellular signaling pathway regulated b by DARPP-32 comprising (a) contacting a potential therapeutic moiety with PP-1 and Thr34-phosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of dephosphorylation of Thr34-phosphorylated DARPP-32; wherein the therapeutic moiety is identified if a decrease in the dephosphorylation of Thr34-phosphorylated DARPP-32 is  
35 detected in the presence of the potential therapeutic moiety.

A similar embodiment provides administering to the patient a therapeutic moiety that increases the dephosphorylation of Thr75-phosphorylated DARPP-32, wherein the therapeutic moiety increases PP2A activity. The therapeutic moiety is identified using the herein disclosed DARPP-32 polypeptide.

5 In accordance with the above, the invention provides a method for identifying therapeutic moiety for use in the treatment a patient presenting a disease or disorder characterized by dysfunctional dopaminergic intracellular signaling pathway regulated b by DARPP-32 comprising (a) contacting the potential therapeutic moiety with PP2A and Thr75-phosphorylated DARPP-32; and (b) detecting the amount of dephosphorylation of Thr75-phosphorylated DARPP-32; wherein an increase in the  
10 dephosphorylation of Thr75-phosphorylated DARPP-32 in the presence of the potential therapeutic moiety is indicative that the therapeutic moiety has therapeutic utility in the treatment of the disorder .

In another embodiment, the invention provides a method for identifying therapeutic moiety for use in the treatment of a psychotic disorder mediated by DARPP-32 in a patient in need of such treatment comprising: (a) contacting a potential therapeutic moiety with Cdk5 and Thr75-  
15 dephosphorylated DARPP-32; and (b) detecting the amount of phosphorylation of Thr75-dephosphorylated DARPP-32; wherein the therapeutic moiety is identified if a decrease in the phosphorylation of Thr75-dephosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

Likewise, an embodiment provides a method for identifying therapeutic moiety to be  
20 tested for an ability to treat a patient presenting symptoms consistent with a disease or disorder characterized by dysfunctional dopaminergic intracellular signaling pathway regulated b by DARPP-32 comprising: (a) contacting a potential therapeutic moiety with dopamine and The34-dephosphorylated DARPP-32 of SEQ ID NO: 2; and (b) detecting the amount of phosphorylation of Thr34-dephosphorylated DARPP-32; wherein an increase in the phosphorylation of Thr34-dephosphorylated  
25 DARPP-32 in the presence of the potential therapeutic moiety indicates that the test therapeutic moiety is capable of treating a dopamine mediated disorder.

In another aspect, the invention provides a method for identifying a potential therapeutic moiety to be tested for an ability to treat a psychotic disorder in a patient in need of such treatment comprising the steps of:

30 (a) contacting, in a transformed cell or one expressing the DARPP-32 of the invention the potential therapeutic moiety with a Thr-75 dephosphorylated DARPP-32 and detecting the amount of phosphorylation of Thr-75 dephosphorylated DARPP-32, or

(b) contacting, in a transformed cell or one expressing the DARPP-32 of the invention the potential therapeutic moiety with Thr-75 phosphorylated DARPP-32 and detecting the  
35 amount of dephosphorylation of Thr-75 phosphorylated DARPP-32, wherein the therapeutic moiety is

identified as a potential atypical anti-psychotic compound if: (i) an increase in the level of phosphorylation of Thr-75 dephosphorylated DARPP-32 is detected in step (a), ii) a decrease in the level of dephosphorylation of Thr-75 phosphorylated DARPP-32 is detected in step (b), respectively, relative to a control level, in the presence of the potential test therapeutic moiety.

5 In another embodiment, the invention provides a method for treating a dysfunctional dopaminergic signaling pathway related disorder in a patient in need thereof comprising administering to the patient therapeutic moiety that decreases the dephosphorylation of Ser137-phosphorylated DARPP-32, wherein the therapeutic moiety decreases PP2C activity.

Accordingly, the method for identifying the therapeutic moiety for use in the treatment of  
10 dysfunctional dopaminergic signaling pathway related disorder in a patient in need of such treatment comprises: (a) contacting a potential therapeutic moiety with PP2C and Ser137-phosphorylated DARPP-32; and (b) detecting the amount of dephosphorylation of Ser137-phosphorylated DARPP-32; wherein the therapeutic moiety is identified if a decrease in the dephosphorylation of Ser137-phosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

15 Another embodiment provides a method for identifying a therapeutic moiety to be tested for an ability to treat a dysfunctional dopaminergic signaling pathway mediated disorder in a patient in need of such treatment comprising: (a) contacting a potential therapeutic moiety with a D1 receptor agonist, such as dopamine and Thr34-dephosphorylated DARPP-32; and (b) detecting the amount of phosphorylation of Thr34-dephosphorylated DARPP-32; wherein a decrease in the phosphorylation of  
20 Thr34-dephosphorylated DARPP-32 in the presence of the potential therapeutic moiety indicated the therapeutic potential of said moiety in its ability to treat said disorder.

The same assay may be performed using Cdk5 instead of dopamine and monitoring a decrease in phosphorylation of Thr75 dephosphorylated DARPP-32 of SEQ ID NO:2.

Compounds identified herein for modulating the activity of PKA, CK1, CK2, Cdk5, PP-  
25 1, PP2C, PP2B and/or PP2A are also encompassed by the invention as are pharmaceutical compositions of the therapeutic moieties (or drugs or compounds) for use in treating disease or disorders due in part to an aberration or dysregulation of an intracellular signaling pathway regulated by DARPP-32.

The invention also encompasses pharmaceutical compositions for treating disorders of brain function mediated by DARPP-32.

30 brief description of the drawings

Figure 1 depicts the results of a sandwich ELISA (sELISA) for rat DARPP-32.

Figure 2 represents the nucleotide sequence (SEQ ID NO:1) encoding a DARPP-32 polypeptide derived from a guinea pig.

Figure 3 depicts the deduced amino acid sequence (SEQ ID NO:2) of the DARPP-32 disclosed herein.

Figure 4 depicts the amino acid sequence alignment between DARPP-32 (SEQ ID NO:2) and the corresponding protein derived from a human, cow, rat and mouse.

5 Figure 5 shows the standard curve for determination of pT34-DARPP-32 by sELISA.

detailed description of the invention

Before the present proteins, nucleotide sequences, and methods are described, it is to be understood that the present invention is not limited to the particular methodologies, protocols, cell lines,  
10 vectors, and reagents described, as these may vary. It is also understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not to limit the scope of the present invention.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

15 All technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention pertains. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of protein chemistry and biochemistry, molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature.

20 Although any machines, materials, and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred machines, materials, and methods are now described. All patents, patent applications, and publications mentioned herein, whether supra or infra, are each incorporated by reference in its entirety.

## 25 Definitions

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single-or double-stranded, and represent the sense or antisense strand. Similarly, "amino  
30 acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence and fragments or portions thereof, of a naturally occurring or synthetic molecule.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

As used herein, "DARPP-32" or "nhDARPP-32" or "DARPP -32 of the invention" or "invention protein" or grammatical equivalents thereof are used interchangeably to refer to "Dopamine- and cyclic AMP (cAMP)-regulated phosphoprotein having a molecular weight of 32 kilodaltons". The term refer to the amino acid sequences of substantially purified DARPP-32 obtained from any species and from any source whether natural, synthetic, semi-synthetic, or recombinant. Preferably the DARPP-3  
5 comprises the amino acid sequence as depicted in SEQ ID NO: 2 and derived from a guinea pig.

As used herein, the term "Thr34 DARPP-32" is used interchangeably with "Thr34 DARPP32," "thr<sup>34</sup> DARPP-32" "Threonine-34 DARPP-32" and "threonine-34 DARPP-32" along with analogous abbreviations and denotes the thirty-fourth amino acid residue of the amino sequence of  
10 DARPP-32 in SEQ ID NO: 2 or in the human counterpart as disclosed by Brene et al. (J. Neurosci. 14:985-998 (1994)) having the GenBank Accession No. of AAB30129.1, which is a threonine residue that can be phosphorylated by the cyclic AMP dependent protein kinase (PKA).

Likewise, the term "phospho-Thr34 DARPP-32," or analogous abbreviations as disclosed above, denotes the phosphorylated form of Thr34 DARPP-32.

As used herein, the term "Thr75 DARPP-32" is used interchangeably with "Thr75 DARPP32," "thr<sup>75</sup> DARPP-32", "Threonine-75 DARPP-32" and "threonine-75 DARPP-32" along with analogous abbreviations, and denotes the seventy-fifth amino acid residue in the amino sequence of  
15 DARPP-32 as shown in SEQ ID NO: 2. or in the human counterpart as disclosed in Brene et al. supra, having the GenBank Accession of AAB30129.1, which is a threonine residue that can be phosphorylated  
20 by Cdk5.

As used herein, the term "phospho-Thr75 DARPP-32," or analogous abbreviations as disclosed above, denotes the phosphorylated form of Thr75 DARPP-32.

As used herein, the term "Ser137 DARPP-32" is used interchangeably with "Ser137 DARPP32," "ser<sup>137</sup> DARPP-32", "Serine-137 DARPP-32" and "serine-137 DARPP-32" along with  
25 analogous abbreviations denotes the one-hundred and thirty-seventh amino acid residue of the amino sequence of DARPP-32 – SEQ ID NO: 2 or as disclosed by Brene et al. (J. Neurosci. 14:985-998 (1994)) having the GenBank Accession No. of AAB30129.1, which is a serine residue that can be phosphorylated by CK1.

Likewise, the term "phospho-Ser137 DARPP-32" or analogous abbreviations as  
30 disclosed above, denotes the phosphorylated form of Ser137 DARPP-32.

As used herein, the terms "CK1," "casein kinase I" or "CKI," are used interchangeably with or "casein kinase 1." CK1 is a member of the serine/threonine protein kinases. CK1 includes, but is not limited to members of the CK1 (CKI) family of multiple isoforms. See Desdouits, F. et al. 1995. J. Biol. Chem. 270:8772-8778; Gross et al., 1998, Cell Signal 10(10): 699-711; Vielhaber et al., 2001,  
35 IUBMB Life 51(2), 73-8).

Fragment of DARPP-32 can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues. Methods of synthesizing fragments are well known to a skilled artisan.

5 A "CK1 phosphorylatable fragment of DARPP-32" refers to a protein fragment of DARPP-32 of SEQ ID NO: 2 that contains a serine residue that, when in the dephosphorylated form, can be phosphorylated by CK1. For the nhDARPP-32 having the amino acid sequence as set forth in SEQ ID NO: 2 the serine residue is preferably Ser137 DARPP-32. Such fragments can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues. Methods of synthesizing fragments are well known to a skilled artisan. A CK1 phosphorylatable fragment of DARPP-32 can be prepared by  
10 any method commonly known in the art, e.g., cleaving (such as with a protease) and dephosphorylating the phosphorylated fragment from a larger fragment of phospho-Ser137 DARPP-32 protein or from the full-length phospho-Ser137 DARPP-32 protein.

As used herein, the term "Ser102-DARPP-32" is used interchangeably with "Ser102 DARPP32," "ser<sup>102</sup> DARPP-32", "Serine-1302 DARPP-32" and "serine-102 DARPP-32" along with  
15 analogous abbreviations denotes the one-hundred and second amino acid residue of the amino sequence of DARPP-32 – SEQ ID NO: 2 or as disclosed by Brene et al. (J. Neurosci. 14:985-998 (1994)) having the GenBank Accession No. of AAB30129.1, which is a serine residue that can be phosphorylated by CK2.

Likewise, the term "phospho-Ser102 DARPP-32" or analogous abbreviations as  
20 disclosed above, denotes the phosphorylated form of Ser102 DARPP-32.

As used herein, the terms "CK2," "casein kinase 2" or "CKI," are used interchangeably with or "casein kinase 2." CK2 is a member of the serine/threonine protein kinases.

Fragment of DARPP-32 can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues. Methods of synthesizing fragments are well known to a skilled  
25 artisan.

A "CK2 phosphorylatable fragment of DARPP-32" refers to a protein fragment of DARPP-32 of SEQ ID NO: 2 that contains a serine residue that, when in the dephosphorylated form, can be phosphorylated by CK2.

The terms "CDK5", "Cdk5" or "cdk5" are used interchangeably with "cyclin-dependent  
30 kinase 5," which is also known as neuronal cyclin-dependent-like protein (Nclk) and tau protein kinase II (TPKII). Cdk5 is a member of the cyclin dependent kinases but atypically Cdk5 employs a non-cyclin cofactor called neuronal cyclin-dependent-like kinase 5 associated protein (Nck5a) rather than a cyclin. It is a protein kinase that phosphorylates DARPP-32 on Threonine-75 but not on Threonine-34.

Likewise, "Cdk5 phosphorylatable fragment of DARPP-32" refers to a protein fragment  
35 of DARPP-32 that contains a threonine residue that when in the dephosphorylated form can be

phosphorylated by Cdk5. For the non-human DARPP-32 having the amino acid sequence of SEQ ID NO:2, the threonine residue is preferably Thr75 DARPP-32. Fragments can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues. For example, in a particular embodiment, the peptide fragment comprises 5 consecutive amino acids from SEQ ID NO: 2 including Thr75.

5           The term "PP2C dephosphorylatable fragment of DARPP-32" refers to a protein fragment of DARPP-32 that contains a serine residue that when in the phosphorylated form can be dephosphorylated by PP2C. For the non-human DARPP-32 having the amino acid sequence of SEQ ID NO:2, the serine residue is preferably Ser137 DARPP-32. Fragments can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues. All of the peptide fragments of DARPP-  
10   32 can be prepared by any method commonly known in the art, e.g., cleaving (such as with a protease) and by phosphorylating the dephosphorylated fragment or by cleaving (such as with a protease) the phosphorylated fragment from a larger fragment of phospho-Ser137 DARPP-32 protein or from the full-length phospho-Ser137 DARPP-32 protein.

          As used herein, the term "PP2B dephosphorylatable fragment of DARPP-32" is a protein  
15   fragment of DARPP-32 that contains a threonine residue that when in the phosphorylated form can be dephosphorylated by PP2B. For the DARPP-32 of SEQ ID NO: 2, the threonine residue is preferably Thr34 DARPP-32. Preferred fragments can be between about 5 and 100 residues, or more preferably between about 10 and 50 residues and may be prepared as any of the above fragments.

          Likewise, the term "PP2A dephosphorylatable fragment of DARPP-32" is a protein  
20   fragment of DARPP-32 that contains a threonine residue that when in the phosphorylated form can be dephosphorylated by PP2A. For the DARPP-32 of SEQ ID NO:2, the threonine residue is preferably Thr75 DARPP-32.

          The amount and/or rate of phosphorylation of DARPP-32 or of a phosphorylatable fragment of DARPP-32, as described hereinabove, as a kinase reaction is "significantly changed" when  
25   the amount and/or rate of phosphorylation of DARPP-32 or the phosphorylatable fragment of DARPP-32 is increased or decreased by at least about 10-25%, relative to the control reaction. Preferably, a significant change in rate of the phosphorylation of DARPP-32 by a molecule of interest (e.g., dopamine) observed in the presence of a potential modulator is at some point correlated with the Michaelis constants (e.g., the  $V_{max}$  or  $K_m$ ) of the reaction. For example, in the case of an inhibitor, a  $K_i$  can be determined.  
30   Thus, in certain embodiments, it may be preferable to study various concentrations of a modulator in a reaction mixture to allow the identification of the potential modulator as a modulator.

          As used herein, the amount and/or rate of dephosphorylation of DARPP-32 or of a dephosphorylatable fragment of DARPP-32, as described hereinabove, in a phosphatase reaction is "significantly changed" when the amount and/or rate of dephosphorylation of DARPP-32 or the  
35   dephosphorylatable fragment of DARPP-32 is increased or decreased by at least about 10-25%, relative to

the control reaction. Preferably, a significant change in rate of the dephosphorylation of DARPP-32 by a molecule of interest (e.g., PP2C, PP2B or PP2A) observed in the presence of a potential modulator is at some point correlated with the Michaelis constants (e.g., the  $V_{max}$  or  $K_m$ ) of the reaction. For example, in the case of an inhibitor, a  $K_i$  can be determined. Thus, in certain embodiments, it may be preferable to study various concentrations of a modulator in a reaction mixture to allow the identification of the potential modulator as a modulator.

As used herein, the term "dopaminergic signaling pathway mediated disorder" is used interchangeably with the terms "dysfunctional dopamine regulated disorder" refers collectively to a disorder characterized by a "dysfunctional" or "dysregulation" of an intracellular signaling pathway, preferably a dopaminergic signaling pathway that is mediated by DARPP-32. Such disorders include, but are not limited to, depression, manic-depressive disorder, obsessive-compulsive disorder, eating disorder, post-traumatic stress syndrome, Parkinson's disease, schizophrenia, or a neurodegenerative disorder. Such a disorder also includes, but is not limited to, a disease (e.g., depression) or a condition (e.g., addiction to cocaine) that involves an aberration or dysregulation of a signal transmission pathway, including, but not limited to, neurotransmission mediated by dopaminergic receptors in excitable cells, tissues or organs (e.g., neurons, brain, central nervous system, etc.). A dysregulated serotonergic pathway is also included within the meaning of this disorder. Preferably, the pathway affected includes the phosphorylation and/or dephosphorylation of DARPP-32, with the corresponding treatment of the dysregulation involving the stimulation and/or inhibition of the phosphorylation and/or dephosphorylation of one or more specific threonine and/or serine residues of DARPP-32 (see, e.g., Greengard et al., Neuron 23:435-447 (1999); Bibb et al., Proc. Natl. Acad. Sci. 97:6809-6814 (2000)).

A "variant" of nhDARPP-32, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

"Alterations" in the polynucleotide of SEQ ID NO:1, as used herein, comprise any alteration in the sequence of polynucleotides encoding the protein of the invention including deletions, insertions, and point mutations that may be detected using hybridization assays. Included within this definition is the detection of alterations to the genomic DNA sequence which encodes nhDARPP-32 (e.g., by alterations in the pattern of restriction fragment length polymorphisms capable of hybridizing to SEQ ID NO:1), the inability of a selected fragment of SEQ ID NO:1 to hybridize to a sample of genomic DNA

(e.g., using allele-specific oligonucleotide probes), and improper or unexpected hybridization, such as hybridization to a locus other than the normal chromosomal locus for the gene encoding DARPP-32 (e.g., using fluorescent in situ hybridization "FISH" to metaphase chromosomes spreads).

5 A "deletion", as used herein, refers to a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent.

An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, respectively, as compared to the naturally occurring molecule.

10 A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

The term "biologically active" as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic nhDARPP-32, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific  
15 antibodies.

As used herein, the term "modulate" or "modulation" shall have its usual meaning, and encompasses the meanings of the words "enhance," "inhibit," and "mimic." "Modulation" of activity may be either an increase or a decrease in protein activity, a change in the degree or amount of phosphorylation, change in binding characteristics, or any other change in the biological, functional, or  
20 immunological properties of DARPP-32.

As used herein, an "agonist" is any compound that acts directly or indirectly through or upon a receptor to produce a pharmacological effect. The terms "antagonist" or "inhibitor" is any moiety or compound that blocks the stimulation of a target molecule, e.g., DARPP-32 and its resulting pharmacological effect.

25 As used herein, an "effective amount" of a modulatory compound is an amount that can be determined by one of skill in the art based on data from studies using methods of analysis such as those disclosed herein. Such data may include, but not be limited to, results from IC50 determinations etc.

The term "derivative", as used herein, refers to the chemical modification of a nucleic acid encoding nhDARPP-32 or the encoded nhDARPP-32. Illustrative of such modifications would be  
30 replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of the natural molecule.

The term "substantially purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are  
35 naturally associated.

The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g., C<sub>0</sub>t or R<sub>0</sub>t analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., membranes, filters, chips, pins or glass slides to which cells have been fixed for in situ hybridization).

The terms "complementary" or "complementarity", as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, for the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between the nucleic acids strands.

The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid; it is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous sequence or probe to the target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target sequence.

As known in the art, numerous equivalent conditions may be employed to comprise either low or high stringency conditions. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in solution or

immobilization, etc.), and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

5               The term "stringent conditions", as used herein, is the "stringency" which occurs within a range from about  $T_m - 5^\circ\text{C}$ . ( $5^\circ\text{C}$ . below the melting temperature ( $T_m$ ) of the probe) to about  $20^\circ\text{C}$ . to  $25^\circ\text{C}$ . below  $T_m$ . As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect identical or related polynucleotide sequences.

10               The term "antisense", as used herein, refers to nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules may be produced by any method, including synthesis by ligating the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a complementary strand. Once introduced into a cell, this  
15 transcribed strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

20               The term "portion", as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein "comprising at least a portion of the amino acid sequence of SEQ ID NO: 2" encompasses the full-length nhDARPP-32 and fragments thereof.

25               "Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various method well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection,  
30 and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host cell chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

              The term "sample", as used herein, is used in its broadest sense. A biological sample  
35 suspected of containing nucleic acid encoding nhDARPP-32 or fragments thereof may comprise a cell,

chromosomes isolated from a cell (e.g., a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for northern blot analysis), cDNA (in solution or bound to a solid support), extract from cells or a tissue, and the like.

5                   As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, F(ab').sub.2, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind nhDARPP-32 polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal can be derived from translated cDNA or synthesized chemically, and can be  
10                   conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

                  The term "humanized antibody", as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a  
15                   human antibody, while still retaining the original binding ability.

                  The term "antigenic determinant" as used herein, refers to that portion of a molecule that makes contact with a particular antibody (i.e., an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or  
20                   structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

                  As used herein, the term "about" means within 10 to 15%, preferably within 5 to 10%. For example an amino acid sequence that contains about 60 amino acid residues can contain between 51 to 69 amino acid residues, more preferably 57 to 63 amino acid residues.

25

#### The nhDARPP-32 Coding Sequences

                  The nucleic and deduced amino acid sequences of nhDARPP-32 are shown in SEQ ID NOS:1 and 2 respectively. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of nhDARPP-32 can be used to generate recombinant molecules which express  
30                   nhDARPP-32.

                  Methods for DNA sequencing are well known to a skilled artisan and may employ such enzymes as the Klenow fragment of DNA polymerase I Sequenase.RTM. (US Biochemical Corp, Cleveland Ohio)), Taq polymerase (Perkin Elmer, Norwalk Conn.), thermostable T7 polymerase (Amersham, Chicago Ill.), or combinations of recombinant polymerases and proofreading exonucleases  
35                   such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg Md.). As well,

methods to extend the DNA from an oligonucleotide primer annealed to the DNA template of interest have been developed for both single-stranded and double-stranded templates. Chain termination reaction products were separated using electrophoresis and detected via their incorporated, labelled precursors. Recent improvements in mechanized reaction preparation, sequencing and analysis have permitted  
5 expansion in the number of sequences that can be determined per day. Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno Nev.), Peltier Thermal Cycler (PTC200; MJ Research, Watertown Mass.) and the ABI Catalyst 800 and 377 and 373 DNA sequencers (Perkin Elmer).

The quality of any particular cDNA library may be determined by performing a pilot  
10 scale analysis of the cDNAs and checking for percentages of clones containing vector, lambda or E. coli DNA, mitochondrial or repetitive DNA, and clones with exact or homologous matches to public databases.

#### Extending the Polynucleotide Sequence:

15 The polynucleotide sequence - SEQ ID NO:1 or biologically equivalent sequences thereof may be extended utilizing partial nucleotide sequence and various methods known in the art to detect upstream sequences such as promoters and regulatory elements. Gobinda et al (1993; PCR Methods Applic 2:318-22) disclose "restriction-site polymerase chain reaction (PCR)" as a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus. According to the  
20 process, initially, a genomic DNA is amplified in the presence of primer to a linker sequence and a primer specific to the known region. Thereafter, the amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR may also be used to amplify or extend the target sequences using divergent  
25 primers based on a known region (Triglia T. et al(1988) Nucleic Acids Res 16:8186). The primers may be designed using Oligo 4.0 (National Biosciences Inc, Plymouth Minn.), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72°C. The method proposes using several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is thereafter circularized by  
30 intramolecular ligation and used as a PCR template.

Capture PCR (Lagerstrom M. et al (1991) PCR Methods Applic 1:111-19) is drawn to a method for PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome (YAC) DNA. Capture PCR also requires multiple restriction enzyme digestions and ligations to place an engineered double-stranded sequence into an unknown portion of the DNA  
35 molecule before PCR.

Likewise, Parker J. D. et al (1991; Nucleic Acids Res 19:3055-60), teach walking PCR, a method for targeted gene walking which permits retrieval of unknown sequence. PromoterFinder™ a new kit available from Clontech (Palo Alto Calif.) uses PCR, nested primers and PromoterFinder libraries to walk in genomic DNA. This process avoids the need to screen libraries and is useful in finding  
5 intron/exon junctions.

Another PCR method, "Improved Method for Obtaining Full Length cDNA Sequences" by Guegler et al, patent application Ser. No. 08/487,112, filed Jun. 7, 1995 and hereby incorporated by reference, employs XL-PCR.TM. (Perkin-Elmer) to amplify and/or extend nucleotide sequences.

Preferred libraries for screening for full length cDNAs are ones that have been size-  
10 selected to include larger cDNAs. Also, random primed libraries are preferred in that they will contain more sequences which contain the 5' and upstream regions of genes. A randomly primed library may be particularly useful if an oligo d(T) library does not yield a full-length cDNA. Genomic libraries are useful for extension 5' of the promoter binding region.

A newer method for analyzing either the size or confirming the nucleotide sequence of  
15 sequencing or PCR products is commonly known as "capillary electrophoresis". Systems for rapid sequencing are available from Perkin Elmer, Beckman Instruments (Fullerton Calif.), and other companies. In general, capillary sequencing employs flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity is converted to electrical  
20 signal using appropriate software (eg. Genotyper™ and Sequence Navigator™ from Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display is computer controlled. Capillary electrophoresis is particularly suited to the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample. The reproducible sequencing of up to 350 bp of M13 phage DNA in 30 min has been reported (Ruiz-Martinez M. C. et al (1993) Anal Chem  
25 65:2851-8).

#### Expression of the Nucleotide Sequence:

In accordance with the present invention, the polynucleotide sequence(s) – SEQ ID NO:1 or biologically equivalent fragment/sequences thereof or functional equivalents thereof, may be used to  
30 generate recombinant DNA molecules that direct the expression of DARPP-32 in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express the invention protein- nhDARPP-32. As will be understood by those of skill in the art, it may be advantageous to produce the nhDARPP-32 -encoding nucleotide sequences possessing non-naturally occurring codons.  
35 Codons preferred by a particular prokaryotic or eukaryotic host (Murray E. et al (1989) Nuc Acids Res

17:477-508) can be selected, for example, to increase the rate of GPG expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequence of SEQ ID NO:1 under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego Calif.) incorporated herein by reference, and confer a defined "stringency" as explained below.

"Maximum stringency" typically occurs at about  $T_m-5^\circ\text{C}$ . ( $5^\circ\text{C}$ . below the  $T_m$  of the probe); "high stringency" at about  $5^\circ\text{C}$ . to  $10^\circ\text{C}$ . below  $T_m$ ; "intermediate stringency" at about  $10^\circ\text{C}$ . to  $20^\circ\text{C}$ . below  $T_m$ ; and "low stringency" at about  $20^\circ\text{C}$ . to  $25^\circ\text{C}$ . below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences. The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J. (1994) Dictionary of Biotechnology, Stockton Press, New York N.Y.) as well as the process of amplification has carried out in polymerase chain reaction technologies as described in Dieffenbach C. W. and G. S. Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview N.Y.) and incorporated herein by reference.

As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent. As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring DARPP-32. As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

Altered DARPP-32 encoding polynucleotide sequences of the invention that may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally/biologically equivalent DARPP-32. The protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent nhDARPP-32. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological activity of a nhDARPP-32 is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with

uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine phenylalanine, and tyrosine.

Also included within the scope of the present invention are alleles of the nhDARPP-32.

As used herein, an "allele" or "allelic sequence" is an alternative form of nhDARPP-32, e.g. the

5 nhDARPP-32 isoform. Alleles result from a mutation, i.e., a change in the nucleic acid sequence, and generally produce altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to deletions, additions or substitutions of amino acids. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given  
10 sequence.

The nucleotide sequences of the present invention may be engineered in order to alter a nhDARPP-32 coding sequence for a variety of reasons, including but not limited to, alterations, which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis to insert new  
15 restriction sites, to alter glycosylation patterns, to change codon preference, etc.

Yet another embodiment of the invention proposes ligating a DARPP-32 natural, modified or recombinant sequence to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for inhibitors of DARPP-32 activity, it may be useful to encode a chimeric DARPP-32 protein expressing a heterologous epitope that is recognized by a commercially  
20 available antibody. A fusion protein may also be engineered to contain a cleavage site located between a DARPP-32 sequence and the heterologous protein sequence, so that the nhDARPP-32 may be cleaved and purified away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of nhDARPP-32 (SEQ ID NO:1) could be synthesized, whole or in part, using chemical methods well known in the art (see  
25 Caruthers M. H. et al (1980) Nuc Acids Res Symp Ser 215-23, Horn T. et al(1980) Nuc Acids Res Symp Ser 225-32, etc). Alternatively, the protein itself could be produced using chemical methods to synthesize a nhDARPP-32 amino acid sequence, whole or in part identical to that embodied in SEQ ID NO:2. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) Proteins Structures And  
30 Molecular Principles, W. H. Freeman and Co, New York N.Y.). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (eg, the Edman degradation procedure; Creighton, supra).

Direct peptide synthesis can be performed using various solid-phase techniques (Roberge J. Y. et al (1995) Science 269:202-204) and automated synthesis may be achieved, for example, using the  
35 ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the

manufacturer. Additionally the amino acid sequence of nhDARPP-32, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequence(s) from other calcium channel subunits, or any part thereof, to produce a variant polypeptide.

5 Expression Systems:

In order to express a biologically active nhDARPP-32 of SEQ ID NO: 2 including fragments, and biologically equivalent fragments thereof, the nucleotide sequence coding for nhDARPP-32, or a functional equivalent, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

10 Conventional methods, e.g., which are well known to those skilled in the art can be used to construct expression vectors containing a nhDARPP-32 coding sequence and appropriate transcriptional or translational controls. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination or genetic recombination. Such techniques are described in Maniatis et al (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview  
15 N.Y. and Ausubel F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York N.Y.

A variety of expression vector/host systems may be utilized to contain and express a nhDARPP-32 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast  
20 transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (eg, baculovirus); plant cell systems transfected with virus expression vectors (eg, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (eg, Ti or pBR322 plasmid); or animal cell systems.

The "control elements" or "regulatory sequences" of these systems vary in their strength  
25 and specificities and are those nontranslated regions of the vector, enhancers, promoters, and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript.RTM.  
30 phagemid (Stratagene, LaJolla Calif.) and ptrp-lac hybrids and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (eg, heat shock, RUBISCO; and storage protein genes) or from plant viruses (eg, viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from the mammalian genes or from mammalian viruses are most appropriate. If it is necessary to

generate a cell line that contains multiple copies of nhDARPP-32, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for nhDARPP-32 of SEQ ID NO:2 or variant or fragment thereof (collectively referred to as "nhDARPP-32". For example, when large quantities of nhDARPP-32 are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli cloning and expression vector Bluescript.RTM. (Stratagene), in which the nhDARPP-32 coding sequence may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke G. & S. M. Schuster (1989) J Biol Chem 264:5503-5509); and the like. pGEX vectors (Promega, Madison Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems are designed to include heparin, thrombin or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the nhDARPP-32 moiety at will.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH may be used. For a review of the vectors and promoters, see Ausubel et al (supra).

In cases where plant expression vectors are used, the expression of a nhDARPP-32 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S or 19S promoters of CaMV (Rhodes C. A. et al (1988) Science 240:204-207) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu N. et al (1987) EMBO J 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi G. et al (1984) EMBO J 3:1671-79; Broglie R. et al (1984) Science 224:838-43); or heat shock promoters (Winter J. and Sinibaldi R. M. (1991) Results Probl Cell Differ 17:85-105) may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Refer to Hobbs S or Murry L E in McGraw Yearbook of Science and Technology (1992) McGraw Hill New York N.Y., pp 191-196 for reviews of such techniques.

An alternative expression system which could be used to express nhDARPP-32 encoding sequence is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The nhDARPP-32 coding sequence may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of nhDARPP-32 will render the polyhedrin gene inactive and produce recombinant virus lacking coat

protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which nhDARPP-32 is expressed (Smith G. et al (1983) *J Virol* 46:584; Engelhard E. K. et al (1994) *Proc Nat Acad Sci* 91:3224-7).

5 In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a nhDARPP-32 coding sequence may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will result in a viable virus capable of expressing nhDARPP-32 in infected host cells. (Logan and Shenk (1984) *Proc Natl Acad Sci* 81:3655-59). In addition, transcription enhancers, such as the rous sarcoma virus (RSV) enhancer,  
10 may be used to increase expression in mammalian host cells.

Specific initiation signals may also be required for efficient translation of an inserted nhDARPP-32 sequence. These signals include the ATG initiation codon and adjacent sequences. In cases where nhDARPP-32, its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where  
15 only coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided. As well, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf D. et al (1994) *Results Probl Cell Differ* 20:125-62; Bittner M. et al (1987) *Methods in Enzymol* 153:516-544).  
20

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "prepro" form of the protein may  
25 also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, etc have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is  
30 preferred. For example, cell lines which stably express nhDARPP-32 may be transformed using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection and its presence allows growth and recovery of cells which

successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler M. et al (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy I. et al (1980) Cell 22:817-23) genes which can be employed in tk<sup>-</sup> or aprt<sup>-</sup> cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler M. et al (1980) Proc Natl Acad Sci 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin F. et al (1981) J Mol Biol 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman S. C. and R. C. Mulligan (1988) Proc Natl Acad Sci 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins,  $\beta$  glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes C. A. et al (1995) Methods Mol Biol 55:121-131).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding nhDARPP-32 is inserted within a marker gene sequence, recombinant cells containing sequences encoding nhDARPP-32 can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with an nhDARPP-32 sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem nhDARPP-32 as well.

Transformed cells containing the polynucleotide sequence encoding nhDARPP-32 can be detected by DNA-DNA or DNA-RNA hybridization or amplified using probes or portions or fragments of polynucleotides encoding nhDARPP-32. Conventional nucleic acid amplification based assays generally involve using oligonucleotides or oligomers based on the nhDARPP-32-encoding sequence to detect transfectants containing DNA or RNA encoding the target sequence, e.g., nhDARPP-32. Consequently, as used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe or amplimer.

A variety of protocols for detecting and measuring the expression of nhDARPP-32, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent

activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the invention protein is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R. et al (1990, Serological Methods, a Laboratory Manual, APS Press, St. Paul Minn.) and Maddox D. E. et al (1983, J Exp Med 158:1211).

Likewise, the prior art is replete with references teachings a wide variety of labels and conjugation techniques useful in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding nhDARPP-32 include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide are detailed in the art. Alternatively, target sequences – those encoding nhDARPP-32, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits (Pharmacia Upjohn, (Kalamazoo, Mich.); Promega (Madison, Wis.) and U.S. Biochemical Corp., Cleveland, Ohio). Suitable reporter molecules or labels, which may be used, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in U.S. Pat. No. 4,816,567 incorporated herein by reference.

#### Purified nhDARPP-32 polypeptides:

Host cells transformed with a nhDARPP-32 encoding nucleotide sequence may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing nhDARPP-32 can be designed with signal sequences which direct secretion of nhDARPP-32 through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join nhDARPP-32 to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll D. J. et al (1993) DNA Cell Biol 12:441-53; see also above discussion of vectors containing fusion proteins). Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle Wash.). The inclusion of a cleavable linker sequences such as those specific for Factor XA

or enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and nhDARPP-32 may be used to facilitate purification. One such expression vector which may be used provides for expression of a fusion protein containing a nhDARPP-32 and a nucleic acid encoding 6 histidine residues followed by thioredoxin and an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC  
5 (immobilized metal ion affinity chromatography as described in Porath, J. et al. (1992) Prot. Exp. Purif. 3: 263-281) while the enterokinase cleavage site provides a means for purifying nhDARPP-32 from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D. J. et al. (1993) DNA Cell Biol. 12:441-453.

On the other hand, suitable host cells that contain the coding sequence for nhDARPP-32  
10 and express nhDARPP-32 may be identified by a variety of procedures known to one of skill in the art. Such procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations, fluorescent activated cell sorting and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of the nucleic acid or protein.

Eukaryotic cells expressing heterologous nhDARPP-32 of the invention may be used in  
15 assays to assay for DARPP-32 modulators. The recombinant cells of the invention may be used to assess D1 or D2 receptor function or DARPP-32 tissue distribution and to identify compounds that modulate the activity of, for example, DARPP-32. Because DARPP-32 is a member of the dopamine regulated signaling cascade and is thus involved in regulating the intracellular effects of dopamine within the nervous system and other fundamental processes, assays designed to assess such activities and assays to  
20 identify modulators of these activities provides a means to understand fundamental physiological processes and also a means to identify new drug candidates for an array of disorders.

In addition to recombinant methods, fragments of nhDARPP-32 may be also produced by direct peptide synthesis using solid-phase techniques (cf Stewart et al. (1969) Solid-Phase Peptide Synthesis, W. H. Freeman Co., San Francisco, Calif.; Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-  
25 2154). In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Various fragments of nhDARPP-32 may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

In another aspect, the recombinant cells of the invention contain heterologous gene(s)  
30 (foreign to the cell- cDNA encoding DARPP-32 of SEQ ID NO:2) with a transcriptional control element, which is active in the cell and responsive to an ion or molecule capable of entering the cell through a functional calcium channel and linked operatively for expression to a structural gene for an indicator protein, can also be employed for assaying a compound for calcium channel agonist or antagonist activity.

The preferred method comprises exposing a culture of such recombinant cells to a  
35 solution of a compound being tested for such activity, together with an ion or molecule, which is capable

of entering the cells through a functional calcium channel and affecting the activity of the transcriptional control element controlling transcription of the genes for the indicator protein, and comparing the level of expression, in the cells of the culture, of the genes for the indicator protein with the level of such expression in the cells of another, control culture of such cells.

5 A "control culture," as clearly understood by the skilled, will be a culture that is treated, in substantially the same manner as the culture exposed to the compound being assayed except that the control culture is not exposed to the compound being assayed. Alternatively, control culture may comprise cells expressing a dysfunctional calcium channel. Levels of expression of the genes for the indicator proteins are ascertained readily by the skilled by known methods, which involve measurements  
10 of the concentration of indicator protein via assays for detectable compounds produced in reactions catalyzed by the indicator protein.

As indicated above, indicator proteins are enzymes which are active in the cells of the invention and catalyze production of readily detectable compounds (e.g., chromogens, fluorescent compounds).

15 The role of DARPP-32 in the mobilization of  $\text{Ca}^{++}$  as part of the signal transduction pathway can be assayed *in vitro*. It requires preloading calcium channel expressing cells with a fluorescent dye such as FURA-2 or BCECF (Universal Imaging Corp, Westchester Pa.) whose emission characteristics have been altered by  $\text{Ca}^{++}$  binding. When the cells are exposed to one or more activating stimuli artificially or physiologically,  $\text{Ca}^{++}$  flux takes place. This flux can be observed and quantified by  
20 assaying the cells in a fluorometer or fluorescent activated cell sorter. The measurement of  $\text{Ca}^{++}$  mobilization in mobilization assays is well known. Briefly, in a calcium mobilization assay, cells expressing the target receptor are loaded with a fluorescent dye that chelates calcium ions, such as FURA-2. Upon addition of a calcium channel modulator to the cells expressing a calcium channel, the target modulator binds to the calcium channel and calcium is released from the intracellular stores. The dye  
25 chelates these calcium ions. Spectrophotometric determination of the ratio for dye:calcium complexes to free dye determine the changes in intracellular calcium concentrations upon addition of the target modulator. Hits from screens and other test compounds can be similarly tested in this assay to functionally characterize them as agonists or antagonists. Increases in intracellular calcium concentrations are expected for compounds with agonist activity while compounds with antagonist  
30 activity are expected to block target modulator stimulated increases in intracellular calcium concentrations. See U.S. patent Number 6,420,137 and similar patents.

#### Proposed Uses of the various DARPP-32 Sequences of the Invention:

35 In another embodiment of the invention, the DARPP-32 protein or fragments thereof detailed herein may be used for therapeutic purposes.

Based on the chemical and structural homology that exists among nhDARPP-32 protein (SEQ ID NO:2) and its human counterpart as disclosed in Brene et al., supra, the DARPP-32 of SEQ ID NO: 2 or a functionally equivalent fragment thereof, this protein is a cAMP-regulated phosphoprotein and is believed to function in the signal transduction pathway of neurotransmitters in brain tissue. From the homology information provided above, it appears that nhDARPP-32 plays a role in the modulation of neurotransmitter signal transduction and cell development. Consequently, the herein provided sequences may be used in assays to identify anti-psychotics for use in treating human disorders.

The collective data suggest that controlling DARPP-32 activity may provide a novel approach to degenerative neuronal disease treatment and may be especially be useful in combination therapy with other, conventional therapeutic moieties. This is so because combinations of therapeutic moieties having different cellular mechanisms of action often have synergistic effects allowing the use of lower effective doses of each therapeutic moiety thus lessening side effects.

Accordingly, in one embodiment of the invention, the modulation of nhDARPP-32 by agonists and antagonists may play a role in reconstructing signal transduction pathways that have been interrupted by degenerative neuronal disease. In another embodiment of the invention, nhDARPP-32 or derivatives thereof, may be used for regenerating and enhancing the survival of nerve cells by supplying nhDARPP-32 or stimulating residual nhDARPP-32 with nhDARPP-32 agonists to stop the degenerative process in certain brain diseases such as Parkinson's and Huntington's disease.

In an alternative therapeutic embodiment, antagonists which block or modulate the effect of DARPP-32 may be used in those situations where such inhibition or modulation is therapeutically desirable. Such situations may include the down-regulation of DARPP-32 activity to regulate cell growth or to suppress abnormal signal transduction in diseased tissue. For example, in one aspect, antibodies which are specific for DARPP-32 (SEQ ID NO:2) may be used as an agonist, antagonist, or as part of a targeting or delivery mechanism so as to bring a pharmaceutical agent to cells or tissue which express DARPP-32.

The antibodies may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and other, may be immunized by injection with the protein of SEQ ID NO:2 or immunologically active fragments thereof or any functionally equivalent fragment or oligopeptide thereof. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet

hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum* are especially preferable. Preferably, the functionally equivalent peptides or fragments thereof used to induce antibodies to nhDARPP-32 have an amino acid sequence consisting of at least 5 amino acids, and more preferably at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Methods of determining antigenic determinants are well known and may be employed to identify those sequences which will induce an appropriate immune response. See Geysen et al. U.S Patent Nos. 5595915, 5998577 including references cited therein. Short stretches of nhDARPP-32 amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Monoclonal antibodies to DARPP-32 of SEQ ID NO: 2 may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Koehler et al. (1975) *Nature* 256:495-497; Kosbor et al. (1983) *Immunol. Today* 4:72; Cote et al. (1983) *Proc. Natl. Acad. Sci.* 80:2026-2030; Cole et al. (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss Inc., New York, N.Y., pp. 77-96).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison et al. (1984) *Proc. Natl. Acad. Sci.* 81:6851-6855; Neuberger et al. (1984) *Nature* 312:604-608; Takeda et al. (1985) *Nature* 314:452-454). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce nhDARPP-32-specific single chain antibodies. Antibodies with related specificity but of distinct idiotypic composition may be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton D. R. (1991) *Proc. Natl. Acad. Sci.* 88:11120-3).

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi et al. (1989) *Proc. Natl. Acad. Sci.* 86: 3833-3837 and Winter et al. (1991), *Nature* 349:293-299).

Antibody fragments which contain specific binding sites for nhDARPP-32 may also be generated using well known techniques. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse et al. (1989) *Science* 256:1275-1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between nhDARPP-32 and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a specific DARPP-32 protein is preferred, but a competitive binding assay may also be employed (Maddox et al. (1983) J. Exp. Med. 158:1211).

Proposed Diagnostic Assays Using DARPP 32 Specific Antibodies of the Invention:

In another embodiment, antibodies which are specific for nhDARPP-32 may be used for the diagnosis of conditions or diseases characterized by expression of DARPP-32, or in assays to monitor patients being treated with DARPP-32, agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for DARPP-32 include methods which utilize the antibody and a label to detect DARPP-32 in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols for measuring DARPP-32 expression, using either polyclonal or monoclonal antibodies specific for the respective protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on nhDARPP-32 is preferred, but a competitive binding assay may be employed.

In order to provide a basis for diagnosing abnormal levels of DARPP-32 expression, normal or standard values for DARPP-32 expression are established. Standard values may be obtained by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to DARPP-32 under conditions suitable for complex formation which are well known in the art. The amount of standard complex formation may be quantified by comparing various artificial membranes containing known quantities of DARPP-32 with both control and disease samples from biopsied tissues. Thereafter, standard values obtained from normal samples may be compared with values obtained from samples from subjects which are symptomatic for the disease. Deviation between standard and subject values establishes the parameters for diagnosing the disease.

In an alternative embodiment of the invention, the polynucleotides encoding nhDARPP-32 (SEQ ID NO:1) may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, antisense RNA and DNA molecules. The polynucleotides may be

used to detect and quantitate gene expression in biopsied tissues in which expression of DARPP-32 may be implicated. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of DARPP-32 relative to normal, and to monitor regulation of DARPP-32 activity levels during therapeutic intervention.

5 In one aspect, hybridization or PCT probes which are capable of detecting polynucleotide sequences, including genomic sequences encoding human DARPP-32 or closely related molecules, may be used to identify nucleic acid sequences which encode DARPP-32. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' region, and the stringency of the hybridization or  
10 amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding DARPP-32, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of these nhDARPP-32 encoding sequences. The hybridization probes of the subject invention may be derived from the nucleotide sequence of SEQ ID  
15 NO:1 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring DARPP-32.

Other means for producing specific hybridization probes for DNAs encoding DARPP-32 include the cloning of nucleic acid sequences encoding nhDARPP-32 or nhDARPP-32 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available,  
20 and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as <sup>32</sup>P or <sup>35</sup>S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

25 Polynucleotide sequences encoding the DARPP-32 of SEQ ID NO: 2 may also be used for the diagnosis of conditions or diseases which are associated with expression of DARPP-32. The polynucleotide sequences encoding DARPP-32 of SEQ ID NO:1 or derivatives thereof may be used in hybridization or PCR assays of fluids or tissues from patient biopsies to detect DARPP-32 expression, e.g., human DARPP-32 based, in part, upon the close homology between the nhDARPP-32 sequences  
30 disclosed herein and the corresponding human sequences. The form of such qualitative or quantitative methods may include Southern or Northern analysis, dot blot, or other membrane-based technologies; PCR technologies; dip stick, pin, chip, and ELISA, all methods which are well known in the art.

Considering the high degree of sequence homology between the sequences disclosed herein and the human DARPP-32 noted *supra*, the nucleotide sequences encoding of the invention may  
35 be useful in assays that detect activation or inactivation of human DARPP-32 associated with various

degenerative neuronal diseases. Accordingly, the nucleotide sequence encoding nhDARPP-32 of SEQ ID NO:1 may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly elevated over that of a comparable control sample, the nucleotide sequence has hybridized with nucleotide sequences in the sample, and the presence of elevated levels of nucleotide sequences encoding DARPP-32 in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with aberrant (high or low levels relative to normal) expression of DARPP-32 for example, in a human, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with DARPP-32, or a fragment thereof, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with a dilution series of DARPP-32 measured in the same experiment, where a known amount of a substantially purified DARPP-32 is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease associated with DARPP-32. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

Additional diagnostic uses for oligonucleotides encoding nhDARPP-32 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'.fwdarw.3') and another with antisense (3'.rarw.5'), employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantification of closely related DNA or RNA sequences.

Methods which may also be used to quantitate the expression of DARPP-32 include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P. C. et al. (1993) J. Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 229-236.) The speed of quantitation of multiple

samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

In other embodiments of the invention, the nucleotide sequences of the invention may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, such as the triplet genetic code, specific base pair interactions, and the like.

In another embodiment of the invention, the nucleic acid sequence of SEQ ID NO:1 may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence encoding human DARPP-32. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. Such techniques include in situ hybridization to chromosomal spreads, flow-sorted chromosomal preparations, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price C. M. (1993) *Blood Rev.* 7:127-134, and Trask B. J. (1991) *Trends Genet* 7:149-154.

The technique of fluorescent *in situ* hybridization of chromosome spreads, as described in Verma et al. (1988) *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York, N.Y., may also be used. Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of *Science* (265:1981f). Correlation between the location of the gene encoding nhDARPP-32 on a physical chromosomal map and a specific disease (or predisposition to a specific disease) may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier, or affected individuals.

*In situ* hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti et al. (1988) *Nature* 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

Alternatively, the DARPP-32 of the invention, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques with the aim of identifying therapeutic moieties useful for treating neurological diseases in humans. . The fragment employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes, between the invention protein and the therapeutic moiety being tested, may be measured.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to nhDARPP-32, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with nhDARPP-32, or fragments thereof, and washed. Bound nhDARPP-32 is then detected by methods well known in the art. Purified nhDARPP-32 can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In yet another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding nhDARPP-32 specifically compete with a test compound for binding nhDARPP-32 or human DARPP-32. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with nhDARPP-32.

In additional embodiments, the nucleotide sequences of the invention (SEQ ID NO:1) may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

#### Methods for Screening for Compounds that Modulate the Activity of Dopamine

Activation of dopamine per se and therapeutic moiety /agents that enhance dopaminergic neurotransmission act on cell-surface receptors. Without wishing to be bound by any particular theory, in one aspect of the invention, dopamine D1 receptors mediate the phosphorylation of DARPP-32 via dopamine D1 receptor intracellular signaling pathways. As noted supra, dopamine via the D1 receptors, activates adenylyl cyclase and increased cAMP, which, in turn, activates protein kinase A (PKA; cAMP-dependent protein kinase), which phosphorylates (or modulates phosphorylation of) downstream elements in intracellular signaling pathways, including but not limited to DARPP-32, cAMP responsive element binding protein (CREB), AMPA receptor (e.g., GluR1 AMPA receptor), cAMP, cGMP, CK1, CK2, Cdk5, PKA, PKG, PP-2C, PP-2B and PP-1. Activation of PKA leads to the phosphorylation of DARPP-32 at Thr34. This phosphorylation event converts DARPP-32 into an inhibitor of PP1. Consequently,

the phosphorylation state of Thr34/137-DARPP-32 can be modulated by modulation of PKA via the dopaminergic intracellular signaling pathway.

Alternatively, dopamine D2 receptor activation leads to adenylyl cyclase inhibition (and decreased cAMP). Intracellular concentration of cGMP also are unchanged or inhibited after D2 receptor activation. cGMP activates protein kinase G (PKG; cGMP-dependent protein kinase), which phosphorylates downstream signal transduction pathway elements, including but not limited to downstream elements in intracellular signaling pathways, including but not limited to, DARPP-32 .

Since dopamine mimics the activity of other substances that modulate DARPP-32 phosphorylation, such as activators of CK1 or CK2, inhibitors of cdk5, inhibitors of PP-1, inhibitors of PP2C, inhibitors of PP2B, or activators of PP2A, and since dopaminergic intracellular signaling pathways are involved in the etiology of Parkinson's disease, depression, schizophrenia, compounds that alter activity of dopaminergic intracellular signaling molecules; preferably PKA but also including, but not limited to CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C , PP2B and/or PP2A, may be identified using the herein disclosed sequences as a means of identifying compounds having anti-psychotic activity. See US Patent Application No. 2003/0109419, which is incorporated herein by reference in its entirety.

Likewise, since dopamine plays an important role in controlling levels of cAMP, and since the cAMP-PKA pathway interacts with many other signaling pathways in the brain, modulation of dopamine will, in certain embodiments, ameliorate the symptoms and/or be used in the treatment of disorders including, but not limited to, Parkinson's disease, Huntington's disease, attention deficit disorder (ADD), attention deficit hyperactivity disorder (ADHD), neurodegenerative disorder, Tourette's syndrome, tic disorder, Lesch-Nyans disease, substance or drug abuse, schizophrenia, depression, manic-depressive disorder and obsessive-compulsive disorder.

For example, dopamine via the D1 receptors, activates PKA formation and activates CK-1. CK-1, in turn, phosphorylates DARPP-32 at Ser137. This phosphorylation event converts DARPP-32 into an inhibitor of PP2B (i.e., calcineurin). Since PP2B dephosphorylates DARPP-32 at Thr34, the serotonin-mediated increase in DARPP-32 phosphorylation at Ser 137 potentiates the serotonin/PKA-mediated phosphorylation at Thr34-DARPP-32 and the subsequent inhibition of PP-1. In other embodiments, the phosphorylation state of Thr34-DARPP-32 can be modulated by modulation of PP2C via the dopaminergic intracellular signaling pathway. According to this embodiment, the phosphorylation of Thr34DARPP-32 increases via a decrease in the activity, e.g., inhibition, of PP2C.

In the following examples, it is understood that the high degree of sequence homology between the nhDARPP-32 protein of SEQ ID NO: 2 and the human DARPP-32 disclosed in Brene et al., supra, suggest that the herein disclosed protein of SEQ ID NO:2 may be used in various assay methods to ultimately identify compounds useful in treating various neurological disorders involving an aberrant dopaminergic signaling pathway regulated DARPP-32.

In a broad aspect, the invention provides a method for modulating activity of an intracellular signaling molecule, preferably, DARPP-32 comprising contacting an amount of a compound sufficient to alter activity of an intracellular signaling pathway, including but not limited to a dopamine D1 receptor, dopamine D2 receptor, serotonin, or glutamate (e.g., NMDA receptor, AMPA receptor).

5 The intracellular signaling molecule may also include any one or more of cAMP responsive element binding protein (CREB), AMPA receptor (e.g., GluR1 AMPA receptor), cAMP, cGMP, CK1, CK2, Cdk5, PKA, PKG, PP-2C, PP-2B, PP-1, calcium channels, Na/K ATPase and NMDA receptor.

Methods of modulating casein kinase 1 ("CK1 " or "CK1"), casein kinase 2 ("CK2"), cyclin-dependent kinase 5 ("Cdk5," "cdk5" or "CDK5"), protein phosphatase 1 ("PP-1), AMPA receptor  
10 ("AMPA"), protein phosphatase-2C ("PP2C"), protein phosphatase-2B ("PP2B") or protein phosphatase-2A ("PP2A") activity in a cell are also encompassed.

A representative embodiment features a method for modulating DARPP-32 activity in a cell comprising contacting said cell with an amount of a compound sufficient to alter activity of an intracellular signaling pathway, including but not limited to the dopamine D1 receptor intracellular  
15 signaling, wherein contact of said cell or tissue with the compound results in modulation of DARPP-32 activity.

Contact, of the cell with the compound results in a modulation of the activity of PKA, CK1, Cdk5, PP-1, PP2C, PP2B and/or PP2A, whose modulation may be quantified via the determination of the rate of phosphorylation or dephosphorylation of DARPP-32 of SEQ ID NO:2 at distinct residues  
20 known to one skilled in the art. It is understood that the cell expresses the protein of SEQ ID NO: 2 or a functionally equivalent fragment thereof. In other embodiments, the phosphorylation of an element downstream in an intracellular signaling pathway, including but not limited to a calcium channel, Na/K ATPase, NMDA receptor, and CREB, is modulated via modulation of dopamine. In certain embodiments, the compound is a compound identified by the methods of the invention, wherein the compound  
25 modulates DARPP-32 activity and wherein modulation this activity results in an alteration in the activity of said intracellular signaling molecule in a cell. In certain embodiments, the compound binds to dopamine. In other embodiments, the compound alters expression of dopamine.

A specific method contemplates detecting the increase (or decrease) in the amount of phosphorylation (or dephosphorylation) of Thr34-phosphorylated DARPP-32, Ser137-phosphorylated  
30 DARPP-32, or Thr75-phosphorylated DARPP-32. Detecting an increase or decrease in the phosphorylation of other residues mediated by the modulation of any one of PKA, CK1, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A are well known to one skilled in the art.

Another embodiment proposes a method for identifying a compound to be tested for its ability to modulate the activity of a dopaminergic intracellular signaling pathway in a cell comprising:

35 (a) determining a first level of dopamine activity in said cell;

(b) contacting said cell with a test compound; and

(c) determining a second level of dopamine activity, respectively, in said cell,

wherein a difference in said first level and said second level of dopamine activity is indicative of the ability of said test compound to modulate the dopaminergic intracellular signaling pathway.

5 Dopamine activity is quantified via the determination of the rate of phosphorylation or dephosphorylation of DARPP-32 of SEQ ID NO:2 at distinct residues known to one skilled in the art. In preferred embodiments, the phosphorylation of Thr34 of DARPP-32 is modulated via modulation of dopamine.

10 In a preferred embodiment, a difference in dopamine activity is indicative of the ability of said test compound to modulate phosphorylation-dependent activation of an intracellular signaling molecule, representative members of which include DARPP-32 (dopamine and cAMP-regulated phosphoprotein-32), cAMP responsive element binding protein (CREB), AMPA receptor (e.g., GluR1 AMPA receptor), CK1, CK2, Cdk5, PKA, PKG, PP-2C, PP-2B, PP-1, calcium channels, Na/K ATPase and NMDA receptor. Preferably, phosphorylation of DARPP-32 is modulated.

15 According to the invention, a control level means a separate baseline level measured in a comparable cell or tissue not contacted with a test compound or a level that is measured in a cell or tissue prior to contacting it with a test compound.

In furtherance of the above, the invention provides an exemplary embodiment that provides a method of identifying a compound that modulates dopamine activity in a dopamine D1  
20 receptor intracellular signaling pathway in a cell or tissue comprising:

(a) determining a level of dopamine activity in said cell or tissue prior to contact with the compound to obtain a first level; and determining a second level of dopamine after contact with said compound to in said cell or tissue, wherein a difference in said first level and said second level of dopamine activity is indicative of the ability of said test compound to modulate dopamine activity. In  
25 certain embodiments, the difference in dopamine activity is indicative of the ability of said test compound to modulate activity of the dopamine D1 receptor intracellular signaling pathway.

However, in a preferred embodiment the difference in dopamine activity is indicative of the ability of the test compound to modulate phosphorylation-dependent activation of an intracellular signaling pathway molecule, wherein said molecule is DARPP-32

30 An alternative embodiment of the invention provides a method of identifying a compound that modulates dopamine activity in a dopamine D1 receptor intracellular signaling pathway in a cell or tissue comprising:

(a) contacting said cell or tissue with a test compound; and (b) determining a level of dopamine activity in said cell or tissue; wherein a difference in said level and a control level of dopamine  
35 activity in a comparable cell or tissue not contacted with the test compound is indicative of the ability of

said test compound to modulate dopamine activity. Preferably, the difference in dopamine activity is indicative of the ability of said test compound to modulate phosphorylation-dependent activation of a DARPP-32. Phosphorylation/dephosphorylation activity of other members such cAMP responsive element binding protein (CREB), AMPA receptor (e.g., GluR1AMPA receptor), cAMP, cGMP, CK1, CK2, Cdk5; PKA, PKG, PP-2C, PP-2B, PP-1, calcium channels, Na/K ATPase and NMDA receptor may also be determined following any one or more of the assays detailed herein. .

Consequently, a specific embodiment provides a method for identifying a therapeutic moiety to be tested for an ability to treat a dopamine related disorder or a dopamine D1 intracellular signaling pathway disorder, in a patient in need of such treatment comprising:

- (a) contacting a potential therapeutic moiety with dopamine and Thr34-dephosphorylated DARPP-32; and
- (b) detecting the amount of phosphorylation of Thr34-dephosphorylated DARPP-32, wherein said therapeutic moiety has therapeutic utility for treating said disorder if an increase in the phosphorylation of Thr34-dephosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

Alternatively, the assay may measure the rate of dephosphorylation of Thr34-phosphorylated DARPP-32 in the presence of PP2B.

Another embodiment provides a method for identifying an therapeutic moiety to be tested for an ability to modulate activity of a dopamine D1 receptor, dopamine D2 receptor, serotonin or , glutamate (e.g., NMDA receptor, AMPA receptor) intracellular signaling pathway in a cell or tissue comprising:

- (a) contacting said cell or tissue with a potential therapeutic moiety; and (b) determining a level of dopamine activity in said cell; wherein a difference in said level and a control level of dopamine activity in a comparable cell or tissue not contacted with the test compound is indicative of the ability of said test compound to modulate of the intracellular signaling pathway. Preferably, modulation of a dopamine D1 receptor intracellular signaling pathway is modulated by dopamine.

In certain embodiments, the method comprises the additional step of: (c) determining whether said intracellular signaling pathway is modulated.

As would be clearly understood by a person of ordinary skill in the art, any and/or all of the embodiments disclosed herein for identifying an therapeutic moiety, drug or compound that can modulate the activity of dopamine including such procedures that incorporate rational drug design, as disclosed herein, can be combined to form additional drug screens and assays, all of which are contemplated by the present invention.

In certain embodiments, the compound modulates the activity of DRPP-32 by binding to DARPP-32. Binding may be measured under any standard art-known physiological conditions, according to methods well known in the art.

5 In another embodiment, the method comprises determining a first level of CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A activity in a cell ; contacting the cell or tissue with a test compound; and determining a second level of CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A activity in the cell or tissue, wherein a difference in the first level and the second level of CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A activity is indicative of the ability of the test compound to modulate CK1, CK2, Cdk5, AMPA receptor, PP-1, PP2C, PP2B and/or PP2A  
10 activity. According to the methods of the invention, patterns and/or levels of DARPP-32 phosphorylation may also be determined both before and after treatment of cells or tissues with a test compound.

One of skill would understand that according to the invention, once a compound is identified as capable of producing, e.g., altered patterns and/or levels of DARPP-32 phosphorylation and/or dephosphorylation similar to known ameliorative compounds, the compound may be used to treat  
15 a dopamine-related disorder, a dopamine D1 receptor intracellular signaling pathway disorder, as well as other conditions in which dopaminergic systems are involved such as a dysfunctional serotonergic signaling mediated disorder exemplified by depression. In the context of the present invention, the compounds identified would be administered as an effective dose or amount which can be determined by one of skill in the art based on data from studies such as presented in this specification. Such data would  
20 include, but not be limited to, results from IC50 determinations.

Methods of treating a subject presenting symptoms consistent with a disorder characterized by aberrant or dysregulation of a intracellular signaling pathway regulated by DARPP-32 are also provided. Preferably, the signaling pathway is a dopaminergic signaling pathway although a serotonergic signaling pathway is also included considering that serotonin has also been shown to mediate  
25 phosphorylation of DARPP-32.

The method proposes administering to a subject in need thereof an amount of a compound sufficient to alter activity of an intracellular signaling pathway such as the dopamine D1 receptor, dopamine D2 receptor, serotonin, or glutamate (e.g., NMDA receptor, AMPA receptor) intracellular signaling pathway.

30 In preferred embodiments, the compound is a compound identified by the methods of the invention, wherein the compound modulates dopamine activity and wherein modulation of dopamine activity results in an alteration in the activity of said intracellular signaling molecule in a cell or tissue.

In another embodiment, the invention provides a method for regulating phosphorylation-dependent activation of an intracellular signaling molecule comprising administering an amount of a  
35 compound sufficient to modulate dopamine activity, wherein modulation of the dopamine activity results

in an alteration in the phosphorylation-dependent activation of said intracellular signaling molecule in the cell, preferably DARPP-32.

In another embodiment, dopamine activity in cells or tissues of interest is modulated in situ or in vivo. The in vitro, in situ and in vivo applications may include, but are not limited to modulating activity in any of the cells disclosed hereinabove.

An exemplary *in vivo* method comprises administering the potential therapeutic moiety to a non-human mammal. The amount (and/or rate) of activation of dopamine is then determined. A therapeutic moiety is identified as capable of modulating the activity of an intracellular signaling pathway, via modulation of dopamine, when the amount (and/or rate) of dopamine activation is increased or decreased in the presence of the therapeutic moiety relative to in the absence of the therapeutic moiety. In preferred embodiments, the non-human mammal is a rodent. Preferably, modulation of dopamine results in an increase or decrease in the phosphorylation of DARPP-32.

Methods of testing a potential therapeutic moiety (e.g., a candidate drug, potential modulator, etc.) in animals or animal models are well known in the art. Thus potential therapeutic moieties can be used to treat whole animals

The potential efficacy of these compounds in relieving pathological symptoms of a disorder, including but not limited to, a dopamine-related disorder and/or a dopamine D1 or D2 intracellular signaling pathway disorder, can be assessed in animal models for disease

A still further aspect of the invention is a method for selecting a therapeutic moiety for possible use in the treatment of a psychotic disorder characterized by an aberrant dopaminergic intracellular signaling pathway regulated by DARPP-32, which comprises administering a suspected therapeutic moiety to an animal model for a disorder and measuring and/or determining the putative therapeutic moiety's effect on any of the phenotypic characteristics outlined above which may be believed to be related to said disorder.

In some embodiments, the therapeutic moiety is administered along with a D1 receptor agonist. The amount (and/or rate) of modulation of dopamine activity is then determined. Since the administration of e.g., a D1 receptor agonist, in the absence of the therapeutic moiety, should result in an increase in DARPP-32 activity, a therapeutic moiety is identified as capable of modulating the activity of dopamine when the amount (and/or rate) of activation is significantly increased or decreased in the presence of the moiety relative to in the absence of the moiety.

In other embodiments, the therapeutic moiety is administered along with a D1 receptor antagonist. The amount (and/or rate) of modulation of dopamine activity is then determined. Since the administration of a D1 receptor antagonist in the absence of the therapeutic moiety should result in a decrease in DARPP-32 activity, a therapeutic moiety is identified as capable of modulating the activity

of dopamine when the amount (and/or rate) of activation is significantly increased or decreased in the presence of the therapeutic moiety relative to in the absence of the therapeutic moiety .

Once a drug candidate is selected, structural variants of the drug candidate can be tested. These compounds can also be scrutinized and modified with parameters such as membrane permeability, specificity of effects, and toxicity. The selected (e.g., the most potent) compounds of this secondary screening can then be evaluated in situ and in animal models to determine whether the selected compounds alter the activity of dopamine, and/or induce predicted behavioral alterations with minimal to no side-effects. Such behavioral abnormalities are well known to a skilled artisan. In specific embodiments, methods for testing for antidepressant efficacy commonly known in the art, e.g., a rodent tail-suspension test, can be used. These tests can be then be followed by human trials in clinical studies. Alternatively, in certain embodiments, human trials in clinical studies can be performed without animal testing. Compounds affecting targets other than dopamine can also be similarly screened, using alternative targets exemplified below.

Alternatively, modulators (e.g., activators or inhibitors) of dopamine activity can be obtained by screening, e.g., a random peptide library produced by recombinant bacteriophage (see, e.g., Scott and Smith, *Science* 249:386-390 (1990); Cwirla et al., *Proc. Natl. Acad. Sci. USA* 87:6378-6382 (1990); Devlin et al., *Science* 249:404-406 (1990)) or a chemical library. Using the "phage method" very large libraries can be constructed (10<sup>6</sup>-10<sup>8</sup> chemical entities). A second approach may be to use chemical methods, of which the Geysen method (Geysen et al., *Molecular Immunology* 23:709-715 (1986); Geysen et al. *J. Immunologic Method* 102:259-274 (1987)) and the method of Fodor et al. (*Science* 251:767-773 (1991)) are examples. Furka et al. (14th international Congress of Biochemistry, Volume 5, Abstract FR:013 (1988); Furka, *Int. J. Peptide Protein Res.* 37:487-493 (1991)), Houghton (U.S. Pat. No. 4,631,211, issued December 1986) and Rutter et al. (U.S. Pat. No. 5,010,175, issued Apr. 23, 1991) disclose methods to produce a mixture of peptides. Such peptides can be tested as potential modulators of dopamine activity.

Synthetic libraries (Needels et al., *Proc. Natl. Acad. Sci. USA* 90:10700-4 (1993); Ohlmeyer et al., *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993); Lam et al., International Patent Publication No. WO 92/00252; Kocis et al., International Patent Publication No. WO 94/28028, each of which is incorporated herein by reference in its entirety), and the like can also be used to screen for modulators of dopamine activation, according to the present invention. Once a potential modulator is identified, chemical analogues can be either selected from a library of chemicals as are commercially available (e.g., from Chembridge Corporation, San Diego, Calif. or Evotec OAI, Abingdon, UK), or alternatively synthesized de novo. The prospective therapeutic moiety (drug) can be placed into any standard assay to test its effect on the activity of PDE1B activation. A drug is then selected that modulates the activity of dopamine activation.

Screens for small molecules, analogs thereof are also encompassed by the invention, as are screens for natural modulators of dopamine, such as those molecules that bind to and inhibit or activate, e.g., D1 receptors or dopamine *in vivo*. Such modulation is preferably determined via phosphorylation or dephosphorylation of DARPP-32 of SEQ ID NO:2. Alternatively, natural products  
5 libraries can be screened using assays of the invention for molecules that modulate e.g., D1 or D2 receptors activation or dopamine activity or DARPP-32 modulation.

Preferably, a potential modulator can be assayed for its ability to modulate the phosphorylation of Thr34 DARPP-32 by PKA or its dephosphorylation by PP2B, or the phosphorylation of Ser845-GluR1 AMPA receptor by PKA, or the dephosphorylation of Ser845-GluR1 AMPA receptor,  
10 either independently, or subsequent to, a binding assay as disclosed herein.

In one such embodiment, the amount and/or rate of phosphorylation or dephosphorylation of Thr34 DARPP-32, or a fragment thereof comprising the Thr34 residue, is determined. Such assays are known in the art. See for example U.S Patent Application No. 20030211040 ('040), which is incorporated by reference herein in its entirety.

For example, various enzymatic assays for kinases and phosphatases are known to a skilled artisan and may be used in determining the amount/rate of phosphorylation or dephosphorylation of a phosphorylated or dephosphorylated DARPP-32 fragment. Kinase activity may be measured as described in Parker, Law, et al., 2000, Development of high throughput screening assays using fluorescence polarization: nuclear receptor-ligand-binding and kinase/phosphatase assays, J. Biomolec.  
15 Screening 5(2): 77-88; Bader et al. (2001, Journal of Biomolecular Screening 6(4): 255-64); Liu, F., X. H. Ma, et al. (2001). "Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors." Proceedings of the National Academy of Sciences of the United States of America 98(20): 11062-8; Evans, D. B., K. B. Rank, et al. (2002). "A scintillation proximity assay for studying inhibitors of human tau protein kinase II/Cdk5 using a 96-well format." Journal of Biochemical &  
20 Biophysical Methods 50(2-3): 151-61.

Likewise, activities of protein phosphatases may be monitored by a variety of methods known to those skilled in the art, e.g., the methods disclosed in Cohen et al.(1988, Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle, Methods Enzymol 159:390-408) or Stewart and Cohen (1988, Protein phosphatase-2B from rabbit skeletal muscle: a Ca<sup>sup.2+</sup>-dependent, calmodulin-stimulated enzyme, Methods Enzymol 159:409-16).  
25 30

The clinical use of neuroleptics (anti-psychotics) has provided a means for treating patients suffering from psychotic disorders. Known neuroleptic agents, regardless of their chemical structures, are pharmacologically active with a large number of central monoaminergic neurotransmitter receptors, including dopaminergic, serotonergic, adrenergic, muscarinic, and histaminergic receptors. It is  
35 believed that the therapeutic and adverse effects of these drugs are mediated by distinct receptor subtypes.

With respect to the dopamine receptor system, current neuroleptic agents generally act on the dopamine receptor as dopamine antagonists. Neuroleptics are generally characterized as an agent that produces sedative or tranquilizing effects, and which also produces motor side effects, such as catalepsy or extrapyramidal symptomatology. The prevailing theory as to the mechanism of action of neuroleptics antipsychotic drugs proposes the antagonism of dopamine D2 receptors. This is based on the observation that these drugs have high affinity for this receptor *in vitro*, and that a correlation exists between their potency to block D2 receptors and their clinical efficacy. See, e.g., Silverstone T., Acta Psychiatr Scand Suppl 1990;358:88-91).

At the present time, nine major classes of antipsychotics have been developed and are widely prescribed to treat psychotic symptoms irrespective of their etiology. Continuous long-term use of neuroleptics is indicated in many psychotic disorders, such as (for more than six months) (i) primary indications such as Schizophrenia, Paranoia, Childhood psychoses, some degenerative or idiopathic neuropsychiatric disorders (notably, Huntington's disease and Gilles de la Tourette's syndrome); (ii) secondary indications such as extremely unstable manic-depressive or other episodic psychoses (unusual), otherwise unmanageable behavior symptoms in dementia, amnesia, or other brain syndromes; and (iii) questionable indications such as chronic characterological disorders with schizoid, "borderline," or neurotic characteristics; substance abuse; or antisocial behavior, recurrent mood disorders. See, e.g., Baldessarini, Chemotherapy in Psychiatry, Revised and Enlarged Edition, Harvard University Press, Cambridge, Mass., (1985), the contents of which is entirely incorporated herein by reference.

However, clinical use of these common neuroleptics is limited, however, not only because of their inability to reduce symptoms in a substantial number of patients, i.e., schizophrenia but also by their side effect profiles. In fact, nearly all of the "typical" or older generation compounds have significant adverse effects on human motor function such as persistent and poorly reversible motoric dysfunctions (e.g., tardive dyskinesia) in a significant number of patients. For example, classical neuroleptic agents, as exemplified by the butyrophenones and phenothiazines, can, upon long-term administration, produce severe motoric symptomatology, termed tardive dyskinesia a movement disorder characterized by involuntary writhing movements of the tongue and oral musculature seen with long-term administration of these agents. Tardive dyskinesia is usually reversible upon discontinuation of the chronic neuroleptic, if the drug is discontinued soon after symptoms of tardive dyskinesia appear. Otherwise symptoms may also persist. Pharmacological intervention for treatment of tardive dyskinesia is only moderately successful. Such motor abnormalities are known to occur in as high as 10% of the patients who are maintained on these drugs for several years; the incidence is much greater in certain groups, such as middle-aged females.

Because of the severity of these side effects and the low therapeutic-to-toxic index of conventional neuroleptics, other neuroleptics, called atypical neuroleptics, have been recently developed.

Atypical neuroleptics have a lower incidence of extrapyramidal symptoms and tardive dyskinesia; however, they are still associated with weight gain and effects on blood pressure and liver function, as observed for conventional neuroleptics. This adds considerably to the cost and limits the availability of this treatment. Also, the mechanism of action of atypical anti-psychotics, is not well understood.

5 Notwithstanding the limitations attending newer atypical anti-psychotics, considerable effort has been expended to find an improved therapeutic moieties with similar antipsychotic properties but without much success.

Consequently, there is an unmet need in the art to provide new methods of screening that can be used to develop novel therapeutic moieties or drugs that can be used to treat psychotic diseases or  
10 disorders. In addition, there is a need for simple tests of intracellular consequences of antipsychotic action. Since all anti-psychotics act upon multiple receptors, with widely varying downstream effects in terms of both effective relief of symptoms and unwanted side effects, analysis of the intracellular integration of these signals will provide a straightforward, cost-effective, and mechanism-based comparison useful for development of the next generation of therapeutic drugs. Also, there is a need to  
15 develop treatments for such diseases or disorders that are due, at least in part, to an aberration or dysregulation of an intracellular signaling pathway regulated by DARPP-32. The herein disclosed sequences aim to overcome the aforementioned drawbacks attending conventional therapeutic moieties and fulfills an the unmet needs noted above.

For example, in one aspect, the herein disclosed sequences may be used to screen for  
20 candidate therapeutic moiety based upon its ability to phosphorylate DARPP-32. Thus, a test therapeutic moiety may be classified as an anti-psychotic based, in part upon its ability and phosphorylation pattern of DARPP-32 of SEQ ID NO:2 when compared the the ability and phosphorylation pattern of a conventional atypical anti-psychotic.

Thus, an increase in the sae the phosphorylataion pattern ability of this moiety to said ,  
25 which relies on determining levels and pattern of phosphorylation of DARPP-32 of SEQ ID NO:2 , by a test compound and by a conventional. Atypical anti-psychotic drug fur use in the proposed assays includes, but is not limited to clozapine, risperidone, iloperidone, olanzapine, quetiapine zotepine, perospirone and ziprasidone.

Thus, in one embodiment, an effective atypical anti-psychotic therapeutic moiety is one  
30 which increases the phosphorylation of a DAARP-32 fragment, e.g., Thr34-dephosphorylated DARPP-32 or decreases the dephosphorylation of Thr34-phosphorylated DARPP-32 relative to a conventional anti-psychotic. In another embodiment , the ability to treat a psychotic disorder is tested so that if the therapeutic moiety ameliorates the psychotic disorder, an atypical anti-psychotic moiety is identified. Preferably, the psychotic disorder is Parkinson's disease, depression or schizophrenia. The ability to

treat a psychotic disorder is tested in one of a Parkinson's disease, depression or a schizophrenic animal model.

Likewise, the sequences of the invention may also be used in methods for classifying drugs with unknown pharmacological activity relative to conventional anti-psychotics, both typical and atypical. For example, cells expressing a functional DARPP-32 of SEQ ID NO:2 are contacted with a therapeutic moiety with unknown pharmacological activity, and the level/pattern of phosphorylation of DARPP-32, in said cell is determined and compared to the pattern of phosphorylation of DARPP-32 by conventional therapeutic moieties whose pattern of phosphorylation and known pharmacological activity are well known, such that identification of a similar pattern of phosphorylation of the unknown therapeutic moiety with a pattern of phosphorylation of a therapeutic moiety with known pharmacological activity results in classification of the unknown drug.

Preferably, treatment of a subject *in vivo* with a potential therapeutic moiety for use as an anti-psychotic drug produces a distinct phosphorylation pattern of intracellular signaling protein DARPP-32 at two sites (Thr34 and Thr75). It being understood that PP2B dephosphorylates DARPP-32 at Thr34, while PKA phosphorylates DARPP-32 at Thr34.

According to the invention, in the case of DARPP-32 phosphorylation, all three categories of drugs (typical anti-psychotic, atypical anti-psychotic and selective dopamine D2 receptor antagonist) preferably will increase phosphorylation at Thr34 site of DARPP-32 of SEQ ID NO:2. With administration of a typical anti-psychotic such as haloperidol, Thr 34 phosphorylation will increase for up to 30 minutes, but at 60 minutes, there will be no statistical difference from controls. However, in the case of phosphorylation at the Thr-75 site of DARPP-32, preferably only treatment with an atypical anti-psychotic, e.g., clozapine, will significantly increase phosphorylation levels of DARPP-32 at 15, 30 and 60 minutes. A selective dopamine D2 receptor antagonist, e.g. eticlopride, preferably will decrease DARPP-32 phosphorylation at Thr75 of DARPP-32 30 minutes after administration, while a typical anti-psychotic e.g., haloperidol, preferably will be without effect.

Determining the levels of phosphoproteins in a cell is well known to a skilled artisan. For example, e.g. cultured neuronal cells, aliquots of brain homogenate or of homogenates of cultured cells, may be separated by SDS/PAGE analysis according to standard methods, e.g., SDS/PAGE analysis using 10% polyacrylamide gels. The separated proteins may be analyzed by any method known in the art. For example, proteins are analyzed by immunoblot analysis. Other methods are well known.

Likewise, the effect of the potential therapeutic moiety, whether known or unknown on the phosphorylation of DARPP-32 at either of two sites (Thr34 and/or Thr75) may be assed using conventional methods including phosphorylation state-specific antibodies.

Whether the cell-based screens measure dephosphorylation or phosphorylation may depend on the extent to which the substrate is normally phosphorylated in the cell. Thus, in some

embodiments, the cell is treated with a compound that results in increased phosphorylation of the DARPP-32 prior to performing the assay. In certain, embodiments of the invention quantitative methods for detecting the extent or rate of dephosphorylation (e.g., ELISA) are employed.

5 An alternative embodiment of the invention provides a cell-based assay for phosphorylation. In a specific embodiment, signal transduction based on protein phosphorylation may be visualized *in vivo*, e.g., in single living cells using fluorescent indicators, using methods such as those disclosed in Sato et al. (2002, Fluorescent indicators for imaging protein phosphorylation in single living cells, Nature Biotechnology 20(3): 287-94). Such sensors consist of two fluorescent protein molecules, separated by a flexible linker. The linker peptide contains a phosphorylation site and a phosphoprotein  
10 recognition element. Phosphorylation of the linker causes a conformational change that brings the two fluorescent proteins into close proximity, allowing FRET to occur and changing the fluorescent output of the system.

#### Pharmaceutical Compositions/Dosage:

15 An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of nhDARPP-32, antibodies to DARPP-32, agonists, antagonists, or inhibitors of DARPP-32. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which  
20 may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial,  
25 intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, transdermal, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details  
30 on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an

effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

A therapeutically effective dose refers to that amount of active ingredient, for example agonist, antibodies to DARPP-32, antagonists, or inhibitors of DARPP-32, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

Further details on techniques for formulation and administration may be found in the latest edition of "Remington's Pharmaceutical Sciences" (Mack Publishing Co, Easton Pa.). Although local delivery is desirable, there are other means, for example, oral; parenteral delivery, including intra-arterial (directly to the tumor), intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

#### EXAMPLE 1

##### Cloning of Guinea Pig DARPP-32

Total RNA (1.2 mg) was prepared from 1.2 g of guinea pig whole brain tissue using the TRIZOL reagent (Invitrogen # 15596-026) according to the manufacturer's instructions. The Oligolex kit (Qiagen # 70022) was used to purify poly-A RNA from 500  $\mu$ g of total RNA with a yield of 12  $\mu$ g according to the manufacturer's instructions.

RACE ready cDNA was synthesized using the SMART RACE cDNA Amplification Kit (BD Bioscience # K1811-1). Guinea pig brain mRNA (1  $\mu$ g in 3  $\mu$ l), 3'-CDS primer (1  $\mu$ l) and RNase-free water (1  $\mu$ l) from the kit were mixed in an 0.5 ml microcentrifuge tube. The contents was incubated at 70°C for 2 min and then cooled on ice for 2 min. First-Strand buffer (2  $\mu$ l of 5x concentrate), 20 mM DTT (1  $\mu$ l), 10 mM dNTP mix (1  $\mu$ l), and PowerScript Reverse Transcriptase from the kit (1  $\mu$ l) were added. The tube was incubated at 42°C for 1.5 hr. The RACE ready cDNA sample was diluted with 250  $\mu$ l of Tricine-EDTA buffer from the kit and stored at - 20°C.

PCR primers for the cloning and amplification of the guinea pig DARPP-32 cDNA were designed based on the 5' and 3' ends of the consensus sequence of the human, mouse and rat DARPP-32 cDNAs. A PCR reaction was carried out using the RACE ready cDNA prepared above as template (3  $\mu$ l), 5' primer (NB426: 5'-ATGGACCCCAAGGACCGCAAGAAG-3' (SEQ ID NO:3), 1  $\mu$ l of a 20  $\mu$ M solution), 3' primer (NB428: 5'-TTATGTGCCGGACTCAGGGGGG-3' (SEQ ID NO:4), 1  $\mu$ l of a 20  $\mu$ M solution), RNase-free water (45  $\mu$ l), and two puREtaq Ready-To-Go PCR beads (Amersham Bioscience # 27-9557-01) in a PCR tube with 30 rounds of PCR (94°C for 10 s, 60°C for 10 s and 72°C for 1 min). An amplicon from this reaction was purified using a Qiaquick column (Qiagen # 28104). The purified amplicon was cloned into PCRscript vector, and four E. coli transformant plasmid DNAs were sent to sequencing. Three of four were the same and contained an open reading frame with high homology to the consensus sequence of known DARPP-32 cDNAs.

Since the PCR primers NB426 and NB428 were designed based on the consensus sequence of known DARPP-32 cDNA sequences, the portions of the cDNA cloned above that are derived from the primer sequences may not exactly equal the guinea pig sequence. In order to verify the 5' and 3' sequences of the cloned cDNA, two non-coding region primers (NB468: 5'-CGAGACCCACGACGCGCGCCCGCCCGCC-3' (SEQ ID NO:5) and NB464: 5'-TTTCCCCAGATCTTAGGGTCCTGCCCTGT-3' (SEQ ID NO:6)) were designed according to the consensus sequences of the 5' and 3' non-coding regions of human, mouse and rat DARPP-32. Two primers internal to the guinea pig DARPP-32 cDNA (NB448: 5'-CTCTGGCTCAGTGAGTGCTGGGC-3' (SEQ ID NO:7) and NB445: 5'-ACCACCTCAAGTCCAAGAGACCCAA-3' (SEQ ID NO:8)) were also used for this verification. The PCR reaction settings were the same as above except that the annealing temperature was 65°C instead of 60°C and 35 instead of 30 cycles were performed. The two amplicons were cloned into TA cloning vector and sequenced. The sequencing results showed that the 5' end primer (NB426) sequence was the same as the guinea pig sequence, but that the 3' end primer (NB428) sequence differed from the guinea pig sequence by two bases. These corrections are incorporated into the reported guinea pig cDNA sequence.

## EXAMPLE 2

DARPP-32 specific antibodies can be used to detect a given target in a variety of standard assay formats. Such formats include immunoprecipitation, Western blotting, ELISA, radioimmunoassay, and immunometric assays. See Harlow & Lane, Antibodies, A Laboratory Manual (CSHP NY, 1988). Immunometric or sandwich assays (sELISA) are a preferred format (see U.S. Pat. No. 4,376,110, 4,486,530, 5,914,241, and 5,965,375). Such assays use one antibody or population of antibodies immobilized to a solid phase, and another antibody or population of antibodies in solution. Typically, the solution antibody or population of antibodies is labeled. If an antibody population is used,

the population typically contains antibodies binding to different epitope specificities within the target antigen. Accordingly, the same population can be used for both solid phase and solution antibody. If monoclonal antibodies are used, first and second monoclonal antibodies having different binding specificities are used for the solid and solution phase. Solid phase and solution antibodies can be contacted with target antigen in either order or simultaneously. If the solid phase antibody is contacted first, the assay is referred to as being a forward assay. Conversely, if the solution antibody is contacted first, the assay is referred to as being a reverse assay. If target is contacted with both antibodies simultaneously, the assay is referred to as a simultaneous assay. After contacting the target with antibody, a sample is incubated for a period that usually varies from about 10 min to about 24 hr and is usually about 1 hr. A wash step is then performed to remove components of the sample not specifically bound to the antibody(ies) being used as a diagnostic reagent. When solid phase and solution antibodies are bound in separate steps, a wash can be performed after either or both binding steps. After washing, binding is quantified, typically by detecting label linked to the solid phase through binding of labelled solution antibody. Usually for a given pair of antibodies or populations of antibodies and given reaction conditions, a calibration curve is prepared from samples containing known concentrations of target antigen. Concentrations of antigen in samples being tested are then read by interpolation from the calibration curve. Analyte can be measured either from the amount of labelled solution antibody bound at equilibrium or by kinetic measurements of bound labelled solution antibody at a series of time points before equilibrium is reached. The slope of such a curve is a measure of the concentration of target in a sample. Suitable detectable labels for use in the above methods include any moiety that is detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, chemical, or other means. See Handbook of Fluorescent Probes and Research Chemicals (6th Ed., Molecular Probes, Inc., Eugene Oreg.). Radiolabels can be detected using photographic film or scintillation counters, fluorescent markers can be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

Referring to Figure 5, detailed therein are the results of a detection Assay –  
Luminescence Measurements using a Sandwich ELISA (sELISA)

Briefly, a black 96-well flat-bottom plate (Costar #3925) was treated with 100  $\mu$ l per well of a 1  $\mu$ g/ml solution of an anti-DARPP-32 antibody, MS2551, in 0.05 M sodium carbonate/bicarbonate pH 9.6 for approximately 16 hr at 4 °C with constant agitation. The antibody MS2551 was prepared according to standard methods under contract with Covance Research Products Inc. by immunizing a rabbit with a peptide corresponding to amino acids 2-13 of rat DARPP-32 coupled to KLH. The reactive antibodies were purified on a column of immobilized peptide antigen. Wells were rinsed three times with

0.01 M phosphate-buffered saline pH 7.4 (PBS) at room temperature (RT), and treated with 250  $\mu$ l per well of 0.2% casein in PBS for 2 hr at RT with constant agitation. Solutions containing the indicated concentrations of purified recombinant rat DARPP-32 with 0.2% casein in PBS plus 0.05% tween-20 (PBST) (100  $\mu$ l per well) were incubated in the wells for 2 hr at RT with constant agitation, followed by  
5 three rinses with 200  $\mu$ l per well of PBST. Next wells were treated with 100  $\mu$ l per well of 1  $\mu$ g/ml of sc-11365-AP in 0.2 % casein, PBST for 2 hr at RT with constant agitation. The antibody, sc-11365-AP was prepared by chemical conjugation of alkaline phosphatase (using a kit from Pierce Chemical Co., #31493) to sc-11365, a commercial purified rabbit polyclonal anti-DARPP-32 antibody raised to a C-terminal portion of DARPP-32 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Wells were rinsed five times  
10 with 200  $\mu$ l per well of PBST at RT, followed by incubation for 30 min at RT with 100  $\mu$ l per well of CDP Star (Applied Biosystems), a solution containing an alkaline phosphatase substrate whose product is luminescent. Luminescence was measured using a LJJL Biosystems Analyst AD96-384 luminometer.

## DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

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## WHAT IS CLAIMED:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding a Dopamine- and cyclic AMP (cAMP)-regulated phosphoprotein having an apparent molecular weight of 32 kilodaltons - DARPP-32, said sequence of nucleotides being selected from the group consisting of:
  - (a) a sequence of nucleotides that encodes DARPP-32 comprising the sequence of nucleotides set forth in SEQ.ID.NO:1;
  - (b) a nucleotide sequence varying from the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code;
  - (c) a sequence of nucleotides having at least 95% sequence identity or is exactly complementary to the nucleotide sequence set forth in SEQ ID NO:1;
  - (d) fragments of (a), (b), or (c) that encodes a polypeptide capable of being activated by dopamine.
2. An amino acid sequence selected from the group consisting of: (i) an amino acid sequence coded by the isolated nucleic acid sequence of claim 1; (ii) homologues of the amino acid sequences of (i) in which one or more amino acids has been added, deleted, replaced or chemically modified in the region, or adjacent to the region, where the amino acid sequences differs from the original amino acid sequence, coded by the DARPP-32 encoding nucleic acid sequence from which the variant has been varied by alternative splicing.
3. A substantially pure polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2.
4. A substantially pure polypeptide comprising an amino acid sequence encoded by the nucleotide sequence as set forth in SEQ ID NO:1.
5. A method for modulating dopamine D1 receptor activity in a cell comprising contacting said cell expressing the polypeptide of SEQ ID NO:2 with an effective amount of a compound that alters the activity of a dopaminergic intracellular signaling molecule, wherein contact of said cell with the compound results in a modulation of the activity of PKA.
6. A method for identifying a therapeutic moiety to be tested for its ability to modulate the activity of a dopaminergic intracellular signaling pathway in a cell comprising: (a)

determining a first level of dopamine activity in said cell; (b) contacting said cell with the therapeutic moiety under investigation; and (c) determining a second level of dopamine activity, respectively, in said cell, wherein a difference in said first level and said second level of dopamine activity is indicative of the ability of said therapeutic moiety to modulate the dopaminergic intracellular signaling pathway.

5

7. The method according to claim 6, wherein the step of determining dopamine activity comprises determining the level of phosphorylation of Thr34-phosphorylated DARPP-32 .

8. A method for selecting a potential therapeutic agent for use in the treatment of a  
10 disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway mediated by DARPP-32 comprising: (a) administering a potential therapeutic agent to a transgenic animal expressing a DARPP-32 of SEQ ID NO:; (b) measuring the response of said animal to dopamine administration; (c) comparing the response to dopamine administration of said animal with that of a control animal to which the potential therapeutic moiety has not been administered; and (d) selecting a  
15 potential therapeutic moiety based on the difference in responses observed between said animal and said control animal.

9. The method of claim 8 wherein the animal is a mouse or rat.

20 10. The method of claim 8 wherein the disorder is Parkinson's disease or depression or schizophrenia.

11. A method for identifying a potential therapeutic moiety being tested for treating a disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway  
25 mediated by DARPP-32 comprising: (a) contacting the therapeutic moiety with PKA and Thr34-dephosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of phosphorylation of Thr34-dephosphorylated DARPP-32; wherein the therapeutic moiety is considered helpful in treating said disorder if an increase in the phosphorylation of Thr34-dephosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

30

12. A method for identifying a potential therapeutic moiety being tested for treating a disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway mediated by DARPP-32 comprising: (a) contacting the therapeutic moiety with PP1 and Thr34-phosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of dephosphorylation of  
35 Thr34-phosphorylated DARPP-32; wherein the therapeutic moiety is considered helpful in treating said

disorder if a decrease in the dephosphorylation of Thr34-phosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

13. A method for identifying a potential therapeutic moiety being tested for treating a  
5 disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway mediated by DARPP-32 comprising: (a) contacting the therapeutic moiety with PPC and Ser137-phosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of dephosphorylation of Ser137-phosphorylated DARPP-32; wherein the therapeutic moiety is considered helpful in treating said disorder if a decrease in the dephosphorylation of Ser137-phosphorylated DARPP-32 is detected in the  
10 presence of the potential therapeutic moiety.

14. A method for identifying a potential therapeutic moiety being tested for treating a disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway mediated by DARPP-32 comprising: (a) contacting the therapeutic moiety with PPC and Thr34-  
15 phosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of dephosphorylation of Thr34-phosphorylated DARPP-32; wherein the therapeutic moiety is considered helpful in treating said disorder if a decrease in the dephosphorylation of Thr34-phosphorylated DARPP-32 is detected in the presence of the potential therapeutic moiety.

20 15. A method for identifying a potential therapeutic moiety being tested for treating a disease or disorder characterized by aberrant or dysregulation of an intracellular signaling pathway mediated by DARPP-32 comprising: (a) contacting the therapeutic moiety with PP2A and Thr75-phosphorylated DARPP-32 of SEQ ID NO:2; and (b) detecting the amount of dephosphorylation of Thr75-phosphorylated DARPP-32; wherein the therapeutic moiety is considered helpful in treating said  
25 disorder if an increase in the dephosphorylation of Thr75-phosphorylated DARPP-32 of SEQ ID NO: 2 is detected in the presence of the potential therapeutic moiety.

16. An isolated antibody or antibody fragment thereof which selectively binds Thr75-phosphorylated DARPP-32 derived from SEQ ID NO:2.

30 17. The antibody or antibody fragment of claim 16, wherein the antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, monoclonal antibody, chimeric antibody, single chain antibody, a Fab fragment or a Fab expression library.

18. An isolated antibody or a binding fragment thereof which selectively binds Ser137-phosphorylated DARPP-32 derived from SEQ ID NO:2.

19. The antibody or binding fragment thereof of claim 18, wherein the antibody is selected from the group consisting of humanized antibodies, chimeric antibodies and single chain antibodies.

20. An isolated antibody or a binding fragment thereof which selectively binds Thr34-phosphorylated DARPP-32 derived from SEQ ID NO:2.

21. The antibody or binding fragment thereof of claim 20, wherein the antibody is selected from the group consisting of humanized antibodies, chimeric antibodies and single chain antibodies

22. An isolated antibody or a binding fragment thereof which selectively binds Ser102-phosphorylated -DARPP-32 derived from SEQ ID NO:2.

23. The antibody or binding fragment thereof of claim 22, wherein the antibody is selected from the group consisting of humanized antibodies, chimeric antibodies and single chain antibodies.

24. An isolated antibody or a binding fragment thereof which selectively binds the amino acid sequence of SEQ ID NO:2.

25. The antibody or binding fragment thereof of claim 24, wherein the antibody is selected from the group consisting of humanized antibodies, chimeric antibodies and single chain antibodies.

26. The antibody or binding fragment thereof of claim 24, wherein the antibody is a monoclonal antibody.

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>Guinea pig DARPP-32 cDNA sequence (includes 3'-non-coding region;  
confirmed by both 5' and 3' RACE)

ATGGACCCCAAGGACCGCAAGAAGATCCAGTTCTCCGTGCCCCGCGCCCCCAGCCAGCTCGACCCCGCCA  
GGTGGAAATGATCCGGCGCAGGAGGCCAACCCCTGCCATGCTGTTCCGGCTCTCAGAGCACTCCTCACCAG  
AGGAGGAGGCCTCGCCCCACCAGAGAGCCTCTGGAGAGGGGCACCACTCAAGTCCAAGAGACCCAACCCC  
TGTGCCTACACACCGCCCTCACTGAAAGCTGTGCAGCGCATTGCAGAGTCCCACCTGCAGACCATCAGCAA  
CCTGGGCTCAGAGGAGGAGGATGAGCTGGGGGAGCTGCGGGAGCTGGGGTACCCGAGAGAGGATGACGAGG  
AGGAAGAGGATGATGAGGAGGAGGAGGAGGAAGAGGACAGCCAGGCTGAGGTCCTGAAGGCCATCAGGGGC  
TCTGCAGGGCAGAAGGTAACCTGTGGCCAGGGTCTGGAGGGGCCCTGGGAGCGTCCACTCCCTCTGGATGA  
GGACCAAAGCCCAGCACTCACTGAGCCAGAGGAGGAACCCAGCGCCCCGACCCCCCTGAGCCTGGCACAT  
AAGTGCACCGCTCTGCATCTTCTGGGAGGAAGTGGAGGGGACATCGCCGTACCCCTGAAAGCCACTCCGTC  
CCTGCCCTAAATCTGGTGCCCTTTCCCATCCCTGCTAGGGCTGCAATTTCTAGAAGACTAACGCTGGTCTG  
TTTGCTTCTTCGCCCACCTTTGGCTGATGCTGGAGTCCCCTGGTGCCCGTCCCTGCCAGCCGTCCCTCCAT  
TCACCCGACGGGAGGTGGGGTGGGAGCGAGCCACGTTGGAAAATTCCGGAAACAAACAAGGATTGGCTTC  
CCAGTTCTCTTCCCCAGACCCTCTCCCCTGGGCACAGGAGACCC

## FIG. 1

>Guinea pig DARPP-32 amino acid sequence

MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSPEEEASPHQRASGEGHHLKSKRPNP  
CAYTPPSLKAVQRIAESHLQTIISNLGSEEEDELGELRELGYPREDDDEEEEDDEEEEEEDSQAENVLKAIRG  
SAGQKVTCGQGLEGPWERPLPLDEDQSPALTEPEEEPQRPDPEPGT

## FIG. 2

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|             |                                                                   |
|-------------|-------------------------------------------------------------------|
| hum_darpp32 | MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSPEEEASPHQRASGE      |
| gui_Darpp32 | MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSPEEEASPHQRASGE      |
| bov_Darpp32 | MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEHSSPEEEASPHQRASGE      |
| rat_darpp32 | MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPALFRVSEHSSPEEESSPHQRTSGE       |
| mus_darpp32 | MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLVSEHSSPEEEASPHQRTSGE     |
|             |                                                                   |
| hum_darpp32 | GHHLKSKRPNPCAYTPPSLKAVQRIAESHLSISNLNENQASEEEDELGELRELGYPREE       |
| gui_Darpp32 | GHHLKSKRPNPCAYTPPSLKAVQRIAESHLSISNLG----SEEEDELGELRELGYPRE-       |
| bov_Darpp32 | GHHLKSKRPNPCAYTPPSLKAVQRIAESHLSISNLGENQASEEEDELGELRELGYPREE       |
| rat_darpp32 | GHHPKSKRPNPCAYTPPSLKAVQRIAESHLSISNLSENQASEEEDELGELRELGYPOED       |
| mus_darpp32 | GHHPKSKRPNPCAYTPPSLKAVQ-----HLQTSISNLSENQASEEEDELGELRELGYPOED     |
|             |                                                                   |
| hum_darpp32 | DEEEEEEDDEEEEEEEEDSQAARVLKVIROQSAGQKTTTCGQGLEGPWERPPPLDESERDGGSED |
| gui_Darpp32 | DDEEEEEEDDEEEEEEEEDSQAARVLKAIIRGSAGQKVTCGQGLEGPWERPLPLD-----ED    |
| bov_Darpp32 | EEEEEEEEDEEEEE--DSQAARVLKGSRGSGAGQKTTYGQGLEGPWERPPPLDGPQRDGGSED   |
| rat_darpp32 | DEEDEDEDEEEEDDEEEDSQAARVLKGSRGTAGQKLTSGQGLEGPWERPPPLDEPQRDGNSED   |
| mus_darpp32 | DEEDEDE--EEDDEEEDSQAARVLKGSRGTVGQKPTCGRGLEGPWERPPPLDEPQRDGNSED    |
|             |                                                                   |
| hum_darpp32 | QVEDPALS-EPGEEPQRPSPEPGT                                          |
| gui_DARPP32 | Q--SPALT-EPEEEPQRPDPEPGT                                          |
| bov_Darpp32 | QVEDPALN-EPGEEPQRPAHPEPGT                                         |
| rat_darpp32 | QGEGRATQSEPGEEPQRHPTPPESGT                                        |
| mus_darpp32 | QVEGRATLSEPGEEPQHPSPP----                                         |

FIG.3

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Amino acid numbering for DARPP-32

| Species    | Thr (PKA) | Thr (CDK5) | Ser (CK2) | Ser (CK1) |
|------------|-----------|------------|-----------|-----------|
| Human      | 34        | 75         | 102       | 137       |
| Guinea pig | 34        | 75         | 98        | 132       |
| Cow        | 34        | 75         | 102       | 135       |
| Rat        | 34        | 75         | 102       | 137       |
| Mouse      | 34        | 75         | 97        | 130       |

FIG.4

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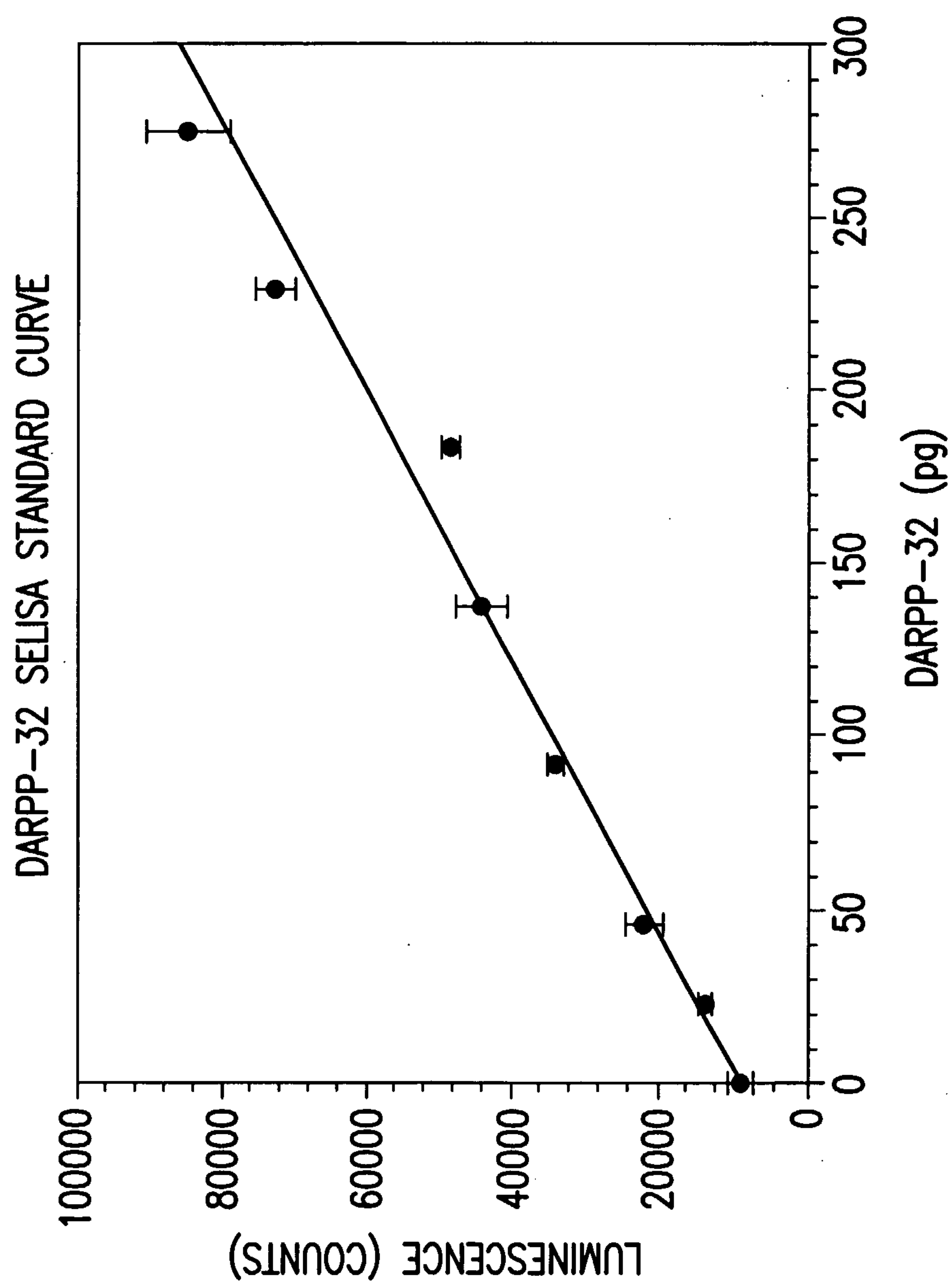


FIG. 5

# DARPP-32 SELISA STANDARD CURVE

