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(54) Title: LINEAGE-RESTRICTED NEURONAL PRECURSORS

(57) Abstract

A self–renewing restricted stem cell population has been identified in developing (embryonic day 13.5) spinal cords that can differentiate into multiple neuronal phenotypes, but cannot differentiate into glial phenotypes. This neuronal–restricted precursor (NRP) expresses highly polysialated or embryonic neural cell adhesion molecule (E–NCAM) and is morphologically distinct from neuroepithelial stem cells (NEP cells) and spinal glial progenitors derived from embryonic day 10.5 spinal cord. NRP cells self renew over multiple passages in the presence of fibroblast growth factor (FGF) and neurotrophin 3 (NT-3) and express a characteristic subset of neuronal epitopes. When cultured in the presence of RA and the absence of FGF, NRP cells differentiate into GABAergic, glutaminergic, and cholinergic immunoreactive neurons. NRP cells can also be generated from multipotent NEP cells cultured from embryonic day 10.5 neural tubes. Clonal analysis shows that E–NCAM immunoreactive NRP cells arise from an NEP progenitor cell that generates other restricted CNS precursors. The NEP–derived E–NCAM immunoreactive cells undergo self renewal in defined medium and differentiate into multiple neuronal phenotypes in mass and clonal culture. Thus, a direct lineal relationship exists between multipotential NEP cells and more restricted neuronal precursor cells present *in vivo* at embryonic day 13.5 in the spinal cord. Methods for treating neurological diseases are also disclosed.

LINEAGE-RESTRICTED NEURONAL PRECURSORS

BACKGROUND OF THE INVENTION

This invention relates to lineage-restricted intermediate precursor cells
5   and methods of making and using thereof. More particularly, the invention
relates to neuronal-restricted precursors (NRP's) isolated from mammalian
embryos, neuroepithelial stem (NEP) cells, or embryonic stem (ES) cells.
These neuronal-restricted precursors are capable of self-renewal and
differentiation into neurons, but not into glia, i.e. astrocytes and
10   oligodendrocytes. Methods of generating, isolating, culturing, transfecting,
and transplanting such neuronal-restricted precursor cells are also described.

Multipotent cells with the characteristics of stem cells have been
identified in several regions of the central nervous system and at several
developmental stages. F.H. Gage et al., Isolation, Characterization and Use of
15   Stem Cells from the CNS, 18 Ann. Rev. Neurosci. 159-92 (1995); M. Marvin &
R. McKay, Multipotential Stem Cells in the Vertebrate CNS, 3 Semin. Cell.
Biol. 401-11 (1992); R.P. Skoff, The Lineages of Neuroglial Cells, 2 The
Neuroscientist 335-44 (1996). These cells, often referred to as neuroepithelial
stem cells (NEP cells), have the capacity to undergo self renewal and to
20   differentiate into neurons, oligodendrocytes, and astrocytes, thus representing
multipotent stem cells.

2

A.A. Davis & S. Temple, A Self-Renewing Multipotential
Stem Cell in Embryonic Rat Cerebral Cortex, 362 Nature
363-72 (1994); A.G. Gritti et al., Multipotential Stem
Cells from the Adult Mouse Brain Proliferate and Self-

5   Renew in Response to Basic Fibroblast Growth Factor, 16 J.
Neurosci. 1091-1100 (1996); B.A. Reynolds et al., A
Multipotent EGF-Responsive Striatal Embryonic Progenitor
Cell Produces Neurons and Astrocytes, 12 J. Neurosci.
4565-74 (1992); B.A. Reynolds & S. Weiss, Clonal and

10   Population Analyses Demonstrate that an EGF-Responsive
Mammalian Embryonic CNS Precursor is a Stem Cell, 175
Developmental Biol. 1-13 (1996); B.P. Williams et al., The
Generation of Neurons and Oligodendrocytes from a Common
Precursor Cell, 7 Neuron 685-93 (1991).

15   The nervous system also contains precursor cells with
restricted differentiation potentials. T.J. Kilpatrick &
P.F. Bartlett, Cloned Multipotential Precursors from the
Mouse Cerebrum Require FGF-2, Whereas Glial Restricted
Precursors are Stimulated with Either FGF-2 or EGF, 15 J.

20   Neurosci. 3653-61 (1995); J. Price et al., Lineage
Analysis in the Vertebrate Nervous System by Retrovirus-
Mediated Gene Transfer, 84 Developmental Biol. 156-60
(1987); B.A. Reynolds et al., supra; B.A. Reynolds & S.
Weiss, supra; B. Williams, Precursor Cell Types in the

25   Germinal Zone of the Cerebral Cortex, 17 BioEssays 391-93
(1995); B.P. Williams et al., supra. The relationship
between multipotent stem cells and lineage restricted
precursor cells is still unclear. In principal, lineage
restricted cells could be derived from multipotent cells,

30   but this is still a hypothetical possibility in the
nervous system with no direct experimental evidence.
Further, no method of purifying such precursors from
multipotent cells has been described.
                As has been shown in copending U.S. Patent

35   Application Serial No. 08/852,744, entitled "Generation,
Characterization, and Isolation of Neuroepithelial Stem
Cells and Lineage Restricted Intermediate Precursor,"
filed May 7, 1997, hereby incorporated by reference in its

3

entirety, NEP cells grow on fibronectin and require
fibroblast growth factor (FGF) and an as yet
uncharacterized component present in chick embryo extract
(CEE) to proliferate and maintain an undifferentiated
5   phenotype in culture. The growth requirements of NEP
cells are different from neurospheres isolated from E14.5
cortical ventricular zone cells. B.A. Reynolds et al.,
supra; B.A. Reynolds & S. Weiss, *supra*; WO 9615226; WO
9615224; WO 9609543; WO 9513364; WO 9416718; WO 9410292;
10   WO 9409119. Neurospheres grow in suspension culture and
do not require CEE or FGF, but are dependent on epidermal
growth factor (EGF) for survival. FGF itself is not
sufficient for long term growth of neurospheres, though
FGF may support their growth transiently. NEP cells,
15   however, grow in adherent culture, are FGF dependent, do
not express detectable levels of EGF receptors, and are
isolated at a stage of embryonic development prior to
which it has been possible to isolate neurospheres. Thus,
NEP cells may represent a multipotent precursor
20   characteristic of the brain stem and spinal cord, while
neurospheres may represent a stem cell more characteristic
of the cortex. Nonetheless, NEP cells provide a model
system for studying the principles of lineage restriction
from multipotent stem cells or precursor cells of the
25   central nervous system. The principles elucidated from
the study of NEP cells are expected to be broadly
applicable to all CNS precursor cells sufficiently
multipotent to generate both neurons and glia. Thus, the
present application is intended to be applicable to any
30   CNS precursor cells regardless of their site of derivation
as long as they are able to differentiate to both neurons
and glial cells.
        U.S. Patent No. 5,589,376, to D.J. Anderson and D.L.
Stemple, discloses mammalian neural crest stem cells and
35   methods of isolation and clonal propagation thereof, but
fails to disclose cultured NEP cells, cultured lineage
restricted precursor cells, and methods of generating,
isolating, and culturing thereof. Neural crest cells

differentiate into neurons and glia of the peripheral nervous system (PNS), whereas the neuroepithelial stem cells differentiate into neurons and glia of the central nervous system (CNS).

U.S. Serial No. 08/909,435, filed July 4, 1997, for "Isolation of Lineage Restricted Neuronal Precursors," hereby incorporated by reference in its entirety, describes neuronal restricted precursor (NRP) cells that are capable of differentiating into neurons, but not into glial cells. It was shown that NRP cells can be isolated from NEP cells, as well as directly from embryonic spinal cords.

U.S. Serial No. 08/980,850, filed November 29, 1997, for "Lineage Restricted Glial Precursors from the Central Nervous System," hereby incorporated by reference in its entirety, describes glial restricted precursor (GRP) cells that are capable of differentiating into oligodendrocytes, A2B5$^+$ process-bearing astrocytes, and A2B5$^-$ fibroblast-like astrocytes, but not into neurons. GRP cells can be isolated from differentiating NEP cells, as well as CNS tissue, and differ from oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells in growth factor requirements, morphology, and progeny.

In U.S. Patent Application Serial No. 09/073,881, filed May 6, 1998, for "Common Neural Progenitor for CNS and PNS," hereby incorporated by reference in its entirety, it was shown that NEP cells can be induced to differentiate into neural crest cells as well as other cells of the CNS and PNS.

The neuron-restricted precursor cells described herein are distinct from the NEP cells, GRP cells, neurospheres, and neural crest stem cells that have been described elsewhere. NEP cells are capable of differentiating into neurons or glia whereas NRPs can differentiate into neurons, but not glia, and NEP cells and NRPs display distinct cell markers. GRP cells can differentiate into glia, but not neurons. As mentioned above, neurospheres grow in suspension culture and do not

5

require CEE or FGF, but are dependent on EGF for survival,
whereas NRP cells grow in adherent culture and do not
express detectable levels of EGF receptors. Further,
neural crest cells differentiate into neurons and glia of
5   the peripheral nervous system (PNS), whereas NRP cells
differentiate into neurons of the central nervous system
(CNS). NRP cells express polysialated or embryonic neural
cell adhesion molecule (E-NCAM), but NEP cells,
neurospheres, GRP cells, and neural crest cells do not.
10   Therefore, NRP cells are different in their proliferative
potential, expression of cell markers, and nutritional
requirements from these other cell types.
        The ability to isolate and grow mammalian neuronal-
restricted precursor cells in vitro allows for of using
15   pure populations of neurons for transplantation, discovery
of genes specific to selected stages of development,
generation of cell-specific antibodies for therapeutic and
diagnostic uses such as for targeted gene therapy, and the
like. Further, NRP cells can be used to generate
20   subpopulations of neurons with specific properties, i.e.
motoneurons and other neuronal cells for analyzing
neurotransmitter functions and small molecules in high
throughput assays. Moreover, the methods of obtaining NRP
cells from NEP cells or embryonic stem (ES) cells provides
25   for a ready source of a large number of post-mitotic
neurons. Post-mitotic cells obtained from a tumor cell
line are already being commercially marketed (e.g.,
Clontech, Palo Alto, CA). The present invention is also
necessary to understand how multipotent neuroepithelial
30   stem cells become restricted to the various
neuroepithelial derivatives. In particular, culture
conditions that allow the growth and self-renewal of
mammalian neuronal-restricted precursor cells are
desirable so that the particulars of the development of
35   these mammalian stem cells can be ascertained. This is
desirable because a number of tumors of neuroepithelial
derivatives exist in mammals, particularly humans.
Knowledge of mammalian neuroepithelial stem cell

development is therefore needed to understand these disorders in humans.

In view of the foregoing, it will be appreciated that isolated populations of mammalian lineage restricted neuronal precursor cells and methods of generating, isolating, culturing, transfecting, and transplanting such cells would 5 be significant advancements in the art.

BRIEF SUMMARY OF THE INVENTION

It is desirable to provide isolated (pure) populations of mammalian neuronal-restricted precursor cells and their progeny.

10 It is also desirable to provide methods of generating, isolating, culturing, and regenerating of mammalian lineage-restricted neuronal precursor cells and their progeny.

It is also desirable to provide a method for the generation of lineage-restricted neuronal precursor cells from a CNS multipotent precursor cell able 15 to generate both neurons and glia.

It is also desirable to provide pure differentiated populations of neuronal cells derived from lineage-restricted neuronal precursor cells.

It is also desirable to provide methods of transfecting and transplanting such neuronal restricted precursor cells.

20 These can be achieved by providing an isolated, pure population of mammalian CNS neuron-restricted precursor cells. Preferably, such neuron-restricted precursor cells are capable of self-renewal, differentiation to CNS neuronal cells but not to CNS glial cells, and expressing embryonic neural cell adhesion molecule (E-NCAM), but not expressing a ganglioside recognized by 25 A2B5 antibody. These neuron-restricted precursor cells may or may not express nestin or β-III tubulin. Thus, embryonic neural cell adhesion molecule (E-NCAM) is a defining antigen for these cells. The NRP cells are able to differentiate into neurons that are capable of releasing and responding to neurotransmitters. These neurons can demonstrate receptors for these 30 neurotransmitters, and such cells are capable of expressing neurotransmitter-synthesizing enzymes. The NRP cells are also capable of differentiating into neurons that can form functional synapses and/or develop electrical activity. The NRP cells are also capable of stably expressing at least one material selected from the group consisting of growth factors for such cells, 35 differentiation factors for such cells, maturation factors for such cells, and combinations of any of these. Further, the present neuron-restricted precursor

cells may be selected, chosen, and isolated from human primates, non-human primates, equines, canines, felines, bovines, porcines, ovines, lagomorphs, and rodents.

In one aspect, the present invention provides an isolated, pure
5   population of mammalian CNS neuron-restricted precursor cells derived from i) a sample of central nervous system tissue from a mammalian embryo at a stage of embryonic development after closure of the neural tube but prior to differentiation of glial and neuronal cells in the neural tube, or ii) mammalian embryonic stem cells, wherein said neuron-restricted precursor cells express
10   embryonic neural cell adhesion molecule and are capable of differentiation to CNS neuronal cells but not to CNS glial cells, and wherein the cells are capable of self-renewal in the presence of FGF.

Preferably, said neuron-restricted precursor cells do not express a ganglioside recognized by A2B5 antibody.
15   Preferably, the cells grow over multiple passages in mass and clonal cultures.

Preferably, the cells are capable of differentiating into neurons in the presence of retinoic acid.

Preferably, astrocyte promoting medium containing FGF and 10% fetal
20   calf serum fails to promote proliferation or differentiation.

Preferably, said neuron-restricted precursor cells do not express nestin.

Preferably, said neuron-restricted precursor cells were obtained from a mammalian embryo selected from the group consisting of human and non-human primates, equines, canines, felines, bovines, porcines, ovines,
25   lagomorphs, and the order Rodentia.

Preferably, said cells are able to differentiate into neurons that are capable of releasing and responding to neurotransmitters. More preferably, said neurons demonstrate receptors for said neurotransmitters, and said cells are capable of expressing neurotransmitter-synthesizing enzymes.
30   Preferably, said cells are capable of differentiating into neurons which can form functional synapses and/or develop electrical activity.

Preferably, said cells are capable of stably expressing at least one material selected from the group consisting of growth factors for said cells, differentiation factors for said cells, maturation factors for said cells, and
35   combinations of any of these.

In a further aspect, the present invention provides a method of isolating a pure population of mammalian CNS neuron-restricted precursor cells comprising the steps of:

(a) isolating a population of mammalian multipotent CNS stem cells capable of generating both neurons and glia;

(b) incubating the multipotent CNS stem cells in a medium configured for inducing said cells to begin differentiating;

(c) removing A2B5+ cells from the differentiating cells via specific antibody capture with an antibody that specifically recognises A2B5;

(d) purifying from the differentiating cells a subpopulation of cells expressing embryonic neural cell adhesion molecule; and

(e) incubating the purified subpopulation of cells in a medium configured for supporting adherent growth thereof,

wherein the cells are derived from i) spinal cords of approximately 13.5 day embryos of rats or equivalent embryonic stages of other mammals, or ii) cells equivalent to i) developed by proliferation and differentiation from early embryonic spinal cord tissue or embryonic stem cells, and wherein the isolated cells are capable of self-renewal in the presence of FGF.

Preferably, the medium configured for inducing said cells to begin differentiation is NEP medium.

Preferably, the method further comprises replating the multipotent CNS stem cells on laminin in NEP medium in the absence of chick embryo extract to induce cell differentiation.

Preferably, said purifying comprises a procedure selected from the group consisting of specific antibody capture, fluorescence activated cell sorting, and magnetic bead capture. More preferably, said procedure uses an embryonic neural cell adhesion molecule antibody that specifically recognises polysialated neural cell adhesion molecule.

Preferably, said isolating a population of CNS neuroepithelial stem cells comprises:

(a) removing a CNS tissue from a mammalian embryo at a stage of embryonic development after closure of the neural tube but prior to differentiation of cells in the neural tube;

(b) dissociating cells comprising the neural tube removed from the mammalian embryo;

(c)   plating the dissociated cells in feeder-cell-independent culture on a
substratum and in a medium configured for supporting adherent
growth of the neuroepithelial stem cells comprising effective
amounts of fibroblast growth factor and chick embryo extract; and

5   (d)   incubating the plated cells at a temperature and in an atmosphere
conducive to growth of the neuroepithelial stem cells.

Preferably, said mammalian embryo is selected from the group
consisting of human and non-human primates, equines, canines, felines,
bovines, porcines, ovines, lagomorphs, and the order Rodentia.

10   Preferably, said substratum is selected from the group consisting of
fibronectin, vitronectin, laminin, and RGD peptides.

Preferably, said medium comprises effective amounts of fibroblast
growth factor and neurotrophin 3.

Preferably, the isolated cells differentiate into CNS neuronal cells upon
15   replacement of adherent growth supporting medium with retinoic acid
containing medium.

Preferably, the isolated cells fail to proliferate or differentiate in astrocyte
promoting medium containing FGF and 10% fetal calf serum.

In another aspect, the present invention provides a method of isolating a
20   pure population of mammalian CNS neuron-restricted precursor cells
comprising the steps of:

(a)   removing a sample of spinal cord of approximately 13.5 day
embryos of rats or equivalent embryonic stages of other mammals,
or ii) cells equivalent to i) developed by proliferation and
25   differentiation from early embryonic spinal cord tissue;

(b)   dissociating cells comprising the sample of CNS tissue removed
from the mammalian embryo;

(c)   removing A2B5+ cells from the differentiating cells via specific
antibody capture with an antibody that specifically recognises
30   A2B5;

(d)   purifying from the dissociated cells a subpopulation expressing
embryonic neural cell adhesion molecule;

(e)   plating the purified subpopulation of cells in feeder-cell-independent
culture on a substratum and in a medium configured for supporting
35   adherent growth of the neuron-restricted precursor cells; and

(f)   incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuron-restricted precursor cells,

wherein the isolated cells are capable of self renewal in the presence of FGF.

Preferably, the isolated cells differentiate into CNS neuronal cells upon
5   replacement of adherent growth supporting medium with retinoic acid containing medium.

Preferably, the isolated cells fail to proliferate or differentiate in astrocyte promoting medium containing FGF and 10% fetal calf serum.

Preferably, said purifying comprises a procedure selected from the group
10   consisting of specific antibody capture, fluorescence activated cells sorting, and magnetic bead capture.

Preferably, said procedure uses an embryonic neural cell adhesion molecule antibody that specifically recognises polysialated neural cell adhesion molecule.

15   Preferably, said mammalian embryo is selected from the group consisting of human and non-human primates, equines, canines, felines, bovines, porcines, ovines, lagomorphs, and the order Rodentia.

In a further aspect, the present invention provides a pure population of mammalian CNS neuron-restricted precursor cells isolated by the method of
20   the invention.

In another aspect, the present invention provides a method of obtaining postmitotic neurons comprising:

(a)   providing neuron-restricted precursor cells according to the invention and culturing the neuron-restricted precursor cells in
25   proliferating conditions; and

(b)   changing the culture conditions of the neuron-restricted precursor cells from proliferating conditions to differentiating conditions, thereby causing the neuron-restricted precursor cells to differentiate into postmitotic neurons.

30   Preferably, said changing the culture conditions comprises adding retinoic acid to basal medium.

Preferably, said changing the culture conditions comprises withdrawing a mitotic factor from basal medium. More preferably, said mitotic factor is fibroblast growth factor.

35   Preferably, said changing the culture conditions comprises adding a neuronal maturation factor to basal medium.

Preferably, said neuronal maturation factor is a member selected from the group consisting of sonic hedgehog, BMP-2, BMP-4, NT-3, NT-4, CNTF, LIF, retinoic acid, brain-derived neurotrophic factor (BDNF), and combinations of any of the above.

5   In yet another aspect, the present invention provides an isolated cellular composition comprising the mammalian CNS neuron-restricted cells according to the invention.

In a further aspect, the present invention provides a pharmaceutical composition comprising a therapeutically effective amount of the composition of
10   the invention and a pharmaceutically acceptable carrier.

In another aspect, the present invention provides a method for treating a neuronal disorder in a mammal comprising administering to said mammal a therapeutically effective amount of the composition of the invention.

In another aspect, the present invention provides a method for treating a
15   neuronal disorder in a mammal comprising administering to said mammal a therapeutically effective amount of the pharmaceutical composition of the invention.

Preferably, said composition is administered by a route selected from the group consisting of intramuscular administration, intrathecal administration,
20   intraperitoneal administration, intravenous administration, and combinations of any of the above.

Preferably, said method also includes the administration of a member selected from the group consisting of differentiation factors, growth factors, cell maturation factors and combinations of any of the above. More preferably, said
25   differentiation factors are selected from the group consisting of retinoic acid, BMP-2, BMP-4, and combinations of any of the above.

In another aspect, the present invention provides for the use of a composition of the invention for the delivery to glial cells of an agent selected from the group consisting of cell growth factors, cell maturation factors, cell
30   differentiation agents, and any combination of the above.

Preferably, for use as a delivery vehicle for the delivery of trophic factors to neurons.

In another aspect, the present invention provides a method for treating neurodegenerative symptoms in a mammal comprising the steps of:
35   (a)   providing a pure population of neuronal restricted precursor cells according to the invention;

    (b)   genetically transforming said neuronal restricted precursor cells with a gene encoding a growth factor, neurotransmitter, neurotransmitter synthesizing enzyme, neuropeptide, neuropeptide synthesizing enzyme, or substance that provides protection against free-radical mediated damage thereby resulting in a transformed population of glial restricted precursor cells that express said growth factor, neurotransmitter, neurotransmitter synthesizing enzyme, neuropeptide, neuropeptide synthesizing enzyme, or substance that provides protection against free-radical mediated damage; and

    (c)   administering an effective amount of said transformed population of neuronal restricted precursor cells to said mammal.
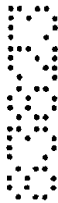
In a further aspect, the present invention provides a method or screening compounds for neurological activity comprising the steps of:

    (a)   providing a pure population of neuronal restricted precursor cells according the invention or derivatives thereof or mixtures thereof cultured *in vitro*;

    (b)   exposing said cells or derivatives thereof or mixtures thereof to a selected compound at varying dosages; and

    (c)   monitoring the reaction of said cells or derivatives thereof or mixtures thereof to said selected compound for selected time periods.

In a further aspect, the present invention provides a method for treating a neurological or neurodegenerative disease comprising administering to a mammal in need of such treatment an effective amount of neuronal restricted precursor cells according to the invention or derivatives thereof or mixtures thereof.

Preferably, said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are caused to proliferate and differentiate *in vitro* prior to being administered.

Preferably, said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are caused to proliferate *in vitro* prior to being administered, and then are caused to further proliferate and differentiate *in vivo* after being administered.

Preferably, said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are caused to proliferate *in vitro* prior to being administered, and then are caused to differentiate *in vivo* after being administered.

In one embodiment, said neuronal restricted precursor cells or
5 derivatives thereof or mixtures thereof are from a heterologous donor. Preferably, said donor is selected from a fetus, a juvenile or an adult.

In another embodiment, said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are from an autologous donor.

Preferably, said derivatives thereof are obtained by differentiation of
10 neuronal restricted precursor cells *in vitro*.

Preferably, said derivatives thereof are obtained by genetic transduction of neuronal restricted precursor cells.

In another aspect, the present invention provides a method of isolating a pure population of mammalian CNS neuron-restricted precursor cells
15 comprising the steps of:

    (a)   providing a sample of mammalian embryonic stem cells;

    (b)   removing A2B5+ cells from the differentiating cells via specific antibody capture with an antibody that specifically recognises A2B5;

20   (c)   purifying from the mammalian embryonic stem cells a subpopulation expressing embryonic neural cell adhesion molecule;

    (d)   plating the purified subpopulation of cells in feeder-cell-independent culture on a substratum and in a medium configured for supporting
25   adherent growth of the neuron-restricted precursor cells; and

    (e)   incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuron-restricted precursor cells,

wherein the isolated cells are capable of self renewal in the presence of FGF.

Preferably, said purifying comprises a procedure selected from the group
30 consisting of specific antibody capture, fluorescence activated cell sorting, and magnetic bead capture.

Preferably, said procedure uses an embryonic neural cell adhesion molecule antibody that specifically recognises polysialated neural cell adhesion molecule.

35 Also provided is a method of isolating a pure population of mammalian CNS neuron-restricted precursor cells comprises the steps of

     (a)   isolating a population of mammalian multipotent CNS stem cells capable of generating both neurons and glia;

     (b)   incubating the multipotent CNS stem cells in a medium configured for inducing such cells to begin differentiating;

5   (c)   purifying from the differentiating cells a subpopulation of cells expressing a selected antigen defining neuron-restricted precursor cells; and

     (d)   incubating the purified subpopulation of cells in a medium configured for supporting adherent growth thereof.

10   A preferred selected antigen defining neuron-restricted precursor cells is embryonic neural cell adhesion molecule. Preferably, the step of purifying the NRP cells comprises a procedure selected from the group consisting of specific antibody capture, fluorescence activated cell sorting, and magnetic bead capture. Specific antibody capture is especially preferred. In a preferred

15   embodiment, the mammalian multipotent CNS stem

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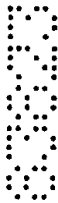
cells are neuroepithelial stem cells. A preferred
procedure for isolating a population of CNS
neuroepithelial stem cells comprises:

        (a)   removing a CNS tissue from a mammalian embryo
5   at a stage of embryonic development after closure of the
neural tube but prior to differentiation of cells in the
neural tube;

        (b)   dissociating cells comprising the neural tube
removed from the mammalian embryo;

10   (c)   plating the dissociated cells in feeder-cell-
independent culture on a substratum and in a medium
configured for supporting adherent growth of the
neuroepithelial stem cells comprising effective amounts of
fibroblast growth factor and chick embryo extract; and

15   (d)   incubating the plated cells at a temperature
and in an atmosphere conducive to growth of the
neuroepithelial stem cells.

        Preferably, the mammalian embryo is selected from the
group consisting of human and non-human primates, equines,
20   canines, felines, bovines, porcines, ovines, lagomorphs,
and rodents. It is also preferred that the substratum is
selected from the group consisting of fibronectin,
vitronectin, laminin, and RGD peptides. In a preferred
embodiment, the medium comprises effective amounts of
25   fibroblast growth factor and neurotrophin 3 (NT-3).

        A method of isolating a pure population of mammalian
CNS neuron-restricted precursor cells comprises the steps
of:

        (a)   removing a sample of CNS tissue from a
30   mammalian embryo at a stage of embryonic development after
closure of the neural tube but prior to differentiation of
glial and neuronal cells in the neural tube;

        (b)   dissociating cells comprising the sample of CNS
tissue removed from the mammalian embryo;

35   (c)   purifying from the dissociated cells a
subpopulation expressing a selected antigen defining
neuron-restricted precursor cells;

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   (d)   plating the purified subpopulation of cells in
feeder-cell-independent culture on a substratum and in a
medium configured for supporting adherent growth of the
neuron-restricted precursor cells; and

5   (e)   incubating the plated cells at a temperature
and in an atmosphere conducive to growth of the neuron-
restricted precursor cells.

   Preferably, the selected antigen defining neuron-
restricted precursor cells is embryonic neural cell

10 adhesion molecule. It is also preferred that the step of
purifying comprises a procedure selected from the group
consisting of specific antibody capture, fluorescence
activated cells sorting, and magnetic bead capture.
Specific antibody capture is especially preferred. It is

15 further preferred that the mammalian embryo is selected
from the group consisting of human and non-human primates,
equines, canines, felines, bovines, porcines, ovines,
lagomorphs, and rodents.

   A method of obtaining postmitotic neurons comprises:

20   (a)   providing neuron-restricted precursor cells and
culturing the neuron-restricted precursor cells in
proliferating conditions; and

   (b)   changing the culture conditions of the neuron-
restricted precursor cells from proliferating conditions

25 to differentiating condition, thereby causing the neuron-
restricted precursor cells to differentiate into
postmitotic neurons.

   The changing of the culture conditions preferably
comprises adding retinoic acid to basal medium or

30 withdrawing a mitotic factor from basal medium. Such a
mitotic factor is fibroblast growth factor. Changing the
culture conditions can also comprise adding a neuronal
maturation factor to basal medium. Preferred neuronal
maturation factors are selected from the group consisting

35 of sonic hedgehog, BMP-2, BMP-4, NT-3, NT-4, CNTF, LIF,
retinoic acid, brain-derived neurotrophic factor (BDNF),
and combinations of any of the above.

10

Another preferred embodiment of the invention
comprises an isolated cellular composition comprising the
mammalian CNS neuron-restricted cells described herein.
Another preferred embodiment of the invention comprises a
5   pharmaceutical composition comprising a therapeutically
effective amount of such composition and a
pharmaceutically acceptable carrier.

A method for treating a neuronal disorder in a mammal
comprises administering to such mammal a therapeutically
10   effective amount of the isolated cellular composition
comprising the mammalian CNS neuron-restricted cells
described herein. Another method for treating a neuronal
disorder in a mammal comprising administering to said
mammal a therapeutically effective amount of such
15   pharmaceutical composition and a pharmaceutically
acceptable carrier. Such composition can be administered
by a route selected from the group consisting of
intramuscular administration, intrathecal administration,
intraperitoneal administration, intravenous
20   administration, and combinations of any of the above.
This method can also include the administration of a
member selected from the group consisting of
differentiation factors, growth factors, cell maturation
factors and combinations of any of the above. Such
25   differentiation factors are preferably selected from the
group consisting of retinoic acid, BMP-2, BMP-4, and
combinations of any of the above.

A method for treating neurodegenerative symptoms in a
mammal comprises the steps of:
30   (a)   providing a pure population of neuronal
restricted precursor cells;
(b)   genetically transforming such neuronal
restricted precursor cells with a gene encoding a growth
factor, neurotransmitter, neurotransmitter synthesizing
35   enzyme, neuropeptide, neuropeptide synthesizing enzyme, or
substance that provides protection against free-radical
mediated damage thereby resulting in a transfored
population of glial restricted precursor cells that

11

express such growth factor, neurotransmitter,
neurotransmitter synthesizing enzyme, neuropeptide,
neuropeptide synthesizing enzyme, or substance that
provides protection against free-radical mediated damage;
5   and

(c)   administering an effective amount of said
transformed population of neuronal restricted precursor
cells to such mammal.

A method or screening compounds for neurological
10   activity comprising the steps of:

(a)   providing a pure population of neuronal
restricted precursor cells or derivatives thereof or
mixtures thereof cultured *in vitro*;

(b)   exposing such cells or derivatives thereof or
15   mixtures thereof to a selected compound at varying
dosages; and

(c)   monitoring the reaction of such cells or
derivatives thereof or mixtures thereof to said selected
compound for selected time periods.

20   A method for treating a neurological or
neurodegenerative disease comprises administering to a
mammal in need of such treatment an effective amount of
neuronal restricted precursor cells or derivatives thereof
or mixtures thereof. Such neuronal restricted precursor
25   cells or derivatives thereof or mixtures thereof can be
from either a heterologous donor or an autologous donor.
The donor can be a fetus, juvenile, or adult.

A method of isolating a pure population of mammalian
CNS neuron-restricted precursor cells comprises the steps
30   of:

(a)   providing a sample of mammalian embryonic stem
cells:

(b)   purifying from the mammalian embryonic stem
cells a subpopulation expressing a selected antigen
35   defining neuron-restricted precursor cells;

(c)   plating the purified subpopulation of cells in
feeder-cell-independent culture on a substratum and in a

medium configured for supporting adherent growth of the neuron-restricted precursor cells; and

    (d) incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuron-restricted precursor cells.

5   Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

10   Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present

15 invention as it existed in Australia before the priority date of each claim of this application.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

    FIG. 1 shows a summary of the immunoreactivities of NEP cells and

20 their progeny, including NRP cells.

    FIG. 2 shows results of RT-PCR amplification of total RNA isolated from rat E-NCAM$^+$ cells for determining expression of choline acetyl transferase (ChAT), p75, islet-1 (Isl-1), calbindin, glutamic acid decarboxylase (GAD), glutaminase, and cyclophilin (a housekeeping gene).

25   FIG. 3 shows a bar graph of the number of cells responding to neurotransmitters on acutely dissociated (unshaded) and differentiated (shaded) cells as measured by fura-2 calcium ion imaging: GABA (γ-amino butyric acid), Gly (glycine), DA (dopamine), Glu (glutamate), Ach (acetyl choline), RR (rat ringers solution), 50 mM K RR (rat ringers solution modified

30 by replacing Na$^+$ with K$^+$).

    FIG. 4 shows an illustrative plot of the ratio (I_{340}/I_{380}) of Ca^{2+} responses over time from an acutely dissociated E-NCAM$^+$ cell.

    FIG. 5 shows an illustrative plot of the ratio (I_{340}/I_{380}) of Ca^{2+} responses over time from a differentiated E-NCAM$^+$ cell.

35   FIG. 6 shows the results of PCR analysis of a single E-NCAM$^+$ clone for expression of markers of mature neurons.

FIG. 7 shows a bar graph of the percentage of cells from four E-NCAM$^+$ clones that responded to neurotransmitters as measured by fura-2 calcium ion imaging: GABA (γ-amino butyric acid), Gly (glycine), DA (dopamine), Glu (glutamate), Ach (acetyl choline), RR (rat ringers solution), 50 mM K RR (rat
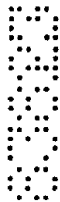
5   ringers solution modified by replacing Na$^+$ with K$^+$).

13

FIGS. 8 and 9 show illustrative traces of the ratio (I_{340}/I_{380}) of Ca^{2+} responses from two cells recorded from one E-NCAM$^+$ clone.

FIG. 10 shows the effect of bone morphogenetic
5   protein 2 (BMP-2) on cell division of E-NCAM$^+$ cells as measured by BRDU incorporation.

FIG. 11 shows the effect of sonic hedgehog (Shh) on cell division of E-NCAM$^+$ cells as measured by BRDU incorporation.

10   FIG. 12 shows results of RT-PCR amplification of total RNA isolated from mouse E-NCAM$^+$ cells for determining expression of (from left to right after the molecular weight markers at the far left) p75, Isl-1, ChAT, calbindin, GAD, and glutaminase.

15   FIG. 13 shows results of RT-PCR amplification of total RNA isolated from differentiated mouse ES cells for determining expression of (from left to right) nestin, N-CAM, neurofilament-M (NF-M), microtubule associated protein 2 (Map-2), GFAP, DM-20/PLP.

20   FIG. 14 shows results of RT-PCR amplification of total RNA isolated from differentiated mouse ES cells for determining expression of (from left to right) ChAT, p75, islet-1, calbindin, GAD, and glutaminase.

25   DETAILED DESCRIPTION

Before the present neuronal-restricted precursor cells and methods of making and methods of use thereof are disclosed and described, it is to be understood that this invention is not limited to the particular configurations,
30   process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended
35   to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

14

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an

5   embryo" includes reference to two or more embryos, reference to "a mitogen" includes reference to a mixture of two or more mitogens, and reference to "a factor" includes reference to a mixture of two or more factors.

In describing and claiming the present invention, the

10   following terminology will be used in accordance with the definitions set out below.

As used herein, "self renewal" refers, for example, to the capability of a neuroepithelial stem cell to divide to produce two daughter cells, at least one of which is a

15   multipotent neuroepithelial stem cell, or to the capability of a neuronal-restricted precursor cell to divide to produce two daughter cells, at least one of which is a neuronal-restricted precursor cell.

As used herein, "clonal density" and similar terms

20   mean a density sufficiently low enough to result in the isolation of single, non-impinging cells when plated in a selected culture dish. An illustrative example of such a clonal density is about 225 cells/100 mm culture dish.

As used herein, "feeder-cell-independent adherent

25   culture" and similar terms mean the growth of cells *in vitro* in the absence of a layer of different cells that generally are first plated on a culture dish to which the cells from the tissue of interest are then added. In feeder cell cultures, the feeder cells provide a

30   substratum for the attachment of cells from the tissue of interest and additionally serve as a source of mitogens and survival factors. The feeder-cell-independent adherent cultures herein use a chemically defined substratum, for example fibronectin, and mitogens or

35   survival factors are provided by supplementation of the liquid culture medium with either purified factors or crude extracts from other cells or tissues. Therefore, in feeder-cell-independent cultures, the cells in the culture

15

dish are primarily cells derived from the tissue of
interest and do not contain other cell types required to
support the growth of cells derived from the tissue of
interest.

5   As used herein, "effective amount" means an amount of
a growth factor or survival factor or other factor that is
nontoxic but sufficient to provide the desired effect and
performance. For example, an effective amount of FGF as
used herein means an amount selected so as to support self

10   renewal and proliferation of NEP cells when used in
combination with other essential nutrients, factors, and
the like. An effective amount of NRP cells or derivatives
thereof or mixtures thereof for transplantation refers to
an amount or number of cells sufficient to obtain the

15   selected effect. NRP cells will generally be administered
at concentrations of about 5-50,000 cells/microliter.
Transplantation will generally occur in volumes up to
about 15 microliters per injection site. However,
transplantation subsequent to surgery on the central

20   nervous system can involve volumes many times this size.
Thus, the number of cells used for transplantation is
limited only by utility, and such numbers can be
determined by a person skilled in the art without undue
experimentation.

25   As used herein, "derivative" of an NRP cell means a
cell derived from an NRP cell *in vitro* by genetic
transduction, differentiation, or similar processes.

As used herein, "administering an NRP cell to a
mammal means transplanting or implanting such NRP cell

30   into CNS tissue or adjacent to such CNS tissue of a
recipient. Such administration can be carried out by any
method known in the art, such as surgery, with an infusion
cannula, needle, and the like.

As used herein, "heterologous" refers to individuals,

35   tissues, or cells different from a transplant recipient.
The transplant donor could be from the same species or a
different species as the transplant recipient. For
example, a heterologous donor of NRP cells for

16

transplantation could be from a different species as the
transplant recipient.

As used herein, "autologous" refers to self-generated
or originating within the body. Thus, for example, an
5   autologous donor of tissue or cells for transplantation is
the same individual that receives the transplant. By way
of further example, autologous cells are cells arising,
transferred, or transplanted within an individual. *In*
vitro manipulation may take place between harvesting of
10   the cells and transplanting such cells or derivatives
thereof, but is not required prior to transplantation.

As used herein, "transforming," "transducing,"
"transfection," and similar terms mean insertion or
transfer of a gene or genes into NRP cells regardless of
15   the method of insertion r transfer. Thus, transformation
can be accomplished by calcium phosphate transfection,
DEAE-dextran transfection, polybrene transfection,
electroporation, lipofection, infection of viruses, and
the like and any other methods known in the art.

20   The present invention is illustrated using neuron-
restricted precursor cells isolated from rats, mice, and
humans. The invention, however, encompasses all mammalian
neuronal-restricted precursor cells and is not limited to
neuronal-restricted precursor cells from rats, mice, and
25   humans. Mammalian neuron-restricted precursor cells can
be isolated from human and non-human primates, equines,
canines, felines, bovines, porcines, ovines, lagomorphs,
and the like.

Pluripotent stem cells in the central nervous system
30   may generate differentiated neurons and glia either
directly or through the generation of lineage-restricted
intermediate precursors. In the developing retina, it
appears that multipotent retinal precursors can generate
any combination of differentiated cells even at their
35   final division, indicating that intermediate precursors do
not exist. In other regions of the central nervous
system, in contrast, retroviral labeling studies have
suggested the existence of lineage-restricted precursors

17

that generate only one type of cell or a limited number of
cell types. Intermediate stage precursors such as the
bipotential oligodendrocyte-type-2-astrocyte precursor (O-
2A) and a neuronal precursor have also been described in
5   tissue culture studies. Yet, the generation of
intermediate lineage-restricted precursors from
pluripotent embryonic or adult stem cells or other stem
cells capable of differentiating into neurons and glia had
not been observed until recently, i.e. U.S. Patent
10   Application Serial No. 08/909,435, filed July 4, 1997.
Thus, the lineal relationship between pluripotent stem
cells identified in culture and the committed precursors
identified in vivo and in vitro had heretofore been
unknown. Possible models of development have included (1)
15   pluripotent and more committed stem cells representing
lineally related cells or (2) such cells representing
independent pathways of differentiation.

The developing rat spinal cord represents an ideal
model for studying this differentiation. At embryonic day
20   10.5 (E10.5), the caudal neural tube appears as a
homogeneous population of nestin-immunoreactive dividing
cells in vivo and in vitro. These initially homogeneous
cells are patterned over several days to generate neurons,
oligodendrocytes, and astrocytes in a characteristic
25   spatial and temporal profile. Neurogenesis occurs first
on a ventro-dorsal gradient, with the earliest neurons
becoming postmitotic on E13.5 in rats. Neurogenesis
continues over an additional two days followed by
differentiation of oligodendrocyte precursors and the
30   subsequent differentiation of astrocytes.

Methods for growing neuroepithelial stem (NEP) cells
isolated from E10.5 rat embryos as undifferentiated cells
for extended periods in vitro have been described in
Serial No. 08/852,744., and it has been shown further that
35   these populations were able to generate the three major
cells types in the CNS. Thus, NEP cells represent a
dividing multipotent stem cell that may differentiate into
neurons either via an intermediate neuroblast or directly

18

as a part of its terminal differentiation. To determine
whether neurons differentiated from NEP cells via
intermediate, more-restricted precursors, a variety of
immunologically defined populations from differentiating
5   cultures of NEP cells were isolated and characterized. It
is shown herein that cells morphologically and
phenotypically identical to NRP's can be isolated from NEP
cell cultures. Clonal analysis shows that individual NEP
cells generate neurons via the generation of neuronal
10   precursors and that individual NEP cells can generate
neuron-restricted and glial-restricted precursors. It is
further shown that E-NCAM$^+$ (embryonic neural cell adhesion
molecule positive) cells are present in E13.5 neural tube
cultures and that these cells are mitotic, self renewing
15   stem cells that can generate multiple neuronal phenotypes,
but not astrocytes or oligodendrocytes. Thus, neuron
restricted precursors (NRPs) are an identifiable stage in
the *in vivo* differentiation of neurons. Moreover, it is
shown that NRPs can be isolated and cultured from mouse
20   embryos, mouse embryonic stem (ES) cells, and from human
embryonic spinal cords. These data provide a
demonstration of a direct lineal relationship between
multipotent and neuron-restricted stem cells and suggest
that neural differentiation involves progressive
25   restriction in developmental fate.

FIG. 1 presents a model for spinal cord
differentiation. This model is similar to that proposed
for hematopoiesis and for differentiation of neural crest
(see review by D.J. Anderson, The Neural Crest Lineage
30   Problem: Neuropoiesis?, 3 Neuron 1-12 (1989)). According
to this model, NEP cells 10 represent a homogeneous
population of cells in the caudal neural tube that express
nestin (i.e. nestin $^+$) but no other lineage marker (lin$^-$).
These cells divide and self renew in culture and generate
35   differentiated phenotypes. Previous data have suggested
intermediate dividing precursors with a more restricted
potential. Such precursors include glial restricted
precursors 14 that generate oligodendrocytes 18 and

19

astrocytes 22, as well as neuronal progenitors 26 that
generate several kinds of neurons 30, 34. The model also
shows that neural crest stem cells 38, which can
differentiate into PNS neurons 42, Schwann cells 46, and
5   smooth muscle cells 50, also descend from NEP cells. The
model therefore suggests that the multipotent precursors
(NEP cells) generate differentiated cells (i.e.,
oligodendrocytes, type 2 astrocytes, type 1 astrocytes,
neurons, motoneurons, PNS neurons, Schwann cells, and
10   smooth muscle cells) through intermediate precursors.
Consistent with this model are the results presented
herein showing the existence of cells with a neuron-
restricted proliferative potential.

     NEP cell cultures provide a large source of transient
15   cells that can be sorted to obtain differentiated cell
types. The results described herein provide direct
evidence to support a model describing initially
multipotent cells undergoing progressive restriction in
developmental potential under extrinsic influence to
20   generate the different phenotypes within the CNS.
Evidence is provided that initially multipotent NEP cells
generate neuron-restricted precursors *in vitro* and that
such neuron-restricted precursors are also present *in
vivo*. It is also shown that NRPs fulfill criteria of
25   blast cells and that a direct lineal relationship between
multipotent stem cells and more restricted NEP cells
exists.

     The results presented herein support that E-NCAM-
immunoreactive cells are restricted in their developmental
30   potential. E-NCAM$^+$ cells failed to differentiate into
oligodendrocytes or astrocytes under any culture
conditions tested. In contrast, NEP cells differentiated
into neurons, astrocytes, and oligodendrocytes, and A2B5-
immunoreactive cells differentiate into oligodendrocytes
35   under identical conditions. For these reasons, E-NCAM-
immunoreactive cells are described herein as neuron-
restricted precursors or NRPs.

20

Immunopanning and double-labeling data demonstrate
that E-NCAM can be used to identify a specific neuronal
sublineage that is generated from multipotential NEP
cells. Like markers for intermediate precursors in the
5   hematopoietic system and neural crest, however, E-NCAM,
and the A2B5 glial precursor marker as well, is not unique
to intermediate precursors. E-NCAM has been shown to
label some astrocytes. Similarly, A2B5 has been shown to
recognize neurons in some species and is transiently
10   expressed by astrocytes in some culture conditions.
Nevertheless, under the specific culture conditions
defined herein these markers can be used to select
intermediate precursors and therefore represent the first
cell surface epitopes that are co-expressed in concordance
15   with a restriction in developmental potential.

The basal medium (NEP medium) used in the experiments
described herein comprises DMEM-F12 (GIBCO/BRL,
Gaithersburg, MD) supplemented with 100 μg/ml transferrin
(Calbiochem, San Diego, CA), 5 μg/ml insulin (Sigma
20   Chemical Co., St. Louis, MO), 16 μg/ml putrescine (Sigma),
20 nM progesterone (Sigma), 30 nM selenious acid (Sigma),
1 mg/ml bovine serum albumin (GIBCO/BRL), plus B27
additives (GIBCO/BRL), 20 ng/ml basic fibroblast growth
factor (bFGF), and 10% chick embryo extract (CEE). In
25   general, these additives were stored as 100X concentrates
at -20°C until use. Normally, 200 ml of NEP medium was
prepared with all additives except CEE and used within two
weeks of preparation. CEE was added to the NEP medium at
the time of feeding cultured cells.
30   FGF and CEE were prepared as described in D.L.
Stemple & D.J. Anderson, *supra*; M.S. Rao & D.J. Anderson,
supra; L. Sommers et al., Cellular Function of the bHLH
Transcription Factor MASH1 in Mammalian Neurogenesis, 15
Neuron 1245-58 (1995), hereby incorporated by reference.
35   FGF is also available commercially (UBI).
Briefly, CEE was prepared as follows. Chick eggs
were incubated for 11 days at 38°C in a humidified
atmosphere. Eggs were washed and the embryos were removed

21

and placed in a petri dish containing sterile Minimal
Essential Medium (MEM with glutamine and Earle's salts)
(GIBCO/BRL) at 4°C. Approximately 10 embryos each were
macerated by passage through a 30-ml syringe into a 50-ml

5   test tube. This procedure typically produced about 25 ml
of medium. To each 25 ml was added 25 ml of MEM. The
tubes were rocked at 4°C for 1 hour. Sterile
hyaluronidase (1 mg/25 g of embryo) (Sigma) was added, and
the mixture was centrifuged for 6 hours at 30,000 g. The

10   supernate was collected, passed through a 0.45 μm filter
and then through a 0.22 μm filter, and stored at -80°C
until use.

        Laminin (Biomedical Technologies Inc.) was dissolved
in distilled water to a concentration of 20 mg/ml and

15   applied to tissue culture plates (Falcon). Fibronectin
(Sigma) was resuspended to a stock concentration of 10
mg/ml and stored at -80°C and then diluted to a
concentration of 250 μg/ml in D-PBS (GIBCO/BRL). The
fibronectin solution was applied to tissue culture dishes

20   and immediately withdrawn. Subsequently, the laminin
solution was applied and plates were incubated for 5
hours. Excess laminin was withdrawn, and the plates were
allowed to air dry. Coated plates were then rinsed with
water and allowed to dry again. Fibronectin was chosen as

25   a growth substrate for NEP cells because NEP cells did not
adhere to collagen or poly-L-lysine (PLL) and adhered
poorly to laminin. Thus, all subsequent experiments to
maintain NEP cells in culture were performed on
fibronectin-coated dishes. Laminin-coated dishes were

30   used, however, to promote differentiation of NEP stem
cells.

        For clonal analysis, cells harvested by
trypsinization were plated at a density of 50-100 cells
per 35 mm dish. Individual cells were identified and

35   located on the dish by marking the position with a grease
pencil. Cells were grown in DMEM/F12 with additives, as
described above, for a period ranging from 10-15 days.

The cells of the present invention may be used in the
preparation of compositions, including pharmaceutical
compositions, which may be appropriately formulated and
administered to treat and correct deficiencies,
5   debilitations, and other dysfunctions that may result from
injury, disease, or other degeneration of relevant neural
tissue. By way of non-limiting examples, suitable cells
prepared in accordance with the present invention may be
administered, e.g., by implantation as a means of
10   effecting cell-replacement therapy, to treat instances
where cell injury or debilitation has taken place. Thus,
for example, the cells may be prepared in appropriate
growth medium such as one for promotion of growth and
differentiation. Suitable medium may include, for
15   example, growth or differentiation factors, e.g., retinoic
acid, BMP-2, BMP-4, or one or more members of the
neurotrophins such as NT-3, NT-4, CNTF, BDNF and the like.
Cells thus suitably prepared in such medium would be
introduced either intrathecally, I.V., I.P., or wherever
20   or by any means by which introduction of the cell
preparation to the target site is best accomplished. The
particulars of administration of this type may vary and
would be within the skill set of the physician or
practitioner.
25   The cells of the present invention are likewise
useful in a variety of diagnostic applications and may,
for example, be prepared for use in a screening assay,
e.g., for identification of neuronal markers and other
binding partners or ligands, modulators or other factors
30   that may function as modulators of cell growth and/or
differentiation. The cells of the present invention may
also be used, e.g., as a positive control in an assay to
identify deficiencies in cell growth and differentiation,
and the factors that may be the cause thereof.
35   The cells of the present invention may be utilized in
a variety of therapeutic applications, including in the
preparation of pharmaceutical compositions and appropriate
carriers, for administration to individuals in need of

23

such therapy, to treat various cellular debilitation,
dysfunctions or other irregularities or abnormalities
associated with injury, disease or genetically caused
neuronal deficits. Maladies or conditions contemplated
5   herein include Parkinson's disease, Huntington's disease,
Alzheimer's disease, dysfunctions resulting from injury or
trauma, amyotrophic lateral sclerosis (ALS or Lou Gehrig's
Disease), and anencephaly.

10   Example 1
To determine if a dividing neuron-restricted
precursor is normally present *in vivo*, sections of E13.5
rat spinal cords were analyzed with a panel of early
neuronal markers. Sections were cut of embryos fresh
15   frozen at 13.5 days gestation and then were labeled by
immunocytochemistry. Staining procedures were carried out
according to methods well known in the art. Cells were
double-labeled with antibodies against E-NCAM
(Developmental Studies Hybridoma Bank, Iowa) and β-III
20   tubulin (Sigma Chemical Co., St. Louis, Missouri) or were
stained with E-NCAM and counterstained with DAPI, a
nuclear marker for identifying all cells. All secondary
monoclonal antibodies were from Southern Biotechnology.
Polysialated or embryonic N-CAM (E-NCAM) appeared to
25   be a likely marker for neuronal precursors. E-NCAM
immunoreactivity was first detected at E13.5. E-NCAM
immunoreactive cells could be seen in the margins of the
neural tube, but not in the proliferating ventricular
zone. Double-labeling with β-III tubulin indicated that
30   most E-NCAM-immunoreactive cells co-expressed this
neuronal marker. A small proportion of cells present more
medially were E-NCAM⁺, but did not express β-III tubulin
immunoreactivity, suggesting that E-NCAM may be an early
and specific marker of differentiation into neuronal
35   precursors that is expressed prior to β-III tubulin.

24

Example 2

To characterize E-NCAM-immunoreactive cells, E13.5
spinal cords were dissociated and E-NCAM-immunoreactive
cells were stained with a panel of antibodies (Table 1).

5   Sprague-Dawley rat embryos were removed at embryonic day
13.5 and placed in a petri dish containing Hanks balanced
salt solutions (HBSS, Gibco). The trunk segments of the
embryos were dissected using tungsten needles, rinsed, and
then transferred to fresh HBSS. Spinal cords were

10   mechanically dissected free from the surrounding
connective tissue using sharpened No. 5 forceps.
Isolated spinal cords were incubated in 0.05% trypsin/EDTA
solution for 20 minutes. The trypsin solution was
replaced with fresh HBSS containing 10% fetal bovine serum

15   (FBS). The segments were gently triturated with a Pasteur
pipette to dissociate cells. Cells dissociated by
trituration were plated in PLL/laminin-coated 35 mm dishes
(Nunc) at high density and stained after 24 hours.

Staining for the cell surface markers, such as A2B5

20   and α-GalC, was carried out with cultures of living cells.
To stain cells with antibodies against internal antigens
such as GFAP, which specifically recognizes astrocytes (A.
Bignami et al., Localization of the Glial Fibrillary
Acidic Protein in Astrocytes, by Immunofluorescence, 43

25   Brain Res. 429-35 (1972)), β-III tubulin (DAKO) and RT-97,
which stain neurons (E. Geisert & A. Frankfurter, The
Neuronal Response to Injury as Visualized by
Immunostaining of Class β-tubulin in the Rat, 102
Neurosci. Lett. 137-41 (1989), nestin, which is a marker

30   for undifferentiated stem cells (U. Lendahl et al., CNS
Stem Cells Express a New Class of Intermediate Filament
Protein, 60 Cell 585-95 (1990)), or 5-bromodeoxyuridine
(BrdU, Sigma), which is a marker for determining the
number of dividing cells, cultures were fixed in ice-cold

35   methanol. Double- or triple-labeling experiments were
performed by simultaneously incubating cells in
appropriate combinations of primary antibodies followed by
non-cross-reactive secondary antibodies, , e.g. M. Mayer

et al., Ciliary Neurotrophic Factor and Leukemia
Inhibitory Factor Promote the Generation, Maturation, and
Survival of Oligodendrocytes, 120 Development 142-53
(1994), hereby incorporated by reference. In triple-label

5   experiments, cultures were incubated with the primary
antibody in blocking buffer for a period of 1 hour, rinsed
with PBS, and incubated with a species-specific secondary
antibody in blocking buffer for 1 hour. Cultures were
rinsed three times with PBS and examined under a

10   fluorescence microscope. For labeling with 4 antibodies
simultaneously, live cells were first incubated with the
surface antibodies A2B5 and α-GalC without the secondary
layers. Cells were then fixed in ice-cold methanol for
ten minutes and stained with α-β-III tubulin and the

15   appropriate secondary antibody. After scoring the results
of this staining, which was usually negative, clones were
stained with GFAP and the secondary layer for the first
set of surface antibodies. Finally, the secondary
antibody for GFAP was added. This procedure allowed

20   staining with four antibodies using only three
fluorescent-color conjugated secondary antibodies.

      E-NCAM-immunoreactive cells constituted 60%±3% of all
cells present in dissociated culture 24 hours after
plating. The majority of the remaining cells were A2B5[+].

25   It has been shown in U.S. Patent Application Serial No.
08/852,744 that at this stage of development, A2B5-
immunoreactive cells are glial precursor cells.
Consistent with these results, β-III tubulin or E-NCAM-
immunoreactive cells did not co-express A2B5. The vast

30   majority of cultured E-NCAM-immunoreactive cells (85%±8%)
co-expressed β-III tubulin immunoreactivity as well as
nestin immunoreactivity, but not markers characteristic of
glial precursor immunoreactivity. Approximately 20% of
the E-NCAM^{+} cells divided in a 24-hour period. Most of

35   the dividing E-NCAM^{+} cells did not co-express β-III
tubulin, indicating that this population of cells could
represent a dividing neuroblast. It is not yet known

Table 1

Antibody/Kind	Source	Antigen Recognized	Cell Type Recognized
Anti-NCAM/mouse IgG	DSHB[a]	Polysialated N-CAM	Neurons
Anti-Nestin	DSHB	Nestin	NEP cells
Anti-β-III tubulin/mouse IgG1	Sigma[b]	Intermediate filament	Neurons
RT-97	DSHB	Neurofilaments	Neurons
Anti-A2B5/mouse IgM	BMB[c]	Ganglioside	Oligodendrocytes and precursors
Anti-GFAP/rabbit IgG	Accurate[d]	Glial fibrillary acid	Astrocytes
Anti-NF60	Chemicon[e]	Neurofilament 60	Neurons
Anti-GalC/mouse IgG	BMB	Galactocerebroside	Oligodendrocytes and precursors
Anti-Peripherin	Chemicon	Peripherin	Motoneurons, PNS Neurons
Anti-MAP kinase	Chemicon	MAP2 kinase	Neurons

a   Developmental Studies Hybridoma Bank, Iowa
b   Sigma Chemical Co., St. Louis, MO
c   Boehringer Mannheim Biochemicals, Gaithersburg, MD
d   Accurate, Westbury, NY
e   Chemicon, Temecula, CA

27

whether a higher percentage of the cells would be observed
to divide under these conditions with longer labeling
periods. However, even if this population were to include

5   a subset of cells sufficiently committed to neuronal
differentiation as to no longer engage in division, these
committed neurons would be eliminated from the population
with expansion and division in tissue culture. Table 2
summarizes results of the antigenic profile of the cells,

10   showing the percentages of E-NCAM⁺ cells from E13.5
embryos that express various other antigens. These
results show that E-NCAM⁺ cells from E13.5 spinal cord
express neuronal, but not glial, markers.

Table 2	
Antigen	% Expression
α-Nestin	98%
α-β-III tubulin	50%
RT-97	95%
α-NF M	100%
α-MAP kinase	100%
A2B5	0%
α-GFAP	0%
α-NF 60	0%
α-GalC	0%
α-Peripherin	0%

Example 3

        To determine the differentiation potential of E-NCAM-

30   immunoreactive cells, E-NCAM⁺ cells were purified by
immunopanning and plated at clonal density in gridded
dishes. E13.5 cells were prepared according to the
procedure of Example 2. An E-NCAM⁺ cell population was
purified from these E13.5 cells using a specific antibody-

35   capture technique according to the procedure of L. Wysocki
& V. Sato, "Panning" for Lymphocytes: A Method for Cell

28

Selection, 75 Proc. Nat'l Acad. Sci. USA 2844-48 (1978);
M. Mayer et al., *supra*, hereby incorporated by reference.
In brief, cells were trypsinized and the resulting cell
suspension was plated on an A2B5-antibody-coated dish to
5   allow binding of all A2B5⁺ cells to the plate. The
supernate was removed, and the plate was washed with DMEM
supplemented with additives described by J. Bottenstein
and G. Sato, Growth of a Rat Neuroblastoma Cell Line in
Serum-free Supplemented Medium, 76 Proc. Nat'l Acad. Sci.
10   USA 514-17 (1979), hereby incorporated by reference,
(DMEM-BS). The supernate was then plated on an E-NCAM-
antibody-coated dish to allow binding of the E-NCAM-
immunoreactive cells. The bound cells were scraped from
the plate and replated on fibronectin/laminin-coated glass
15   coverslips in 300 ml DMEM-BS ± growth factors at 5000
cells/well.

The A2B5 and E-NCAM antibodies for coating the plates
were used at concentrations of 5 μg/ml. Cells were
allowed to bind to the plate for 20-30 minutes in a 37°C
20   incubator. Growth factors were added every other day at a
concentration of 10 ng/ml. Recombinant bFGF and
neurotrophin 3 (NT-3) were purchased from PeproTech, and
retinoic acid (RA) was obtained from Sigma.

After 24 hours, some immunopanned E-NCAM⁺ cells were
25   assayed by immunocytochemistry according to the procedure
of Example 2. Greater than 95% of the cells were E-NCAM⁺
at that time. Purified and stained cells were plated on
gridded clonal dishes, and individual E-NCAM⁺ cells were
identified and followed over time by immunocytochemistry
30   according to the procedure of Example 2.

Of all the cytokines tested, optimum growth was
observed when cells were cultured in FGF (10 ng/ml) and
NT-3 (10 ng/ml). In the presence of FGF and NT-3, single
E-NCAM⁺ cells divided in culture to generate colonies
35   ranging from one to several hundred cells. By day 5, most
colonies contained between 20 and 50 daughter cells that
continued to express E-NCAM immunoreactivity. Daughter
cells appeared phase bright and had short processes. At

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this stage, most E-NCAM-positive cells did not express β-III tubulin or neurofilament-M immunoreactivity.

To promote differentiation of E-NCAM⁺ clones, the FGF- and NT-3-containing medium was replaced with medium containing retinoic acid (RA) and from which the mitogen, bFGF, was withheld. In this differentiation medium, E-NCAM⁺ cells stopped dividing and elaborated extensive processes and started to express neuronal markers. Quadruple-labeling of clones with neuronal and glial markers and DAPI histochemistry, to identify all cells, showed that all clones contained β-III tubulin-immunoreactive cells and neurofilament-M (NF-M) immunoreactive cells and that none of the E-NCAM⁺ clones differentiated into oligodendrocytes or astrocytes.

Table 3 summarizes the results obtained by quadruple labeling of 124 E-NCAM⁺ clones with DAPI, α-β-III tubulin, A2B5, and α-GFAP.

Table 3	
Antigen Expressed	% of Clones
α-β-III tubulin	100%
A2B5	0%
α-GFAP	0%

Example 4

In this example, immunopanned A2B5⁺ cells derived from dissociated E13.5 spinal cords according to the procedure of Example 2 were cultured in neuron-promoting medium, i.e. basal medium plus FGF and NT-3. Cultures were grown for 5 days and then switched to RA-containing medium as described in Example 3 , and sister plates were stained for either E-NCAM or A2B5 immunoreactivity.

No A2B5 immunopanned cell expressed E-NCAM immunoreactivity when grown under conditions that promote growth of neuronal cells. All A2B5 immunopanned cells, however, continued to express A2B5 immunoreactivity, indicating that neuron-promoting conditions do not affect

30

the survival and proliferation of glial precursor cells.
Thus, the inability to detect oligodendrocyte and
astrocyte differentiation in Example 3 was unlikely to be
due to the death in neuronal cultures of oligodendrocytes
5   and astrocytes that might have differentiated from E-NCAM⁺
precursors since A2B5 glial precursor cells purified and
grown in parallel in the presence of FGF and NT-3
continued to express A2B5 without apparent cell death and
generated healthy oligodendrocytes and astrocytes after 10
10   days in culture. In addition, A2B5⁺ cells never generated
neurons in the presence of FGF and NT-3 and showed no
expression of E-NCAM at any time tested. Thus, E-NCAM
immunoreactive cells, unlike A2B5-immunoreactive glial
restricted precursors, could not differentiate into
15   oligodendrocytes and appeared limited to neuronal
differentiation when compared to multipotential E10.5
neuroepithelial cells.

                              Example 5
20   While it has been clearly shown in the present system
that E-NCAM identifies neuronally restricted precursor
cells, it has been reported that certain glial precursors
at later stages of development can also express E-NCAM
immunoreactivity. This observation raises the possibility
25   that some E-NCAM⁺ cells identified by the presently
described methods may be bi-potential. To test this
possibility, E-NCAM⁺ cells were plated clonally in either
neuron-promoting medium (FGF + NT-3) or in glial-promoting
medium (FGF + 10% fetal calf serum) and compared for their
30   development. Medium containing FGF with 10% fetal calf
serum was chosen for glial differentiation since this
medium promotes astrocyte differentiation of both spinal
cord NEP cells as well as A2B5 immunoreactive A2B5 glial
precursor cells, as shown in U.S. Patent Application
35   Serial No. 08/ 852,744. All E-NCAM⁺ clones (24/24) that
were grown in neuron-promoting medium contained only β-III
tubulin⁺ cells after 8 days, while the clones grown in
serum-containing medium did not generate astrocytes or

31

proliferate. From a total of 97 E-NCAM⁺ cells grown in
glial-promoting conditions, 90 clones (92%) consisted of a
single dead cell after 24 hours, while the remaining 7
clones (8%) contained 1 or 2 dead cells after 48 hours.

5   Thus, E-NCAM immunoreactive cells, in contrast with glial
precursor cells, fail to proliferate or differentiate in
astrocyte-promoting conditions.

Example 6

10   To determine whether the restriction of E-NCAM⁺ cells
to generation of neurons also includes a restriction to
generation of certain subtypes of neurons, E-NCAM⁺ clones
grown in RA and NT-3 in the absence of FGF were examined
for the expression of different neurotransmitters. The

15   antibodies used in this example are described in Table 4.

Table 4			
Antibody/Kind	Source	Antigen Recognized	Cell Type Recognized
Anti-ChAT/goat IgG	Chemic on	Choline acetyl transferase	Motoneurons
Anti-Glutamate/rabb it IgG	Chemic on	Glutamate	Excitatory neurons
Anti-GABA/rabbit IgG	Chemic on	Gamma amino butyric acid	Inhibitory neurons

These results indicate that individual clones could
generate GABA-ergic, glutaminergic, and cholinergic

30   neurons. Of ten clones tested, all contained
glutaminergic, GABAergic, and cholinergic neurons. Thus,
E-NCAM-immunoreactive cells, while limited to
differentiating neurons, are capable of generating
excitatory, inhibitory, and cholinergic neurons.

35

Example 7

Primary clones of E-NCAM⁺ cells grown in FGF and NT-3
according to the procedure of Example 5 grew to large

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sizes of several hundred cells after 7 to 10 days in
culture, indicating some degree of self renewal. To
demonstrate prolonged self renewal of the E-NCAM$^+$
population, selected clones were followed by secondary and
5   tertiary subcloning. Individual E-NCAM$^+$ cells from E13.5
spinal cord were plated in fibronectin/laminin and
expanded for 7 days in the presence of FGF and NT-3. Five
individual clones were randomly selected and replated at
clonal density using the same expansion conditions. The
10   number of secondary clones was counted, and large clones
were selected and replated. The number of tertiary clones
obtained was counted, and clones were then induced to
differentiate into postmitotic neurons by replacing FGF
and RA.
15   All clones examined generated numerous daughter
clones that subsequently generated tertiary clones. Small
clones and very large clones showed similar self renewal
potential. When tertiary clones were switched to a medium
containing RA and lacking FGF, the majority of cells in a
20   clone differentiated into post-mitotic neurons expressing
β-III tubulin. Thus, E-NCAM$^+$ cells are capable of
prolonged self renewal and can generate multiple daughter
cells capable of generating neurons.
            These results suggest that E-NCAM immunoreactivity
25   identifies a neuroblast cell that can differentiate into
multiple neuronal phenotypes in culture, even after
multiple passages. NT-3 and FGF are required to maintain
the blast cell in a proliferative state, while RA promotes
differentiation.
30

Example 8

It has been shown previously that individual NEP
cells derived from E10.5 spinal cord are an E-NCAM-
immunonegative, multipotent, self renewing population of
35   cells that can generate neurons, astrocytes, and
oligodendrocytes (U.S. Patent Application Serial No.
08/852,744). To determine if neuronal differentiation
from NEP precursors involved the generation of an E-NCAM$^+$

intermediate neuronal precursor cell, NEP cell cultures
that were induced to differentiate *in vitro* were examined
for the presence of E-NCAM⁺ immunoreactive cells.

NEP cells were prepared according to the method
described in Serial No. 08/852,744. Briefly, Sprague
Dawley rat embryos were removed at E10.5 (13-22 somites)
and placed in a petri dish containing Ca/Mg-free Hanks
balanced salt solution (HBSS, GIBCO/BRL). The trunk
segments of the embryos (last 10 somites) were dissected
using tungsten needles, rinsed, and then transferred to
fresh HBSS. Trunk segments were incubated at 4°C in 1%
trypsin solution (GIBCO/BRL) for a period of ten to twelve
minutes. The trypsin solution was replaced with fresh
HBSS containing 10% fetal bovine serum (FBS, GIBCO/BRL).
The segments were gently triturated with a Pasteur pipette
to release neural tubes free from surrounding somites and
connective tissue. Isolated neural tubes were transferred
to a 0.05% trypsin/EDTA solution (GIBCO/BRL) for an
additional period of ten minutes. Cells were dissociated
by trituration and plated at high density in 35 mm
fibronectin-coated dishes in NEP medium. Cells were
maintained at 37°C in 5% CO_2/95% air. Cells were replated
at low density, i.e. ≤5000 cells per 35 mm plate, one to
three days after plating. Cells from several dishes were
then harvested by trypsinization (0.05% trypsin/EDTA
solution for two minutes). Cells were then pelleted,
resuspended in a small volume, and counted. About 5000
cells were plated in a 35 mm dish (Corning or Nunc).

NEP cells derived from E10.5 embryos were expanded in
the presence of FGF and CEE for 5 days and differentiated
by replating on laminin in the presence of CEE.
Differentiating NEP cells were triple-labeled with
antibodies to E-NCAM, GFAP, and GalC. This showed that E-
NCAM-immunoreactive cells that differentiated from NEP
cells did not express astrocytic (GFAP) or
oligodendrocytic (GalC) markers. A sister plate was
double-labeled with antibodies to E-NCAM and nestin. This
showed that E-NCAM immunoreactive cells that

34

differentiated from NEP cells co-express nestin.
Differentiating NEP cells were incubated for 24 hours with
BrdU and subsequently double-labeled with an antibody
against BrdU and E-NCAM. This showed that most E-NCAM-

5   immunoreactive cells divided in 24 hours. This higher
labeling rate may reflect differences in the isolate
procedure as compared to the previous example. Table 5
summarizes the antigenic profile of E-NCAM⁺ cells derived
from E10.5 NEP cells. Note that NEP-derived E-NCAM⁺ cells

10   are antigenically similar to E13.5 E-NCAM⁺ cells and, like
E13.5 E-NCAM⁺, do not express any of the glial markers
examined.

Table 5	
Antigen	Expression
α-Nestin	+/-
α-β-III tubulin*	+
A2B5	-
α-GFAP	-
α-GalC	-

15

20

    *   A subset of cells express this marker.

    Thus, induced NEP cultures comprise multiple
phenotypes, including E-NCAM⁺ cells. Like the E13.5 E-

25   NCAM⁺ cells, NEP-derived E-NCAM⁺ cells did not express
glial markers, but co-expressed β-III tubulin (20-30%) and
nestin (70-80%) immunoreactivity. Ninety percent of
panned E-NCAM⁺ cells incorporated BrdU in culture and
generated neurons after addition of RA or NT-3 and thus

30   appeared similar to the E13.5 E-NCAM-immunoreactive cells.

                          Example 9
    To determine whether single NEP-derived E-NCAM⁺ cells
were also restricted to neurons in their differentiation

35   potential, cells were studied in clonal culture. NEP
cells were induced to differentiate by replating on
laminin and withdrawal of CEE, as described in U.S. Patent

35

Application Serial No. 08/852,744. NEP cells derived from
E10.5 embryos were expanded in the presence of FGF and CEE
for 5 days and differentiated by replating on laminin in
the absence of CEE. Immunopanned E-NCAM-immunoreactive
5   cells were then plated on clonal-grid dishes (Greiner
Labortechnik) coated with fibronectin/laminin, and single
cells were followed in culture. After 5 days, clones were
switched to RA and FGF was withdrawn. Clones were allowed
to grow for an additional 3 days, fixed with
10   paraformaldehyde, and triple-labeled with A2B5 and
antibodies against GFAP and β-III tubulin. In addition,
cells were counterstained with DAPI to show individual
cell nuclei. Table 6 summarizes the results of the
staining of all 47 clones studied (8 of 47 clones did not
15   survive replating). Note that no clone contained
astrocytes (GFAP$^+$) cells or glial precursor cells (A2B5$^+$).

	Table 6
Antigen Expressed	% of Clones
α-β-III tubulin	100%
A2B5	0%
α-GFAP	0%

25   Forty-eight hours after cells were induced to
differentiate, 10-30% of the cells had begun to express E-
NCAM immunoreactivity. NEP-cell-derived E-NCAM$^+$ cells
were selected by immunopanning according to the procedure
of Example 3, and individual E-NCAM$^+$ cells were plated in
30   medium containing FGF and NT-3 and clones were analyzed
after 10 days.

All clones contained only E-NCAM$^+$/β-III-tubulin$^+$
cells, but not GFAP or A2B5 immunoreactive cells. In
addition, individual E-NCAM$^+$ cells failed to differentiate
35   into oligodendrocytes or astrocytes under culture
conditions that promoted astrocytic and oligodendroglial
differentiation from the parent NEP cell population. E-
NCAM$^+$ cells could be maintained as dividing precursor

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cells in defined medium in the presence of high
concentrations of FGF (10 ng/ml) and NT-3 (10 ng/ml). E-
NCAM$^+$ cells maintained for up to three months could
readily differentiate into β-III tubulin$^+$ mature neurons

5   that expressed a variety of neurotransmitter phenotypes
when exposed to RA grown on laminin. Thus, E-NCAM$^+$ cells
are similar to E13.5 neuronal precursors in their
differentiation potential, antigenic profile, and in the
conditions optimal for extended growth as a dividing

10   precursor cell population.

                            Example 10
        Differentiation of the E-NCAM$^+$ population from an
apparently homogeneous Nestin$^+$/E-NCAM$^-$ NEP cell population

15   suggests a progressive restriction in developmental fate.
It was thought possible, but unlikely, that individual NEP
cells could be pre-committed to generating neuroblasts or
glioblasts. To rule out this possibility, individual NEP
clones were examined for their ability to generate E-NCAM-

20   immunoreactive cells and A2B5-immunoreactive cells. A2B5
and E-NCAM were chosen since it had previously been shown
that A2B5 immunoreactivity is unique to oligodendrocyte-
astrocyte precursors at this stage of development. NEP
cells derived from E10.5 embryos were expanded in the

25   presence of FGF and CEE for 5 days, harvested by
trypsinization, and replated at clonal density in gridded
clonal dishes. After 7 days in culture, individual clones
were double-labeled with antibodies against E-NCAM and
A2B5 according to the procedure of Example 2. Of 112 NEP

30   clones that were followed in culture, 83% generated both
A2B5 and E-NCAM immunoreactive cells. Five percent of the
clones consisted of only A2B5 immunoreactive cells, and
12% of the clones showed no convincing staining for either
A2B5 or E-NCAM immunoreactivity. In all clones tested, E-

35   NCAM and A2B5 were expressed in non-overlapping
populations. That is, no cell co-expressed both markers.
Table 7 summarizes the results obtained with 112 clones.

37

Table 7		
Antigen Expressed	% of Clones	Number of Clones
E-NCAM⁺/A2B5⁺	83%	93
A2B5⁺ alone	5%	6
E-NCAM⁻/A2B5⁻	12%	13

5

Thus, the majority of NEP cells appear to be capable of generating precursors for glial restricted cells as well as neuronal restricted precursors.

10

Example 11

To test if most neurons were generated via an E-NCAM⁺ intermediate neuroblast, complement-mediated cell lysis was utilized to selectively kill E-NCAM⁺ cells. Twenty-

15 four hours after replating NEP cells in differentiating conditions, E-NCAM-immunoreactive cells were killed using an IgM antibody to E-NCAM and guinea pig complement. In sister plates, glial precursors were killed using an anti-A2B5 IgM antibody and complement. At this stage in

20 development, most E-NCAM+ cells do not express β-III tubulin. Treated plates were allowed to differentiate for an additional three days, and the development of neurons was monitored. E-NCAM-mediated lysis significantly reduced the number of β-III tubulin-immunoreactive cells

25 that developed when compared to cultures treated with A2B5 (219 ± 35 versus 879 ± 63, respectively) suggesting that neuronal differentiation from NEP cells *in vitro* requires a transition through an E-NCAM immunoreactive state.

30   ### Example 12
Differentiated E-NCAM⁺ Cells Can Be Distinguished from Acutely Dissociated NRP Cells

ENCAM⁺ cells were isolated by immunopanning according

35 to the procedure of Example 3, plated in 35 mm dishes, and allowed to grow for 24 hours (acutely dissociated) or 10 days (differentiated). Cultured cells were then analyzed for cell division by BRDU incorporation, E-NCAM

38

expression, NF-M expression, and synaptophysin expression
according to the procedure of Example 2. About 70% of
acutely dissociated E-NCAM⁺ cells incorporated BRDU,
showing that such cells were dividing in culture, whereas

5   after 10 days in differentiation promoting medium few or
no cells incorporated BRDU, and had therefore stopped
dividing. Double-labeling for E-NCAM and NF-M
immunoreactivity showed that very few acutely dissociated
cells expressed NF-M, whereas nearly all differentiated

10   cells expressed this protein. Similarly, synaptophysin, a
protein specifically associated with synaptic vesicles and
functional synapses, see T.C. Sudhof, The Synaptic Vesicle
Cycle: A Cascade of Protein-Protein Interactions, 375
Nature 645-653 (1995), was expressed by differentiated but

15   not acutely dissociated ENCAM⁺ cells. Although
synaptophysin protein expression was associated with
synaptic vesicles, early expression could also be detected
in the cell bodies and throughout the lengths of the
processes where it was initially expressed during

20   neurogenesis. M. Fujita et al., Developmental Profiles of
Synaptophysin in Granule Cells of Rat Cerebellum: An
Immunocytochemical Study, 45 J. Electron Microsc. Tokyo
185-194 (1996); D. Grabs et al., Differential Expression
of Synaptophysin and Synaptoporin during Pre- and

25   Postnatal Development of the Rat Hippocampal Network, 6
Eur. J. Neurosci. 1765-1771 (1994). These results show
that acutely dissociated E-NCAM⁺ cells are immature,
dividing cells that mature in culture. These results
suggest that if NRP cells are induced to differentiate by

30   RA and the removal of mitogen, they acquire many
morphological and immunological properties of mature
neurons.

                              Example 13

35   Numerous Neuronal Phenotypes Can Be Detected in
       Differentiated but Not Acutely Dissociated E-NCAM⁺ Cells

            It was shown above that NRP cells can differentiate
into postmitotic neurons, but not into oligodendrocytes or

39

astrocytes. To determine if NRPs can differentiate into
all of the major neuronal phenotypes present in the spinal
cord, or whether they are more limited in their
differentiation potential, the expression of
5   neurotransmitter synthesizing enzymes and cell type
specific markers for mature neurons was examined after
inducing NRPs to differentiate. In addition, the
expression of p75, Q. Yan & E.J. Johnson, An
Immunocytochemical Study of the Nerve Growth Factor
10   Receptor in Developing Rats, 8 J. Neurosci. 3481-3498
(1988), and Islet-1, T. Tsuchida et al., Topographic
Organization of Embryonic Motor Neurons Defined by
Expression of LIM Homeobox Genes, 79 Cell 957-970 (1994),
which are characteristic of motoneurons in the spinal
15   cord, and calbindin, which is often co-expressed with
GABA, C. Batini, Cerebellar Localization and
Colocalization of GABA and Calcium Binding Protein-D28K,
128 Arch. Ital. Biol. 127-149 (1990), were examined.
         E-NCAM$^+$ cells from E13.5 rat neural tube were
20   isolated by immunopanning according to the procedure of
Example 3, plated in 35 mm dishes, and cultured in
differentiation-promoting medium. After 10 days in
culture, total RNA was isolated from these cells and the
ability to synthesize the neurotransmitters acetylcholine
25   (Ach), GABA, and glutamate was assessed by the expression
of their synthesizing enzymes by RT-PCR. Total RNA was
isolated from cells or whole tissues by a modification of
the guanidine isothiocyanate-phenol-chloroform extraction
method (TRIZOL, Gibco/BRL). For cDNA synthesis, 1-5 μg of
30   total RNA was used in a 20 μl reaction using SUPERSCRIPT
II (Gibco/BRL), a modified Maloney murine leukemia virus
reverse transcriptase (RT), and oligo(dT)$_{12-18}$ primers
according to the Gibco/BRL protocol.
         For PCR amplification of the cDNA, aliquots of cDNA,
35   equivalent to 1/20 of the reverse transcriptase reaction,
were used in a 50 μl reaction volume. PCR amplification
was performed using ELONGASE polymerase (Gibco/BRL).
Primer sequences and cycling temperatures used for PCR

amplification of receptors are shown in Table 8. The reactions were run for 35 cycles, and a 10-minute incubation at 72°C was added at the end to ensure complete extension. The PCR products were purified using the ADVANTAGE PCR-PURE kit (Clontech, Palo Alto, CA) and sequenced to confirm their identities.

Table 8		
Gene	Product Size (bp)	Primers (sense, antisense)
p75	329	SEQ ID NOS:1 and 2
ChAT	377	SEQ ID NOS:3 and 4
Isl-1	350	SEQ ID NOS:5 and 6
GAD_{65}	327	SEQ ID NOS:7 and 8
calbindin28	276	SEQ ID NOS:9 and 10
glutaminase	560	SEQ ID NOS:11 and 12
cyclophilin	302	SEQ ID NOS:13 and 14

As shown in FIG. 2, all of these were present in differentiated cells (labeled "D"). In contrast, none of these markers of neurotransmitter phenotypes could be detected from cells that were examined within 24 hours of isolation (termed "acutely dissociated;" labeled as "AD" in FIG. 2), even though expression of the housekeeping gene, cyclophilin, could be readily detected from both cell populations. These data show that NRP cells mature in culture and that NCAM expression and neuronal fate determination precede neurotransmitter synthesis.

41

The expression of neurotransmitter synthesizing
enzymes was also examined by immunocytochemistry to
determine whether all cells, or only a subset of
differentiated cells, express these markers. Cells were
5   grown in culture for 10 days and allowed to differentiate,
fixed, and processed by immunocytochemistry according to
the procedure of Example 2 to detect expression of choline
acetyltransferase (ChAT), glutamic acid decarboxylase
(GAD), tyrosine hydroxylase (TH), glycine, and glutamate.
10   Antibodies to ChAT, TH, and GAD were obtained from
Chemicon; antibodies to glutamate and glycine were from
Signature Immunologicals. Virtually 100% of the
differentiated cells expressed detectable glutamate
levels. A much smaller percentage expressed glycine and
15   GAD. Exact percentages varied between experiments from
10-50%. The percentage of ChAT and TH$^+$ cells were even
smaller and ranged between 1-5%. However, substantially
larger numbers could be seen by altering culture
conditions. Since virtually 100% of the cells synthesized
20   glutamate, it is likely that at least some cells
synthesized more than one neurotransmitter. Nevertheless,
these results clearly show that upon differentiation, E-
NCAM$^+$ cells are capable of maturing into a heterogeneous
population with respect to their neurotransmitter
25   phenotype.

In contrast to the results obtained with
differentiated cells, neither ChAT, GAD, TH, nor glycine
could be detected in acutely dissociated cells. Glutamate
was detected in a small subset of such cells (less than
30   10%). Glutaminase, however, could not be detected in
these cells by RT-PCR (FIG. 2), which suggests that
glutamate was being taken up by these cells from the
medium.

35

42

Example 14

The Neurotransmitter Receptor Profile of E-NCAM⁺ Cells
Changes with Maturation

5   Another important characteristic of mature neurons is
their ability to respond to multiple neurotransmitters by
expressing appropriate neurotransmitter receptors on their
surfaces. To examine the ability of differentiated E-
NCAM⁺ cells to respond to glutamate, glycine, dopamine,
10   and acetylcholine, fura-2 Ca²⁺ imaging techniques were
used. E13.5 E-NCAM⁺ cells were grown in culture for 10
days and allowed to differentiate. They were then loaded
with fura-2, and the depolarizing response to
neurotransmitter application was monitored.

15   Cells were loaded with 5 μM Fura-2/AM, D. Grynkiewicz
et al., A New Generation of Calcium Indicators with
Greatly Improved Fluorescence Properties, 260 J. Biol.
Chem. 3440-3450 (1985), hereby incorporated by reference,
plus PLURONIC F127 (80 μg/ml) in rat ringers (RR) at 23°C
20   in the dark for 20 minutes followed by 3 washes in RR and
a 30-minute desterification. Relative changes in
intracellular calcium concentration were measured from the
background-corrected ratio of fluorescence intensity by
excitation at 340/380 nm. Response was defined as a
25   minimum rise of 10% of the ratioed baseline value. A
Zeiss-Attofluor imaging system and software (Atto
Instruments Inc., Rockville, MD) were used to acquire and
analyze the data. Data points were sampled at 1 Hz.
Neurotransmitters were made in RR and delivered by bath
30   exchange using a small volume loop injector (200 μl). RR
contained 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10
mM HEPES, and 10 mM glucose. In addition, 500 μM ascorbic
acid was added to dopamine solutions to prevent oxidation.
Control application of 500 μM ascorbic acid had no effect.
35   The pH of all solutions was adjusted to 7.4 with NaOH.
Further, 50 mM K⁺ RR was made by substituting equimolar K⁺
for Na⁺ in the normal RR.

          FIG. 3 shows a bar graph of the number of cells
responding to application of the indicated

43

neurotransmitter on acutely dissociated and differentiated
cells. In general, the number of cells responding to
neurotransmitters and the amplitude of the
neurotransmitter-induced Ca^{2+} responses increased in the

5   differentiated cells. The most striking example was
dopamine, where only 10% of the acutely dissociated cells
responded to 500 μM dopamine with increases in internal
Ca^{2+} compared to 76% of differentiated cells, a net
increase of 66%. Similar, but less striking, changes in

10   the number of cells responding were seen for other
excitatory neurotransmitters. The exceptions to this
trend were the Ca^{2+} responses to GABA and glycine.
Interestingly, 46% of the acutely dissociated cells
responded to GABA compared to only 8% of the

15   differentiated cells. Similarly, Ca^{2+} flux in response to
glycine decreased from 20% in the acutely dissociated
cells to 0% in the differentiated cells. This change in
the inhibitory neurotransmitter profile probably reflects
the decrease in internal chloride ion concentration with

20   maturation that accounts for the shift from depolarizing
to hyperpolarizing GABA and glycine responses. W. Wu et
al., Early Development of Glycine and GABA-Mediated
Synapses in Rat Spinal Cord, 12 J. Neurosci. 3935-3945
(1992). The possibility cannot be excluded, however, that

25   chloride ion levels remain elevated and fewer GABA and
glycine receptors are expressed in the differentiated
cells. Representative plots of the ratio of (I_{340}/I_{380}) Ca^{2+}
responses over time from an acutely dissociated and
differentiated cell are shown in FIG. 4 and FIG. 5,

30   respectively. The acutely dissociated cell responded to
GABA and glutamate, whereas the differentiated cell from
the same embryo responded to dopamine, glutamate, and
acetylcholine, but not to GABA or glycine. Comparison of
Ca^{2+} responses to the various transmitters in adjacent

35   cells revealed that there is heterogeneity in the response
profiles among cells, indicating that not only are the E-
NCAM⁻ cells heterogeneous in their ability to synthesize
neurotransmitters, they are also selected in terms of

44

transmitter receptor expression. In addition to
neurotransmitters, elevated K^+ in rat ringers (50 mM K^+ RR)
was applied to depolarize the cells and allow Ca^{2+} entry
through voltage-gated channels. In acutely dissociated

5   cells, 49% responded to 50 mM K^+ RR compared to 85% of
differentiated cells, suggesting that more of the
differentiated cells were electrically competent than were
the acutely dissociated cells.

        Thus, the contrast between the various properties of

10   acutely dissociated E-NCAM⁺ cells and fully differentiated
E-NCAM⁺ cells, which are summarized in Table 9, is
striking. Immature cells are mitotically active, but
differentiated cells are not. Immature cells do not
express any mature neuronal proteins such as NF-M,

15   synaptophysin, or neurotransmitter synthetic enzymes,
whereas all of these can be detected in differentiated
cells. Moreover, acutely dissociated cells are overall
less responsive than differentiated cells to
neurotransmitter-induced Ca^{2+} responses.

Table 9		
Property	Acutely Dissociated	Differentiated
Mitotic Status	Mitotic	Postmitotic
Cell Size	Comparatively smaller	Comparatively larger
Process Outgrowth	Little or none	Extensive
Neuronal Markers	NCAM, βIII-tubulin, MAP-2 kinase, nestin	NCAM, βIII-tubulin, MAP-2 kinase, NF-M, synaptophysin, peripherin
Neurotransmitters, neurotransmitter synthetic enzymes, or other phenotypic specific markers	None, except for a small amount of glutamate immunoreactivity.	Glutamate, glycine, glutaminase, GAD, ChAT, Isl-1, p75, calbindin
Response to neurotransmitters	Weak, in a small subset of cells.	Robust and in virtually all cells.
Depolarizing response to GABA and glycine	All responses measured were depolarizing.	Few or no depolarizing responses were detected.

Example 15

Individual E-NCAM⁺ Cells Can Generate Multiple
Neurotransmitter Phenotypes

Mass culture experiments described above showed that
the E-NCAM⁺ population can generate multiple
neurotransmitter phenotypes. There existed the
possibility, however, that individual cells could be pre-
committed to generating specific neuronal phenotypes. To
determine whether the differentiation potential of NRPs in
mass culture reflected the potential of an individual NRP,
clonal analysis of E-NCAM⁺ cells was performed. E-NCAM⁺
cells were immunoselected according to the procedure of
Example 3, plated at clonal density, and grown in FGF and
NT-3, conditions that promote proliferation. Clones grew

46

to sizes of several hundred cells after 10 days in
culture, after which their differentiation was promoted by
withdrawal of FGF and addition of RA in the medium.

Three different techniques were used to determine

5 whether clones generated from individual NRP cells were
composed of heterogeneous populations of neurons: RT-PCR
according to the procedure of Example 13,
immunocytochemistry according to the procedure of Example
2, and calcium imaging according to the procedure of

10 Example 14. Six clones were examined by RT-PRC analysis.
Five of the six clones expressed multiple neurotransmitter
phenotypes: one clone expressed all six markers tested, 3
clones expressed four markers, and 1 clone expressed three
markers. Therefore, all but one clone were composed of

15 heterogeneous populations of cells. One clone expressed
detectable levels of only p75 and Isl-1, but not ChAT.
This likely represented an immature clone that had not
fully differentiated. FIG. 6 shows results from a
representative clone that expressed all neurotransmitter

20 markers tested. These results demonstrate that individual
clones express multiple neurotransmitter synthetic enzymes
or other phenotypic markers, and that most clones were
composed of a heterogeneous population.

To confirm the PCR results and to show heterogeneity

25 at the protein level, clones were analyzed for expression
of p75. No clone (0/17) consisted of exclusively p75
immunoreactive cells, but all clones (17/17) contained p75
immunoreactive cells as well as other neurons. Similarly,
staining for either glutamate or glycine immunoreactivity

30 showed that each transmitter was expressed by only a
subset of cells in the same clonal population, indicating
that clones are a heterogeneous population.

Heterogeneity was demonstrated not only by the
synthesis of different neurotransmitters, but also by

35 heterogeneity in the receptors expressed by the cells.
The response profiles of differentiated clonal cells to
application of GABA, glycine, dopamine, glutamate,
acetylcholine, and 50 mM K+ RR, as evidence by increased

47

intracellular calcium concentrations, were examined. Ca^{2+}
measurements were taken from as many as 113 cells from 4
different clones. All clones examined (4/4) displayed
heterogeneity in their response profiles, which varied
5   somewhat between individual clones. FIG. 7 shows a bar
graph of the percentage of cells from all 4 clones that
responded to each of the applied neurotransmitters.

As with the mass cultures of differentiated E-NCAM[+]
cells, high percentages of clonal cells responded to
10   glutamate (93%), acetylcholine (96%), 50 mM K+ RR (70%),
and dopamine (50%), whereas few cells responded to GABA
(27%) and glycine (1%). FIGS. 8 and 9 show representative
traces of the ratio (I_{340}/I_{380}) of Ca^{2+} responses from two
cells recorded from one clone. This heterogeneous
15   expression of receptors also suggested a multipotential
characteristic of individual NRP cells. Thus, the
maturation of clonal populations of cells closely
resembled the maturation of cells in mass culture.

By multiple independent methods, this clonal analysis
20   demonstrates the multipotential characteristic of
individual NRP cells. This analysis confirms the mass
culture results that clearly define the developmental
potential of the NRP cell. Although committed to
generating neurons, the particular phenotypes of its
25   progeny are dictated at some later stage in their
development. Thus, the existence has been established of
a neuronal precursor cell that can be purified and
subsequently manipulated to define the transition between
lineage restricted neuronal precursor and differentiated
30   neuronal progeny.

Example 16
Extracellular Signals Influence the Fate of NRP Cells
The results disclosed herein show that neuronal
35   precursors can develop *in vitro* into mature neurons of
multiple phenotypes in both mass and clonal cultures and
that either application of RA or removal of FGF can
promote differentiation into multiple phenotypes. In

48

normal development, however, differentiation is spatially
and temporally regulated, with motoneurons being generated
ventrally and sensory neurons being generated dorsally,
suggesting that specific environmental signals may bias

5   differentiation of neuronal precursors. In this example,
the effects of two potentially regulatory molecules that
are expressed in the spinal cord at the time of
neurogenesis and have been shown to bias cells to either
dorsal (BMP-2/4; J.M. Graff, Embryonic Patterning: To BMP

10   or Not to BMP, That is the Question, 89 Cell 171-174
(1997)) or ventral (Shh; M.J. Fietz et al., The Hedgehog
Gene Family in Drosophila and Vertebrate Development,
Development (Suppl.) 43-51 (1994)) phenotypes.

When BMP-2 was added to cultures of E-NCAM$^+$ cells, a

15   dramatic reduction in cell division was seen. The effect
of BMP-2 overrode the effect of the mitogen, FGF, and even
in the presence of FGF, caused a 60% reduction in cell
division (FIG. 10). Identical effects were seen with BMP-
4. BMP-2 was not a survival factor, since cells grown in

20   BMP-2 alone did not survive. The decrease in mitosis was
accompanied by the appearance of differentiated cells.
Cell size increased and cells put out extensive processes.
Cells grown in BMP-2 for 48 hours were also examined for
neurotransmitter expression. Glutamatergic, GABAergic,

25   dopaminergic, and cholinergic neurons were detected. The
number of cholinergic neurons was signficantly larger than
in untreated controls (5-10% v. 0-1%), however, there
appeared to be no bias towards ventral phenotypes since
the promotion of all other phenotypes was also

30   significantly larger. Thus, BMP-2 acted as an antimitotic
agent and promoted differentiation of E-NCAM$^+$ NRP cells,
but did not appear to inhibit ventral fates.

In contrast to the antimitotic and differentiation
promoting effect of BMP, Shh appeared to be a mitogen.

35   The mitotic effect of Shh at 100 ng/ml (the maximal
response) was three-fold higher than controls, but was
less than the effect of FGF at 10 ng/ml (FIG. 11).
Experiments with Shh were done in the presence of NT-3,

49

which acts as a survival agent, Y.A. Barde, Neurotrophins:
A Family of Proteins Supporting the Survival of Neurons,
390 Prog. Clin. Biol. Res. 45-56 (1994), and not a
mitogen, because Shh itself did not appear to be a
5   survival factor for E-NCAM⁺ cells, i.e. E-NCAM⁻ cells grown
in Shh alone did not survive. The effect of Shh on
mitosis was only apparent after two days of exposure and
was maintained over 5 days of the assay. No difference in
cell division was seen during the first 24 hours.
10   Shh did not appear to promote motoneuron
differentiation over the 5 days of the assay. Cells
continued to proliferate and no p75 or ChAT immunoreactive
neurons could be detected. The failure to see cholinergic
neurons was not due to an inability of the E-NCAM⁺ cells
15   to differentiate into p75 or ChAT positive cells, as
sister cultures readily differentiated into ChAT and p75
immunoreactive cells when treated with a differentiation
agent such as BMP-2 or RA. Thus, E-NCAM⁺ cells respond to
Shh by proliferating. Shh unexpectedly did not promote
20   motoneuron differentiation, at least over the time period
tested.
            These results indicate that extracellular signaling
molecules Shh and BMP modulate the phenotypic
differentiation of E-NCAM⁺ cells. BMP-2 inhibits cell
25   proliferation and promotes differentiation and does not
inhibit the differentiation of ventral phenotypes. In
contrast, Shh promotes proliferation and inhibits the
differentiation of any neuronal phenotypes, including p75
and ChAT immunoreactive neurons.
30

Example 17
Mouse Neural Tubes Contain E-NCAM Immunoreactive Neural
Precursors

35   To determine whether NRPs are present in mouse neural
tubes, E11 mouse spinal cords were dissociated and
examined for properties of E-NCAM immunoreactive cells,
according to the procedures of Example 2.

50

A large number of E-NCAM immunoreactive cells were
found at E11, and these cells comprised about 60% of the
total population of cells. E-NCAM-positive cells appeared
morphologically similar to neurons with extensive
5   processes. At this stage of development, no co-expression
of E-NCAM with either Gal-C or GFAP was observed in
double-labeling experiments, suggesting that E-NCAM
immunoreactivity may identify neuronal precursors.

To determine if mouse E-NCAM-positive cells, like
10   their rat counterparts, underwent cell division, cells
were pulse labeled with BRDU and then double-labeled to
detect cells that co-expressed BRDU and E-NCAM
immunoreactivity. Results showed that E-NCAM-positive
cells divided for at least three days in culture. E-NCAM
15   -positive cells, thus, appeared similar to the NRPs
previously described in rats. To confirm that E-NCAM-
positive cells could generate multiple neuronal
phenotypes, immunoselected E-NCAM cells prepared according
to the procedure of Example 3 were allowed to
20   differentiate in culture for 10 days. Plates were then
harvested, and cDNA was prepared according to the
procedure of Example 13 to assess neurotransmitter
synthesis. As can be seen in FIG. 12, expression of p75,
islet-1, ChAT, calbindin, GAD, and glutaminase were
25   readily detected in differentiated populations. Thus,
mouse E-NCAM immunoreactive cells can generate neurons
that express cholinergic, excitatory, and inhibitory
phenotypes.

30   Example 18
E-NCAM Immunoreactive Neuroblasts Can Be Generated from ES
Cells

In Example 17 it was shown that mouse spinal cords
35   contain E-NCAM immunoreactive NRPs that are similar to rat
NRP cells. To determine if similar lineage restricted
precursors could be generated from ES cells, mouse ES
cells were obtained from the Developmental Studies
Hybridoma Bank (DSHB; University of Iowa, Iowa City, Iowa)

51

and were then grown in culture and examined for the
expression of E-NCAM, A2B5, and other neuroglial markers.
As has been previously described, undifferentiated ES
cells did not express detectable immunoreactivity for any
5   of the markers tested. In contrast, when ES cells were
plated in neural differentiation conditions ES cells
altered their morphology and began to express multiple
neuronal and glial markers (FIG. 13). Differentiated ES
cells were harvested and total RNA prepared for RT-PCR
10   according to the procedure of Example 13. Of particular
importance is the early expression of E-NCAM (early
neuronal marker) and PLP/DM20 genes (known to be expressed
by embryonic glial precursors). Consistent with the
detection of early neuronal and glial markers by PCR, high
15   polysialiated NCAM expressing cells represented a small
percentage of the total cells. Less than 5% of cells in
culture expressed E-NCAM immunoreactivity after 5 days in
culture. The percentage of A2B5 immunoreactive cells was
significantly higher; about 10% of differentiated cells
20   expressed this marker.

    To determine if E-NCAM immunoreactive cells
represented neuronal precursors, the co-expression of
neuronal and glial markers was examined. E-NCAM
immunoreactive cells co-expressed MAP-2 and β-III tubulin
25   immunoreactivity, but did not co-express GFAP and nestin
immunoreactivity. E-NCAM-positive cells did not express
Gal-C or other oligodendrocytic markers. Thus, E-NCAM
immunoreactive cells that were derived from mouse ES cells
appeared similar to spinal-cord-derived E-NCAM-positive
30   NRPs.

    To confirm that ES-cell-derived neuronal precursors
could generate multiple kinds of neurons, E-NCAM
immunoreactive cells were immunoselected according to the
procedure of Example 3 and such purified cells were
35   allowed to differentiate for 10 days. Cells were then
harvested and analyzed by immunocytochemistry and RT-PCR
for the expression of phenotypic markers. FIG. 14 shows
the results of an illustrative PCR experiment wherein

52

ChAT, p75, islet-1, calbindin, GAD, and glutaminase
expression was readily detected in differentiated
populations. Thus, ES-cell-derived E-NCAM immunoreactive
cells differentiated into postmitotic neurons that
5   expressed multiple neurotransmitters, including
cholinergic, excitatory, and inhibitory phenotypes.
Therefore, ES cells can be used as a source of lineage
restricted NRPs.

10   Example 19
NRPs in Human Neural Tubes
            To determine if NRPs are present in human neural
tubes, human embryonic spinal cords were dissociated and
the phenotypes of cells when grown in DMEM/F12 in a high
15   concentration of FGF were examined according to the
procedure of Example 2.
            Human spinal cord cells (HSCs) initially appeared
morphologically similar to rat and mouse spinal cords, but
rapidly differentiated into fibroblastic appearing cells
20   with a significant proportion of cells having a neuronal
morphology. HSCs continued to divide rapidly and most
cells (95%) were nestin immunoreactive. At this stage,
cultures did not contain astrocytes, oligodendrocytes, or
their precursors as detected by the expression of GFAP or
25   O4/Gal-C immunoreactive cells. A substantial number of E-
NCAM immunoreactive cells were present, however, and
constituted about 40% of the total population. E-NCAM
immunoreactive cells appeared morphologically similar to
neurons, although some flat E-NCAM immunoreactive cells
30   were also present. Both populations of E-NCAM-positive
cells were MAP2K immunoreactive and also expressed a
variety of other early neuronal markers, as summarized in
Table 10.

Table 10	
Antigen	% E-NCAM⁺ Cells
Nestin	100
MAP2	100
Neurofilament H	80
Neurofilament-M	Occasional Cell
β-III tubulin	100
Gal-C/O4	0
GFAP	0

At this stage of development, no co-expression of E-
NCAM with either Gal-C or GFAP was observed in double-
labeling experiments, suggesting that E-NCAM
immunoreactivity identifies neuronal precursors. That is,
E-NCAM immunoreactive human spinal cord cells expressed
neuronal but not non-neuronal antigens. To determine if
human E-NCAM⁺ cells, like their rat counterparts, underwent
cell division, mixed cultures of HSCs were pulse labeled
with BRDU and then double labeled to detect cells that co-
expressed BRDU and E-NCAM immunoreactivity. The results of
this experiment showed that E-NCAM-positive cells divided
for at least three days in culture. Consistent with the
results of Table 10 that E-NCAM immunoreactive cells also
express NF-H, BRDU-incorporating cells also co-expressed
neurofilament-H. Thus, as in fetal rodent spinal cord
cultures, dividing nestin immunoreactive precursor cells
from humans are present and E-NCAM immunoreactive cells
represent a significant fraction of total precursor
population at this age. E-NCAM⁺ cells appear similar to the
NRPs previously described for rats and mice.

Transplanted cells can be administered to any animal,
including humans, with abnormal neurological or
neurodegenerative symptoms obtained in any manner,

54

including as a result of chemical electrolytic lesions,
experimental destruction of neural areas, or aging
processes. Transplantation can be bilateral, or, for
example in patients suffering from Parkinson's Disease,
5   can be contralateral to the most-affected side. Surgery
is preferably performed such that particular brain regions
are located, such as in relation to skull sutures, and
surgery performed with stereotactic techniques.
Alternatively, cells can be implanted in the absence of
10   stereotactic surgery. Cells can be delivered to any
affected neural areas using any method of cell injection
or transplantation known in the art.

In another embodiment of the invention, NRP cells are
transplanted into a host, and induced to proliferate
15   and/or differentiate in that host by (1) proliferation
and/or differentiation *in vitro* prior to being
administered, or (2) differentiation *in vitro* prior to
being administered and proliferation and differentiation
in vivo after being administered, or (3) proliferation *in*
20   *vitro* prior to being administered and then differentiation
in vivo without further proliferation after being
administered, or (4) proliferation and differentiation *in*
vivo after being injected directly after being freshly
isolated.
25   NRP cells can also be used for delivery of
therapeutic or other compounds. Methods for bypassing the
blood-brain barrier for purposes of delivery of
therapeutic compounds include implanting cells in an
encapsulation device according to methods known in the art
30   or directly implanting genetically engineered cells such
that the cells themselves produce the therapeutic
compound. Such compounds may be small molecules,
peptides, proteins, or viral particles. Cells can be
genetically transduced by any means known in the art,
35   including calcium phosphate transfection, DEAE-dextran
transfection, polybrene transfection, electroporation,
lipofection, infection of viruses, and the like. Cells
are first genetically manipulated to express a therapeutic

substance and then transplanted either as free cells able
to diffuse and incorporate within the CNS parenchyma or
are contained within an encapsulation device. R.P. Lanza
& W.L. Chick, Encapsulated Cell Therapy, Sci. Amer.: Sci.
5   & Med., July/Aug, 16-25 (1995); P.M. Galletti,
Bioartificial Organs, 16 Artificial Organs 55-60 (1992);
A.S. Hoffman, Molecular Engineering of Biomaterials in the
1990s and Beyond: A Growing Liaison of Polymers with
Molecular Biology, 16 Artificial Organs 43-49 (1992); B.D.
10   Ratner, New Ideas in Biomaterials Science - A Path to
Engineered Biomaterials, 27 J. Biomed. Mat. Res. 837-850
(1993); M.J. Lysaght et al., Recent Progress in
Immunoisolated Cell Therapy, 56 J. Cell Biochem. 196-203
(1994), hereby incorporated by reference.
15   Transplanted cells can be identified by prior
incorporation of tracer dyes such as rhodamine or
fluorescein-labeled microspheres, fast blue, bis-
benzamide, or genetic markers incorporated by any genetic
transduction procedure known in the art to allow
20   expression of such enzymatic markers as β-galactosidase or
alkaline phosphatase.
          Any expression system known in the art can be used to
express the therapeutic compound, so long as it has a
promoter that is active in the cell, and appropriate
25   internal signals for initiation, termination, and
polyadenylation. Examples of suitable expression vectors
include recombinant vaccinia virus vectors including
pSC11, or vectors derived from viruses such as simian
virus 40 (SV40), Rous Sarcoma Virus (RSV), mouse mammary
30   tumor virus (MMTV), adenovirus, herpes simplex virus
(HSV), bovine papilloma virus, Epstein-Barr virus,
lentiviruses, or any other eukaryotic expression vector
known in the art. Many of such expression vectors are
commercially available.
35   Cells can also be transduced to express any gene
coding for a neurotransmitter, neuropeptide,
neurotransmitter-synthesizing enzyme or neuropeptide

56

synthesizing enzyme for which expression in the host is
desired.

NRP cells and/or their derivatives cultured *in vitro*
can be used for the screening of potentially

5   neurologically therapeutic compositions. These
compositions can be applied to cells in culture at varying
dosages, and the response of the cells monitored for
various time periods. The induction of expression of new
or increased levels of proteins such as enzymes,

10   receptors, and other cell surface molecules, or of
neurotransmitters, amino acids, neuropeptides, and
biogenic amines can be analyzed with any technique known
in the art that can identify the alteration of the level
of such molecules, including protein assays, enzymatic

15   assays, receptor binding assays, enzyme-linked
immunosorbent assays, electrophoretic analysis, analysis
with high performance liquid chromatography, Western
blots, and radioimmune assays. Nucleic acid analysis,
such as Northern blots, can be used to examine the levels

20   of mRNA coding for these molecules, or for the enzymes
that synthesize these molecules. Alternatively, cells
treated with these pharmaceutical compositions can be
transplanted into an animal and their survival, ability to
form neurons, and to express any of the functions of these

25   cell types can be analyzed by any procedure available in
the art.

NRP cells can be cryopreserved by any method known in
the art.

30   Example 20
Use of NRP Cells and/or Their Derivatives for Treatment of
Abnormal Neurological or Neurodegenerative Symptoms

NRP cells are isolated by the methods of Examples 2,
35   3, 8, 18, or 19. Cells are obtained from human embryonic
or adult CNS or from xenographic sources from which
immunorejection of cells is not a clinical problem, such
as pigs genetically engineered so as not to present a
foreign stimulus to the human immune system. Cells

collected from embryos are obtained by dissection of CNS tissue following routine abortion procedures and tissue collection in a sterile collection apparatus. Cells from the postnatal CNS are obtained by digestion of tissue

5   following routine autopsy. Tissue is prepared, cells are immunopurified, and the resulting purified cells are cultured as in Example 2.

Cells can be transplanted directly or can first be expanded *in vitro* prior to transplantation. Populations

10   expanded *in vitro* can further be expanded in conditions that enhance the generation of neurons or cells committed to the generation of neurons.

Transplantation is routinely carried out at cell suspensions of 5-50,000 cells/μl in physiological salt

15   solutions, such as PBS. Cells can be transplanted into or near any CNS regions affected by the disease or condition. Transplantation procedures, with appropriate modifications for use in human patients, are in their essence similar to procedures well known to those skilled in the art of

20   transplantation of O-2A progenitor cells, e.g., A.K. Groves et al., Repair of Demyelinated Lesions by Transplantation of Purified O-2A Progenitor Cells, 362 Nature 453-455 (1993), hereby incorporated by reference.

More specifically, transplantation is performed using

25   a computed tomographic stereotaxic guide. The patient is operated on using any of the procedures known in the art. In cases where precisely localized transplantation is desirable, the patient undergoes CT scanning to establish the coordinates of the region to receive the transplant.

30   The injection cannula can be in any configuration used by those skilled in the relevant arts. The cannula is then inserted into the brain to the correct coordinates, then removed and replaced with a 19-gauge infusion cannula that has been preloaded with cell suspension in a small

35   selected volume. The cells are then slowly infused, at rates generally of 1-10 ml per minute as the cannula is withdrawn. For some diseases in which it is desirable to spread cells over the largest possible area, multiple

58

stereotactic needle passes may be made throughout the
area. Patients are examined post-operatively for
hemorrhage or edema. Neurological evaluations are
performed at various post-operative intervals, as well as
5   PET scans if these can be used to determine the metabolic
activity of the implanted cells. These and similar
procedures can be used for any implantation of NRP cells
for any of the purposes indicated in this invention.
        Success of the procedure is determined by non-
10   invasive analysis with, for example, nuclear magnetic
resonance image scanners, and/or by analysis of functional
recovery according to methods well known in the art.

                            Example 21
15   Use of Genetically Engineered NRP Cells and/or Their
        Derivatives for Transplantation

            In this example, NRP cells are genetically modified
        ex vivo before introduction into or near regions of
20   disease to express gene products that will make the
        transplanted cells resistant to destruction in vivo and/or
        to express gene products that provide trophic support to
        the host cells and/or to express gene products that limit
        destructive processes occurring in the host. Genetic
25   modification is carried out by any of the techniques known
        to those skilled in the art, including but not limited to
        calcium phosphate transfection, DEAE-dextran transfection,
        polybrene transfection, electroporation, lipofection,
        infection of viruses, and the like. Gene products that
30   would make cells resistant to destruction in vivo and/or
        to express gene products that provide trophic support to
        host cells and/or to express gene products that limit
        destructive processes occurring in the host include but
        are not limited to insulin-like growth factor-I, decay
35   accelerating factor, catalase, superoxide dismutase,
        members of the neurotrophin family, glial-derived
        neurotrophic factor, ciliary neurotrophic factor, leukemia
        inhibitory factor, fas ligand, cytokines that inhibit
        inflammatory processes, receptor fragments that inhibit

inflammatory processes, antibodies that inhibit
inflammatory processes, and so forth.

Example 22

5   Use of NRP Cells and/or Their Derivatives for the
     Screening of Potentially Neurologically Therapeutic
     Compositions

          · NRP cells or derivatives thereof or mixtures thereof
10   cultured *in vitro* can be exposed to compositions of
     interest at varying dosages, and the response of the cells
     monitored for various time periods. The induction of
     expression of new or increased levels of proteins such as
     enzymes, receptors, and other cell surface molecules or of
15   neurotransmitters, amino acids, neuropeptides, and
     biogenic amines can be analyzed with any technique known
     in the art that can identify the alteration of the level
     of such molecules, including protein assays, enzymatic
     assays, receptor binding assays, enzyme-linked
20   immunosorbent assays, electrophoretic analysis, analysis
     with high performance liquid chromatography, Western
     blots, and radioimmune assays. Nucleic acid analysis,
     such as Northern hybridization can be used to examine the
     levels of mRNA coding for these molecules, or for the
25   enzymes that synthesize these molecules. Cells can also
     be used to screen for compounds able to promote the
     division of NRP cells and/or their derivatives by
     determining the ability of compounds to cause increases in
     NRP cell number or to promote DNA synthesis, as measured
30   by, e.g. incorporation of bromodeoxyuridine or tritiated
     thymidine. Cells can also be used to screen for compounds
     that promote survival of NRP cells and/or their
     derivatives by applying compounds to cells in conditions
     where they would be expected to die (e.g., exposure to
35   neurotoxic agents, withdrawal of all trophic factors) and
     examining cell survival using any of the techniques well
     known to practitioners of the art. Cells can also be used
     to screen for compounds that specifically inhibit binding
     to particular receptors, by looking at the ability of said

60

blocking compounds to block the response elicited by
binding of agonist to said receptors. Cells can also be
used to screen for compounds able to activate particular
receptors using ligand binding assays well known to
5   practitioners of the art, or by looking at such
physiological alterations as are associated with
activation of the receptor, such as fluxes in calcium
levels, or other alterations well known to practitioners
of the art. Alternatively, cells treated with these
10   pharmaceutical compositions can be transplanted into an
animal and their survival, ability to form neurons and to
express any of the functions of these cells types can be
analyzed by any procedures available in the art.

15   Example 23
        In this example, cells were harvested from E13.5 rat
spinal cords, and E-NCAM immunoreactive neuronal
restricted precursor cells were isolated by immunopanning
according to the procedure of Example 3. These cells were
20   then labeled with a cell tracker and were transplanted to
different cortical regions using a glass microelectrode.
Animals were sacrificed after 3.5, 10 or 21 days, and the
brain was sectioned according to methods well known in the
art. Such transplanted cells were shown to survive and
25   differentiate at all three times.

                         Example 24
        In this example, cells were harvested, isolated, and
plated in a 35 mm dish as described in Example 3. Cells
30   were then incubated with a retroviral construct containing
a green fluorescent protein (GFP) reporter gene under a
cytomegalovirus (CMV) promoter. Cells were allowed to
recover for 8 hours and then were analyzed for GFP
expression. GFP expression was detected as early as 24
35   hours after infection, and GFP expression persisted for up
to two weeks, at which time the experiment was concluded.
These results show that ectopic genes can be expressed in
NRPs under a heterologous promoter, and that infected

cells continue to stably express the ectopic protein for several weeks.

SEQUENCE LISTING

<110>   Rao, Mahendra S.
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<120>   Lineage-Restricted Neuronal Precursors

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   A method of isolating a pure population of mammalian CNS
neuron-restricted precursor cells comprising the steps of:

5   (a)   isolating a population of mammalian multipotent CNS stem cells
                capable of generating both neurons and glia;

        (b)   incubating the multipotent CNS stem cells in a medium
                configured for inducing said cells to begin differentiating;

        (c)   removing A2B5+ cells from the differentiating cells via specific
10   antibody capture with an antibody that specifically recognises
                A2B5;

        (d)   purifying from the differentiating cells a subpopulation of cells
                expressing embryonic neural cell adhesion molecule; and

        (e)   incubating the purified subpopulation of cells in a medium
15   configured for supporting adherent growth thereof,

        wherein the cells are derived from i) spinal cords of approximately 13.5
day embryos of rats or equivalent embryonic stages of other mammals, or ii)
cells equivalent to i) developed by proliferation and differentiation from early
embryonic spinal cord tissue or embryonic stem cells, and wherein the
20 isolated cells are capable of self-renewal in the presence of FGF.

2.   The method of claim 1, wherein the medium configured for
inducing said cells to begin differentiation is NEP medium.

25   3.   The method of claim 2 or claim 3, wherein the method further
comprises replating the multipotent CNS stem cells on laminin in NEP
medium in the absence of chick embryo extract to induce cell differentiation.

4.   The method according to any one of claims 1 to 3, wherein said
30 purifying comprises a procedure selected from the group consisting of
specific antibody capture, fluorescence activated cell sorting, and magnetic
bead capture.

5.   The method of claim 4, wherein said procedure uses an
35 embryonic neural cell adhesion molecule antibody that specifically
recognises polysialated neural cell adhesion molecule.

6.   The method according to any one of claims 1 to 5, wherein said isolating a population of CNS neuroepithelial stem cells comprises:

(a)   removing a CNS tissue from a mammalian embryo at a stage of embryonic development after closure of the neural tube but prior to differentiation of cells in the neural tube;

(b)   dissociating cells comprising the neural tube removed from the mammalian embryo;

(c)   plating the dissociated cells in feeder-cell-independent culture on a substratum and in a medium configured for supporting adherent growth of the neuroepithelial stem cells comprising effective amounts of fibroblast growth factor and chick embryo extract; and

(d)   incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuroepithelial stem cells.

7.   The method of claim 6, wherein said mammalian embryo is selected from the group consisting of human and non-human primates, equines, canines, felines, bovines, porcines, ovines, lagomorphs, and the order Rodentia.

8.   The method of claim 6 or claim 7, wherein said substratum is selected from the group consisting of fibronectin, vitronectin, laminin, and RGD peptides.

9.   The method according to any one of claims 1 to 8, wherein said medium comprises effective amounts of fibroblast growth factor and neurotrophin 3.

10.   The method according to any one of claims 1 to 9, wherein the isolated cells differentiate into CNS neuronal cells upon replacement of adherent growth supporting medium with retinoic acid containing medium.

11.   The method according to any one of claims 1 to 10, wherein the isolated cells fail to proliferate or differentiate in astrocyte promoting medium containing FGF and 10% fetal calf serum.

12. A method of isolating a pure population of mammalian CNS neuron-restricted precursor cells comprising the steps of:

(a) removing a sample of spinal cord of approximately 13.5 day embryos of rats or equivalent embryonic stages of other mammals, or ii) cells equivalent to i) developed by proliferation and differentiation from early embryonic spinal cord tissue;

5

(b) dissociating cells comprising the sample of CNS tissue removed from the mammalian embryo;

(c) removing A2B5+ cells from the differentiating cells via specific antibody capture with an antibody that specifically recognises A2B5;

10

(d) purifying from the dissociated cells a subpopulation expressing embryonic neural cell adhesion molecule;

(e) plating the purified subpopulation of cells in feeder-cell-independent culture on a substratum and in a medium configured for supporting adherent growth of the neuron-restricted precursor cells; and

15

(f) incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuron-restricted precursor cells,

20 wherein the isolated cells are capable of self renewal in the presence of FGF.

13. The method according to claim 12, wherein the isolated cells differentiate into CNS neuronal cells upon replacement of adherent growth supporting medium with retinoic acid containing medium.

25

14. The method according to claim 12 or claim 13, wherein the isolated cells fail to proliferate or differentiate in astrocyte promoting medium containing FGF and 10% fetal calf serum.

30 15. The method according to any one of claims 12 to 14, wherein said purifying comprises a procedure selected from the group consisting of specific antibody capture, fluorescence activated cells sorting, and magnetic bead capture.

16. The method of claim 15, wherein said procedure uses an embryonic neural cell adhesion molecule antibody that specifically recognises polysialated neural cell adhesion molecule.

5   17. The method according to any one of claims 12 to 16, wherein said mammalian embryo is selected from the group consisting of human and non-human primates, equines, canines, felines, bovines, porcines, ovines, lagomorphs, and the order Rodentia.

10   18. A method of isolating a pure population of mammalian CNS neuron-restricted precursor cells comprising the steps of:

    (a) providing a sample of mammalian embryonic stem cells;

    (b) removing A2B5+ cells from the differentiating cells via specific antibody capture with an antibody that specifically recognises

15   A2B5;

    (c) purifying from the mammalian embryonic stem cells a subpopulation expressing embryonic neural cell adhesion molecule;

    (d) plating the purified subpopulation of cells in feeder-cell-

20   independent culture on a substratum and in a medium configured for supporting adherent growth of the neuron-restricted precursor cells; and

    (e) incubating the plated cells at a temperature and in an atmosphere conducive to growth of the neuron-restricted precursor cells,

25 wherein the isolated cells are capable of self renewal in the presence of FGF.

19. The method of claim 18, wherein said purifying comprises a procedure selected from the group consisting of specific antibody capture, fluorescence activated cell sorting, and magnetic bead capture.

30

20. The method of claim 19, wherein said procedure uses an embryonic neural cell adhesion molecule antibody that specifically recognises polysialated neural cell adhesion molecule.

35   21. A pure population of mammalian CNS neuron-restricted precursor cells isolated by the method according to any one of claims 1 to 20.

22. A method of obtaining postmitotic neurons comprising:

(a) providing neuron-restricted precursor cells according to claim 21
and culturing the neuron-restricted precursor cells in proliferating
conditions; and

5   (b) changing the culture conditions of the neuron-restricted precursor
cells from proliferating conditions to differentiating conditions,
thereby causing the neuron-restricted precursor cells to
differentiate into postmitotic neurons.

10   23. The method of claim 22, wherein said changing the culture
conditions comprises adding retinoic acid to basal medium.

24. The method of claim 22, wherein said changing the culture
conditions comprises withdrawing a mitotic factor from basal medium.
15

25. The method of claim 24, wherein said mitotic factor is fibroblast
growth factor.

26. The method of claim 22, wherein said changing the culture
20 conditions comprises adding a neuronal maturation factor to basal medium.

27. The method of claim 26, wherein said neuronal maturation factor
is a member selected from the group consisting of sonic hedgehog, BMP-2,
BMP-4, NT-3, NT-4, CNTF, LIF, retinoic acid, brain-derived neurotrophic
25 factor (BDNF), and combinations of any of the above.

28. An isolated cellular composition comprising the mammalian CNS
neuron-restricted cells according to claim 21.

30   29. A pharmaceutical composition comprising a therapeutically
effective amount of the composition of claim 28 and a pharmaceutically
acceptable carrier.

30. A method for treating a neuronal disorder in a mammal
35 comprising administering to said mammal a therapeutically effective amount
of the composition of claim 28.

31. A method for treating a neuronal disorder in a mammal comprising administering to said mammal a therapeutically effective amount of the pharmaceutical composition of claim 29.

5   32. The method of claim 31, wherein said composition is administered by a route selected from the group consisting of intramuscular administration, intrathecal administration, intraperitoneal administration, intravenous administration, and combinations of any of the above.

10   33. The method of claim 31 or claim 32, wherein said method also includes the administration of a member selected from the group consisting of differentiation factors, growth factors, cell maturation factors and combinations of any of the above.

15   34. The method of claim 33, wherein said differentiation factors are selected from the group consisting of retinoic acid, BMP-2, BMP-4, and combinations of any of the above.

35. The composition of claim 28 for use as a delivery vehicle for the
20   delivery to glial cells of an agent selected from the group consisting of cell growth factors, cell maturation factors, cell differentiation agents, and any combination of the above.

36. The composition of claim 28 for use as a delivery vehicle for the
25   delivery of trophic factors to neurons.

37. A method for treating neurodegenerative symptoms in a mammal comprising the steps of:
    (a) providing a pure population of neuronal restricted precursor cells
30   according to claim 21;
    (b) genetically transforming said neuronal restricted precursor cells with a gene encoding a growth factor, neurotransmitter, neurotransmitter synthesizing enzyme, neuropeptide, neuropeptide synthesizing enzyme, or substance that provides
35   protection against free-radical mediated damage thereby resulting in a transformed population of glial restricted precursor cells that

express said growth factor, neurotransmitter, neurotransmitter synthesizing enzyme, neuropeptide, neuropeptide synthesizing enzyme, or substance that provides protection against free-radical mediated damage; and

5   (c) administering an effective amount of said transformed population of neuronal restricted precursor cells to said mammal.

38. A method for screening compounds for neurological activity comprising the steps of:

10   (a) providing a pure population of neuronal restricted precursor cells according to claim 21 or derivatives thereof or mixtures thereof cultured *in vitro*;

      (b) exposing said cells or derivatives thereof or mixtures thereof to a selected compound at varying dosages; and

15   (c) monitoring the reaction of said cells or derivatives thereof or mixtures thereof to said selected compound for selected time periods.

39. A method for treating a neurological or neurodegenerative disease
20 comprising administering to a mammal in need of such treatment an effective amount of neuronal restricted precursor cells according to claim 21 or derivatives thereof or mixtures thereof.

40. The method of claim 39, wherein said neuronal restricted
25 precursor cells or derivatives thereof or mixtures thereof are caused to proliferate and differentiate *in vitro* prior to being administered.

41. The method of claim 39, wherein said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are caused to
30 proliferate *in vitro* prior to being administered, and then are caused to further proliferate and differentiate *in vivo* after being administered.

42. The method of claim 39, wherein said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are caused to
35 proliferate *in vitro* prior to being administered, and then are caused to differentiate *in vivo* after being administered.

43.   The method according to any one of claims 39 to 42, wherein said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are from a heterologous donor.

5   44.   The method of claim 43, wherein said donor is a fetus.

45.   The method of claim 43, wherein said donor is a juvenile.

46.   The method of claim 43, wherein said donor is an adult.

10

47.   The method according to any one of claims 39 to 42, wherein said neuronal restricted precursor cells or derivatives thereof or mixtures thereof are from an autologous donor.

15   48.   The method of claim 47, wherein said donor is a fetus.

49.   The method of claim 47, wherein said donor is a juvenile.

50.   The method of claim 47, wherein said donor is an adult.

20

51.   The method according to any one of claims 39 to 42, wherein said derivatives thereof are obtained by differentiation of neuronal restricted precursor cells *in vitro*.

25   52.   The method according to any one of claims 39 to 42, wherein said derivatives thereof are obtained by genetic transduction of neuronal restricted precursor cells.

Dated this thirtieth day of October 2002

The University of Utah Research
Foundation
Patent Attorneys for the Applicant:

F B RICE & CO
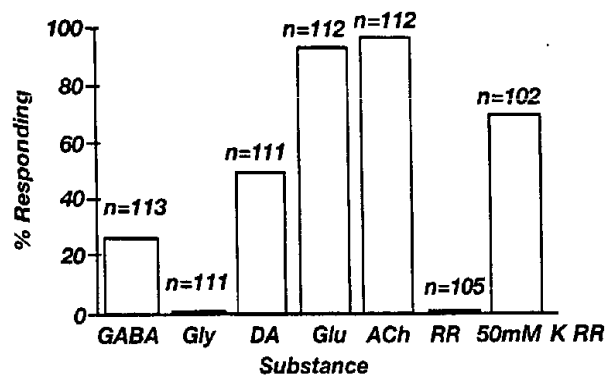
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Fig. 1

Fig. 2

3/7

□ Acutely Dissociated
▨ Differentiated

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

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