Title: DOPAMINE AND AGONISTS AND ANTAGONISTS THEREOF FOR TREATMENT OF NEURODEGENERATIVE DISEASES

Abstract: An agent selected from: (i) dopamine; (ii) a dopamine precursor; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist); (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist); (v) a combination of (i) and (ii); or (vi) a combination of (i), (ii) or (iii) with (iv), down-regulates the suppressive activity of CD4+CD25+ regulatory T cells (Treg) on CD4+CD25- effector T cells (Teff) and is useful in methods and compositions for treating a neurodegenerative condition, disorder or disease other than Parkinson’s disease.
DOPAMINE AND AGONISTS AND ANTAGONISTS THEREOF FOR TREATMENT OF NEURODEGENERATIVE DISEASES

FIELD OF THE INVENTION

The present invention relates to methods and compositions for treatment of neurodegenerative diseases, disorders and conditions. The body protects itself against neurodegeneration by anti-self immunity, namely, a T-cell response against self-antigens residing in sites of central nervous system (CNS) damage is needed to fight off neuronal degeneration. The ability to evoke this response is normally suppressed by naturally occurring CD4⁺CD25⁺ regulatory T cells (Treg). No physiological compound that controls Treg activity has yet been identified. Here we show that the physiological stress-related compound dopamine, acting via type-1 dopamine receptors (found here to be preferentially expressed by Treg), reduces the suppressive activity of Treg on effector T cells (Teff), indicating that dopamine delivers a physiological signal to the immune system, triggering (via its effect on the regulatory T cells) the autoimmunity required for tissue repair.

Abbreviations: APC: antigen-presenting cells; BSA: bovine serum albumin; CNS: central nervous system; CSPG: chondroitin sulfate proteoglycans; CTLA-4: cytotoxic T lymphocyte-associated antigen receptor 4; DA: dopamine; D-R: a dopamine receptor; D1-R: dopamine receptor type 1; D2-R: dopamine receptor type 2; ERK: extracellular signal-regulated kinase; FITC: Fluorescein isothiocyanate; IL: interleukin; MDC: macrophage-derived chemokine; PBS: phosphate-buffered saline; PE: phycoerythrin; SDF-1: stromal-derived factor-1; Teff: effector T-cells; TGF-β: transforming growth factor-β; Treg: regulatory T-cells.
BACKGROUND OF THE INVENTION

It is becoming increasingly clear that the body, to protect itself against tumor growth or CNS neurodegeneration, needs to elicit an autoimmune response against self-antigens associated with tumors (Dummer et al., 2002) or against self-antigens residing in the site of neurodegeneration (WO 99/60021; Moalem et al., 1999; Mizrahi et al., 2002; Schori et al., 2001a, 2001b; Kipnis et al., 2002b), respectively.

Injury to the CNS triggers the immediate death of injured neurons, and this is inevitably followed by a series of destructive processes, collectively termed secondary degeneration (Yoles and Schwartz, 1998), which result in the gradually spreading degeneration and death of initially undamaged adjacent neural cells. The processes of secondary degeneration are mediated mainly by destructive self-compounds that emanate from the directly damaged neurons and render the extracellular environment hostile to recovery. Until very recently, the prevailing view was that the CNS, being an immune-privileged site, cannot benefit from immune intervention and that all immune activity is detrimental. Studies by our group showed, however, that one way in which the hostility of the environment at the damaged site can be circumvented is by eliciting a systemic defensive activity that homes to the lesion site and helps the innate arm of the immune system to fight off the toxicity. This assistance is provided by the spontaneous recruitment of T cells specific to CNS-related self-antigens (WO 99/60021; Kipnis et al., 2002b). The autoimmune T cells home to the site of the lesion and become activated there by encountering their specific antigens, which are presented to them by antigen-presenting cells (APCs), e.g. activated microglia. Thus, contrary to the prevailing belief that the immune system is always harmful to the CNS, our work suggested that the CNS withstands injurious post-injury conditions by eliciting a protective autoimmunity (Moalem et al., 1999).

Further studies by our group showed that adult rats or mice deficient in mature T cells, or deprived (as a result of immunization at birth with spinal cord homogenate) of T cells specific to self-antigens residing in the site of damage, are unable to withstand injurious conditions in the CNS (Kipnis et al., 2001; Schori et
al., 2002). The T cells that participate in protection were found to possess a phenotype characteristic of Th1 cells (Kipnis et al., 2002b). These and related results led us to formulate the concept of "protective autoimmunity" as a physiological mechanism of protection against destructive self-compounds (Schwartz and Kipnis, 2001, 2002). Our group showed that this physiological response can be boosted by injection (passive transfer) of activated autoimmune T cells (Moalem et al., 1999; Kipnis et al., 2002b; Hauben et al., 2000a, 2000b) or by active vaccination with self- or self-related antigens (Hauben et al., 2001).

Normally, autoimmunity is suppressed by naturally occurring regulatory CD4⁺CD25⁺ T cells (Treg) (Shevach et al., 2001; Sakaguchi et al., 1995). Therefore, to elicit the desired autoimmune response for anti-tumor therapy or for protection of CNS neurons at risk of degeneration, the Treg-imposed suppression must be alleviated. Depletion of Treg promotes survival of neurons after CNS insults (Kipnis et al., 2002a) and boosts spontaneous anti-tumor autoimmunity (Sakaguchi et al., 2001).

Treg-imposed suppression is a multi-factorial process, involving cell-to-cell contacts (Nakamura et al., 2001) and the activity of soluble factors, which presumably include IL-10 (Sundstedt et al., 2003) and TGF-β (Piccirillo et al., 2002). Studies have shown that the suppressive activity of Treg can be inhibited by addition of exogenous IL-2 (Thornton and Shevach, 1998), or blocking of CTLA-4 (Nakamura et al., 2001; Takahashi et al., 2000), or activation of the newly discovered glucocorticoid-induced TNF-α receptor (GITR) (McHugh et al., 2002; Shimizu et al., 2002).

Some key adhesion molecules are more abundant on the surfaces of Treg than of effector (CD4⁺CD25⁻) T cells (Teff) (Kohm et al., 2002). The ability of Treg to enter tissues might help prevent autoimmune disease progression. In fighting off neurodegeneration or cancer, however, the presence of Treg is a liability. Compounds capable of reducing the trafficking ability (adhesion and migration) of Treg, or their suppressive activity, or both, might therefore be promising candidates for therapy against both cancer and CNS insults. As a
corollary, compounds capable of up-regulating the inhibitory or trafficking activity
of Treg, or both, might be potential candidates for therapy against autoimmune
diseases. A fine balance would then be needed in order to fight off the conditions
leading to tumor development or neuronal degeneration without creating conditions
that foster autoimmune diseases. Up to now, however, no physiological compounds
have been discovered that can control the activity of Treg.

In an attempt to identify physiological compounds potentially capable of
controlling the Treg activity as needed, we postulated that since stress- or pain-
related physiological compounds are increased after CNS injury (Thiffault et al.,
2000; Malcangio et al., 2000; Rothblat and Schneider, 1998), one or more of them
might transmit an early signal to Treg, with consequent reduction of the latter’s
trafficking or suppressive activity or both. We reasoned that likely candidate
compounds might be key neurotransmitters such as dopamine, norepinephrine,
serotonin, and substance P, all of which have been shown to participate in
interactions between the brain and the immune system (Swanson et al., 2001; Edgar
et al., 2002).

**Dopamine and dopamine agonists and antagonists**

Dopamine (3,4-dihydroxyphenylethylamine or 3-hydroxytyramine) is a
catecholamine formed in the body by the decarboxylation of dopa (3,4-
dihydroxyphenylalanine) and acts as a neurotransmitter in the CNS. Inside the
brain, dopamine acts as a neurotransmitter within the synapse of the nerve cell, and
outside the brain (or more specifically outside the blood-brain barrier), it acts as a
hormone (like most neurotransmitters) and affects the constriction/dilation of blood
vessels. Low-dose dopamine (0.5-3.0 μk/kg/min) infusion is used in hospitals in the
treatment of acute renal disease/failure (reviewed in Saxena, 2002). The
hydrochloride salt of dopamine (Inotropin) is used intravenously for treatment of
hypotension, septic shock and severe congestive heart failure such as in
cardiogenic shock.
In Parkinson’s disease, a progressive degenerative disease caused principally by the degeneration of the dopaminergic cells in the substantia nigra pars compacta, there is consequent loss of dopamine terminals in the striatum. Since dopamine taken orally is rapidly degraded in the intestine and blood and it does not penetrate from the blood into the brain, the most widely used treatment for Parkinson’s disease is pharmacotherapy, mainly by dopamine replacement, administering the precursor L-dopa (levodopa) that is converted to dopamine in the blood and in the brain. The effectiveness of L-dopa is maximized by combination with a medicine such as carbidopa, which blocks the conversion of L-dopa to dopamine in the blood only, thus transporting more L-dopa into the brain, where it is converted to dopamine.

Due to the side effects of the treatment with L-dopa or with the combination L-dopa/carbidopa, dopamine agonists have been developed or are in development for the treatment of Parkinson’s disease and other diseases or conditions in which dopamine is involved. Contrary to levodopa, that is converted to dopamine in the body, the dopamine agonists mimic the activity of dopamine by directly activating the dopamine receptor rather than by replacing dopamine as levodopa does.

The receptors for dopamine are primarily found in the striatum. There are at least five subtypes of dopamine receptors, called D1 through D5; the D1 and D5 subtypes belong to the dopamine receptor type 1 family and are referred to as "D1-like" or "D1-R" while the D2, D3, and D4 belong to the dopamine receptor type 2 family and are referred to as "D2-like" or "D2-R". The receptors are grouped in this manner because of the common properties of the receptor effects.

The different dopamine agonists may have affinity to both D1 and D2 families, albeit with different strength, or they may be specific to the D1 or the D2 family or to one of the receptors within one of the families. Dopamine agonists having varying activities at the different dopamine receptors are known, or being investigated, that exhibit subtly different effects. Some of the dopamine agonists in use for treatment of Parkinson’s disease include apomorphine (D1 and D2 agonist), the ergoline derivatives bromocriptine (D2 agonist), lisuride (D2 agonist), pergolide
(D2/D3 strong agonist), and cabergoline (D2 agonist), and the non-ergoline derivatives ropinirole (D2 agonist) and pramipexole (D2/D3 agonist). Bromocriptine and quinpirole protected cortical neurons from glutamate toxicity via the phosphatidylinositol 3 kinase cascade (Kihara et al., 2002). Other dopamine agonists under investigation include the D1 agonists dihydrexidine (DHX, the first high affinity full D1 dopamine receptor agonist), SKF-38393, SKF-81297, and SKF-82958, and the D2 agonists quinpirole, LY 172555, PPHT and quinelorane. SKF-38393 and quinpirole were shown to exhibit neuroprotective effects against malonate-induced lesion in the rat striatum, a model of focal ischemial (Fancellu et al., 2003), and in a Parkinson animal model (Olsson et al., 1995).

Besides their use in the treatment of Parkinson’s disease, some dopamine agonists have been proposed for different indications. The D2-R agonists bromocriptine, lisuride, cabergoline, and pergolide have been shown to suppress prolactin secretion and can be used as prolactin inhibitor and in the treatment of pituitary tumors secreting prolactin (usually benign tumors) including macroprolactinomas (Liuzzi et al., 1985; Kleinberg et al., 1983; Colao et al., 1997). Bromocriptine and cabergoline lower serum growth hormone levels in acromegaly patients and can be used for treatment of acromegaly. US Patent No. 5,744,476 discloses the D1-R agonist dihydrexidine either alone or together with levodopa or with a D2-R agonist, for raising extracellular brain acetylcholine levels to improve cognition in a human having senile or presenile dementia associated with neurodegeneration.

Dopamine antagonists have been developed for several indications, particularly D2 antagonists such as sulpiride, spiperone, haloperidol, spioperidol, clozapine, olanzapine and sertindole for use as antipsychotic agents. Clozapine has also been disclosed for controlling dyskinesias in people with severe Parkinson's disease (Durif et al., 1997).

WO 03/037247 of the same applicant of the present application discloses a method of regulating activity of a T-cell population, the method comprising exposing the T-cell population with a molecule selected capable of regulating a
Dopamine receptor activity or the expression of a gene encoding a Dopamine receptor of T-cells of the T-cell population, thereby regulating Dopamine mediated activity in the T-cell population. The method is indicated for treating or preventing a T-cell related disease or condition characterized by abnormal T-cell activity by administration of Dopamine and specific Dopaminergic receptor functional analogs and, more particularly, upregulating Dopamine analogs such as 7-OH-DPAT, (D3/D2 receptor agonist), SKF 38393 (D1-R agonist), quinpirole (D2-R agonist), and PD-168077 (D4-R agonist).

Reference is made to copending International Patent Application No. PCT/IL2004/……. entitled “Dopamine and agonists and antagonists thereof for modulation of the suppressive activity of CD4+CD25+ regulatory T cells” filed by applicant at the Israel PCT Receiving Office (RO/IL) on the same date, the contents thereof being explicitly excluded from the scope of the present invention.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

It has now been found in accordance with the present invention that dopamine reduces the suppression imposed by Treg on Teff in vitro and protects mice from CNS injury and glutamate toxicity and this effect is reproduced by SKF-38393, a D1-R agonist, and by clozapine, a D2-R antagonist, or by a combination of dopamine and clozapine.

The present invention thus provides, in one aspect, a method for treating a neurodegenerative condition, disorder or disease excluding Parkinson’s disease, said method comprising administering to an individual in need an agent that down-regulates the suppressive activity of CD4+CD25+ regulatory T cells (Treg) on
CD4⁺CD25⁻ effector T cells (Teff), whereby said agent protects said individual from neurotoxic conditions, wherein said agent is selected from the group consisting of:

(i) dopamine or a pharmaceutically acceptable salt thereof;
(ii) a dopamine precursor or a pharmaceutically acceptable salt thereof;
(iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof;
(iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof;
(v) a combination of (i) and (ii); and
(vi) a combination of (i), (ii) or (iii) with (iv).

In another aspect, the present invention relates to a pharmaceutical composition for treatment of a neurodegenerative condition, disorder or disease other than Parkinson’s disease, comprising a pharmaceutically acceptable carrier and an agent that down-regulates the suppressive activity of CD4⁺CD25⁻ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff), wherein said agent is selected from an agent (i) to (vi) as defined hereinabove.

In a further aspect, the present invention relates to the use of an agent that down-regulates the suppressive activity of CD4⁺CD25⁻ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff) for the manufacture of a pharmaceutical composition for treatment of a neurodegenerative condition, disorder or disease other than Parkinson’s disease, wherein said agent is selected from an agent (i) to (vi) as defined hereinabove.

In a still further aspect, the present invention relates to an article of manufacture comprising a container containing an agent that down-regulates the suppressive activity of CD4⁺CD25⁻ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff) and instructions for the use of said agent for treatment of a neurodegenerative condition, disorder or disease other than Parkinson’s disease, wherein said agent is selected from an agent (i) to (vi) as defined hereinabove.
BRIEF DESCRIPTION OF THE FIGURES

Figs. 1a-1d show that dopamine (DA) reduces the suppressive activity mediated by CD4\(^+\)CD25\(^+\) regulatory T cells (Treg). Proliferation of effector T cells (Teff, a CD4\(^+\)CD25\(^-\) population) was assayed by incorporation of \([\text{H}]\)-thymidine into Teff co-cultured with naturally occurring Treg. Recorded values are from one representative experiment out of three and are expressed as means ± SD of four replicates. (1a) Treg were activated by incubation for 24 h with anti-CD3 antibodies in the presence of mouse recombinant interleukin (mrIL)-2. Incubation of the activated Treg for 2 h with dopamine (10\(^{-5}\) or 10\(^{-7}\) M) prior to their co-culturing with Teff reduced their suppression of Teff compared to that obtained with Treg not exposed to dopamine. (1b) Dopamine (10\(^{-5}\), 10\(^{-7}\) or 10\(^{-9}\) M) added to freshly purified Treg. Dopamine (10\(^{-5}\) M and 10\(^{-7}\) M) had a similar effect on activity of naïve Treg to that of activated Treg, whereas the effect of dopamine at 10\(^{-9}\) M on Treg-mediated suppression was not significant. (1c) Activation of Treg for 96 h, followed by the addition of dopamine (10\(^{-5}\) M) for 2 h at the end of activation, significantly reduced the suppressive activity of Treg on Teff. Incubation of Teff with dopamine (10\(^{-5}\) M) for 2 h did not affect their susceptibility to Treg-induced suppression. (1d) Addition of norepinephrine (NE) (10\(^{-5}\) or 10\(^{-7}\) M) to Treg for 2 h after their activation for 24 h did not affect the suppressive activity of Treg.

Significant differences between groups were analyzed by Student’s t-test (p < 0.001). In all experiments: Teff - 50x10\(^3\) cells (const.); Treg – from 3x10\(^3\) to 50x10\(^3\) cells.

Figs. 2a-2m show the molecular mechanism underlying the effect of dopamine on Treg. (2a) The inhibitory effect of dopamine on the suppressive activity of Treg was mimicked by SKF-38393, a specific agonist of the D1-type family. The D2-type agonist quinpirole did not alter the effect of dopamine on Treg. SCH 23390, a specific D1-type antagonist, wiped out the dopamine effect on the suppressive activity of Treg. Each experiment was performed at least five times and representative results are shown. (2b) Incubation of Treg or Teff with dopamine did not cause apoptosis, as shown by propidium iodide (PI) staining for DNA content
and FACS analysis of Treg and Teff, 48 h after their incubation for 2 h with dopamine. (2c) Staining for apoptosis with annexin V for phosphatidylserine on a surface membrane. No increase in annexin V-labeled cells was detected upon incubation of Treg with dopamine (middle panel) or with the D1-type agonist SKF-38393 (right panel). (2d, 2e) Semi-quantitative RT–PCR analysis for D1-R and D5-R and TGF-β1 expression. mRNA was extracted from freshly isolated Teff and Treg, incubated for 2 h with or without dopamine, and subjected to semi-quantitative RT–PCR. The results of one representative experiment out of five are shown (2d). The housekeeping gene β-actin was used for quantitative analysis of the PCR products. Results are expressed by mean ± SEM of 3 independent experiments (2e). (2f) Quantitative real-time PCR using primers for D1-R and D5-R to verify the differences in the expression of dopamine receptors on Teff and Treg. The results presented in the figure are arbitrary units and are from one representative experiment of three performed. (2g, 2h) Semi-quantitative RT–PCR for D2-R, D3-R and D4-R expression. mRNA was extracted from freshly purified Teff and Treg. The housekeeping gene β-actin was used for quantitative analysis. The results of one representative experiment out of five are shown. (2i) Representative micrographs of D1-R-immunoreactive T cells using fluorescence and confocal microscopy. Also shown are micrographs stained with Hoechst and visualized by fluorescence microscopy. D1-R-immunoreactivity was observed in Treg but not in Teff. (2j) Expression of CTLA-4. Treg were activated for 24 h, then incubated for 2 h with dopamine or SKF-38393 (control cells were activated but were not incubated with either dopamine or SKF-38393; note, different cell preparations were used for each treatment and therefore the controls used for each treatment were not the same), and were stained 24 h later for CTLA-4 on cell surfaces. CTLA-4 expression was reduced after exposure to dopamine or to SKF-38393. Representative results of one of five independent experiments with each treatment are shown. (2k) Production of IL-10. Treg were activated for 24 h with anti-CD3 and IL-2 in the presence of lethally irradiated splenocytes (APCs) and then for an additional 2 h with dopamine. Conditioned media were collected after
24, 48, or 72 h of culture and were assayed for IL-10 using a sandwich ELISA. At any given time, significantly less IL-10 was detected in media conditioned by dopamine-treated Treg than in media conditioned by Treg not exposed to dopamine. Statistical significance was verified using a student's T-Test analysis (**, p < 0.01; *, p < 0.05). The results shown are of one of three independent experiments, performed at each time point. (2i) Lack of IL-2 production by Treg. Treg and Teff were activated separately for 48 h with anti-CD3 and anti-CD28 (without mrlIL-2) with or without dopamine. Conditioned media were collected after 48 h and subjected to ELISA. Treg with or without dopamine did not secrete detectable levels of IL-2. Production of Il-2 by Teff was not affected by dopamine. (2m) Foxp3 expression in Treg. Treg were activated for 24 h with anti-CD3 and anti-CD28 in the presence of IL-2, then exposed to dopamine for 2 h, washed, and analyzed 30 min later for Foxp3 expression. No changes in Foxp3 were detected after 30 min of dopamine treatment of naïve Treg.

Figs. 3a-3e show the correlation between activity of Treg and activation state of ERK1/2. (3a) Treg (12x10^3, 25x10^3 or 50x10^3 cells) were activated by incubation for 30 min with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and in the presence or absence of tyrosine kinase inhibitor (genistein), and were then co-cultured with Teff (50x10^3 cells). The suppression of Teff by Treg was significantly reduced in the presence of genistein. (3b) Similarly, incubation of activated Treg (25x10^3 or 50x10^3 cells) with the specific MEK inhibitor PD98059, which inhibits the ERK1/2 signaling pathway, almost completely abolished their suppression of Teff (50x10^3 cells). (3c, 3d) Western blot analyses of Treg lysates after activation for 20 min with anti-CD3 and anti-CD28, in the presence or absence of dopamine (3c) or SKF-38393 (3d). After activation, the amounts of phospho-ERK1/2 seen in Treg are larger than in Teff (3c), but are reduced by dopamine (3c) or by SKF-38393 (3d). Dopamine did not cause a significant change in phospho-ERK1/2 levels in Teff (c); (3e) Quantitative analysis of phospho-bands using NIH Image 1.62.

Figs. 4a-4i show that dopamine alters the adhesive and migratory activities of Treg. (4a) Treg and Teff were activated for 24 h with anti-CD3 and anti-CD28
and were then incubated, with or without dopamine (10^{-5}–10^{-9}M), for 2 h. In the absence of dopamine, adhesion of Treg to the CSPG matrix was significantly stronger than that of Teff. Incubation with dopamine significantly reduced the adhesion of Treg in a concentration-dependent manner. The effect of dopamine on Treg adhesion could be mimicked by SKF-38393, a specific agonist of the D1-type family. The dopamine effect was blocked by SCH-23390, a D1-type antagonist. Dopamine did not significantly alter the adhesion of Teff. A Mann-Whitney nonparametric test was used for statistical analysis. (4b) In the absence of dopamine, adhesion of Treg to fibronectin was only slightly (but still significantly) stronger than that of Teff. However, dopamine did not significantly alter the adhesion of either Treg or Teff. A Mann-Whitney nonparametric test was used for statistical analysis. (4c) Treg were activated for 30 min in the presence or absence of the ERK1/2 signaling pathway inhibitor PD98059, and then subjected to an adhesion assay towards MDC. Adhesion of Treg incubated with PD98059 was significantly weaker than that of control Treg cells. (4d) CD44 expression in Treg and in Teff. FACS analysis showed that significantly larger amounts of CD44 are expressed in Treg than in Teff. After incubation with dopamine, CD44 expression was significantly decreased in Treg, but was not affected in Teff. (4e) The total population of purified CD4^{+} T cells was subjected to a migration assay towards SDF-1 or MDC. The percentage of CD4^{+}CD25^{+} T cells in the total population after migration towards CCL22 (MDC) was significantly higher than in the original population. Exposure of Treg to dopamine significantly decreased their migration towards MDC. Migration of Treg towards SDF-1 was not significantly affected by exposure to dopamine. A Mann-Whitney nonparametric test was used for statistical analysis. (4f, 4g) Migration of purified Treg towards MDC was significantly decreased after incubation of Treg with dopamine. Treg in the lower (post-migration) chamber were collected and counted by FACS for a defined time period after staining for membrane CD4 marker. Values are representative results of the FACS analysis (4f) and mean number of cells from triplicates of the same experiment are shown in (4g). (4h, 4i) Semi-quantitative RT–PCR for CCR-4
expression in Treg and Teff. mRNA was isolated from Teff and Treg, incubated for 2 h with or without dopamine. The PCR products were quantified (4i) relative to a housekeeping gene (β-actin). Results of one representative experiment are shown (4h). Each experiment was performed in triplicate and repeated at least three times. 

***, p < 0.01; **, p < 0.01.

**Fig. 5** shows that systemic injection of dopamine increases neuronal survival after optic nerve crush injury. Balb/c mice were injected with dopamine (0.4 mg/kg) immediately after being subjected to a partial crush injury of the optic nerve. Two weeks later their retinas were excised and the numbers of surviving neurons determined (see Materials and Methods). Significantly more neurons survived in dopamine-injected mice than in vehicle-injected controls (p < 0.01; Student’s t-test). Bars represent mean numbers of retinal ganglion cells (RGC)/m² of the retina. Each experiment was performed twice; n = 6–8 mice in each group. A two-tailed Student’s t-test was used for statistical analysis; ***, p < 0.001; **, p < 0.01.

**Figs. 6a-6b** show that exposure of Treg to dopamine in vitro reduces their suppressive activity in vivo. (6a) Neuronal survival was significantly worse in Balb/c mice that were inoculated (immediately after their exposure to a toxic excess of intraocular glutamate) with activated Treg than in Teff-inoculated mice. Neuronal loss is expressed as a percentage of the number of neurons in untreated glutamate-injected controls. Neuronal survival in Balb/c mice that were exposed to a toxic excess of intraocular glutamate and then treated with activated Treg that were incubated for 2 h with 10⁻⁵ M dopamine before being administered in vivo did not differ from that in vehicle-treated glutamate-injected mice. (6b) Representative micrographs of retinas from mice injected with glutamate and either Teff or Treg. Each experiment was performed twice; n = 6–8 mice in each group. A two-tailed Student’s t-test was used for statistical analysis; ***, p < 0.001.

**Fig. 7** shows that the D1-R agonist SKF-38393 improves neuronal survival after CNS insult by glutamate toxicity in mice.
**Fig. 8** shows that administration of the D2-R antagonist clozapine alone or together with dopamine increases neuronal survival after glutamate-induced neuronal cell death in mice.

**Figs. 9a-9b** show the effect of systemic injection of clozapine/dopamine on mice with spinal cord contusion. (9a) Injection of clozapine/dopamine immediately after partial spinal cord contusion (200 DPI for 1 sec) confers significant neuroprotection in male C57 mice (n=6/7 in each group) as compared with PBS injected controls. The neuroprotective effect of this treatment was statistically significant (*, p < 0.05 and **, p <0.01, tow-tailed student’s t test). (9b) The scores recorded are those of individual mice at the last time point that was examined.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention is based on the surprising finding by the inventors that dopamine blocks the suppressive activity of naturally occurring CD4+CD25+ cells, which comprise about 10% of the total CD4+ population.

The CD4+CD25+ cells, so-called regulatory T cells (hereinafter designated "Treg"), originally called suppressor T cells, express the transmembrane protein called CD25, that is the α chain of the receptor for IL-2 (Sakaguchi et al., 1995). When activated, Treg begin to secrete large amounts of IL-10 and often some TGF-β as well. Both these lymphokines are powerful immunosuppressants, inhibiting Th1 help for cell-mediated immunity and inflammation and Th2 help for antibody production.

The antigenic peptides recognized by the T-cell receptors of Treg tend to be self-peptides and, perhaps, the major function of Treg cells is to inhibit other T cells (effector cells, hereinafter “Teff”) from mounting an immune attack against self components, namely, to protect the body against autoimmunity. Indeed, it has been confirmed that naturally occurring Treg suppress autoimmunity (Shevach et al., 2001; Sakaguchi et al., 1995).
As mentioned above, recent evidence provided by the present inventors indicate that autoimmunity, that has long been viewed as a destructive process, is the body’s endogenous response to CNS injury and its purpose is in fact beneficial (Schwartz and Kipnis, 2001; Yoles et al., 2001). This neuroprotective autoimmunity was shown by the inventors to be inhibited by naturally occurring CD4⁺CD25⁺ cells, that suppressed an endogenous T-cell mediated neuroprotective mechanism to achieve maximal activation of autoimmunity and, therefore, to withstand injury to the CNS (Kipnis et al., 2002a).

In one embodiment, the present invention provides a method for down-regulation of the suppressive activity of CD4⁺CD25⁺ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff) in an individual suffering from a neurodegenerative condition, disorder or disease other than Parkinson’s disease, which comprises administering to said individual in need of such a treatment an effective amount of an agent selected from the group consisting of (i) dopamine; (ii) a dopamine precursor: (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist): (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist); (v) a combination of (i) and (ii); and (vi) a combination of (i), (ii) or (iii) with (iv), and wherein said agent protects said individual from neurotoxic conditions.

As used herein, the terms “protection from neurotoxic conditions” and “protection from “neurodegeneration” are used interchangeably and are meant to include protection of the central nervous system or peripheral nervous system from any injury, disorder or disease independently from their primary cause. These neurotoxic conditions include, but are not limited to, CNS injuries, glutamate toxicity, several diseases and exposure to nerve gases as will be detailed hereinafter.

As used herein, the terms “dopamine”, “D1-R agonist” and “D2-R antagonist” are meant to include the compounds themselves as well as their pharmaceutically acceptable salts.
In one most preferred embodiment, the agent is dopamine or a pharmaceutically acceptable salt thereof such as the hydrochloride or hydrobromide salt, and is preferably dopamine hydrochloride.

It should be understood that the present invention uses an approach different from that used in treatment of Parkinson’s disease. Parkinson’s disease is caused by a loss of dopamine-producing cells in specific areas of the brain, including those that control movement. These cells continue to be lost as the disease progresses and as a result the brain loses its ability to produce dopamine, a neurotransmitter which is essential to transmit signals within the brain necessary for the brain to coordinate and control body movement. Thus, in the treatment of Parkinson’s disease, dopamine should be provided to the brain. Since dopamine does not cross the blood brain barrier, treatment is carried out with its precursor, levodopa, that has the ability to cross the blood brain barrier and in the brain is transformed into dopamine.

According to the present invention, dopamine will not replace the dopamine in the brain but will down-regulate the suppressive effect of Treg cells on Teff cells in the periphery, thus allowing the Teff cells to exhibit the protective autoimmunity that is necessary to prevent neuronal degeneration.

In another preferred embodiment, the agent is dopamine in combination with its precursor levodopa, optionally in further combination with carbidopa. This combination can be used even for the treatment of Parkinson’s disease, or for treatment of a Parkinson’s patient in need of a treatment of another non-Parkinsonian neurodegenerative condition.

In another preferred embodiment, the agent is a dopamine D1-R agonist selected from any such agonist known or to be developed in the future and includes, without being limited to, a D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine. Preferably, the D1-R agonist is SKF-38393 and its hydrochloride salt [(+/-)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol.HCl].
In a further preferred embodiment, the agent is a dopamine D2-R antagonist selected from any such antagonist known or to be developed in the future and includes, without being limited to, a D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2. Preferably, the D2-R antagonist is clozapine.

In a further preferred embodiment, the agent is a combination of dopamine with a dopamine D2-R antagonist, preferably dopamine and clozapine.

In still a further embodiment, the agent is a combination of dopamine D1-R agonist with a dopamine D2-R antagonist, preferably a combination of SKF-38393 and clozapine.

When a combination of compounds is used, the two agents may be administered concomitantly (in a mixture) or subsequently to each other.

In one embodiment of the invention, the neuronal degenerative condition, disorder or disease is a neuronal degeneration, in particular a secondary neuronal degeneration, resulting from an injury that has caused primary neuronal damage.

Thus, in one preferred embodiment, the method of the present invention comprises administering to an individual in need for treating neurodegenerative effects caused by a primary injury, an agent that down-regulates the suppressive activity of Treg on Teff selected from the group consisting of an agent (i) to (vi) as defined hereinabove, in an amount effective to reduce neuronal degeneration caused by said primary injury. The agent is preferably dopamine. The neuronal degeneration is more particularly a secondary neuronal degeneration caused by a primary injury including, without being limited to, spinal cord injury, blunt trauma such as those caused by dangerous sports, penetrating trauma such as gunshot wounds, hemorrhagic stroke, or ischemic stroke blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke, cerebral ischemia, and injury caused by surgery such as tumor excision.
In another preferred embodiment, the present invention provides a method for treating a neurodegenerative disease, disorder or condition caused or exacerbated by glutamate toxicity, which comprises administering an agent that down-regulates the suppressive activity of Treg on Teff selected from the group consisting of an agent (i) to (vi) as defined hereinabove, to the individual in need in an amount effective to ameliorate the neurodegeneration caused or exacerbated by glutamate toxicity.

Glutamate toxicity was found to play a major role in triggering neurodegeneration following CNS insult regardless of the initial cause, whether acute or chronic. We use herein an in vivo model of glutamate toxicity for assessing neuroprotective agents. Intraocular injection of glutamate into the eye of a mouse exposes the RGCs (the retinal neurons that project the visual information to the brain through their axons, the optic nerve, and are part of the CNS) to temporary elevation of glutamate concentration, leading to RGC death as measured 7 days later. The effectiveness of the neuroprotective agent in protecting neurons is measured by counting the surviving RGCs.

In another preferred embodiment, the method of present invention comprises administering an agent that down-regulates the suppressive activity of Treg on Teff selected from the group consisting of an agent (i) to (vi) as defined hereinabove, to an individual in need for treating neurodegenerative effects caused by a condition, disorder or disease associated with the eye, such as non-arteritic optic neuropathy, age-related macular degeneration, a retinal disorder or a disease associated with elevated intraocular pressure, e.g. glaucoma, in an amount effective to reduce neuronal degeneration caused by said condition, disorder or disease associated with the eye. The ophthalmic disease or disorder may be caused or exacerbated by glutamate toxicity but may also have a different etiology.

In a preferred embodiment, the invention relates to a method for lessening RGC death and/or lessening damage to the optic nerve arising from a condition selected from the group consisting of glaucoma, increased intraocular pressure, and glutamate toxicity, comprising administering to an individual in need of such
treatment an effective amount of an agent selected from the group consisting of (i) to (vi) as defined hereinabove, thereby lessening RGC death and/or lessening damage to the optic nerve. In a most preferred, the agent is administered for lessening, preventing or reducing optic nerve degeneration in glaucoma patients.

In another embodiment, the disease, disorder or condition caused or exacerbated by glutamate toxicity may be a neurodegenerative disease such as a senile dementia of both Alzheimer's type and non-Alzheimer's type, a non-Parkinsonian neurodegenerative condition or disorder in a Parkinson patient, facial nerve (Bell's) palsy, Huntington's chorea, a motor neurone disease such as amyotrophic lateral sclerosis (ALS), Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, Guillain-Barré syndrome, and a prion disease such as Creutzfeldt-Jakob disease. The disease may also be chronic progressive multiple sclerosis (MS), a term used to describe cases in which symptoms continue to worsen slowly without remission, and include secondary-progressive MS, in which a progressive course of nerve and muscle deterioration occurs with occasional acute flare-ups, remissions, and plateaus.

Thus, the agent that suppresses Treg activity may be used to ameliorate the effects of disease or disorder that result in a degenerative process, e.g. degeneration occurring in either gray or white matter (or both) as a result of the chronic neurodegenerative diseases recited in the previous paragraph or as a result of further diseases, disorders and conditions. For example, in a patient suffering from Huntington's disease, the agent is administered in an amount therapeutically effective to reduce disease progression and/or to protect the patient from neurodegeneration and/or from glutamate toxicity. In a patient suffering from a motor neurone disease, the agent is administered in an amount effective to reduce disease progression, and/or to protect the patient from motor nerve degeneration and/or from glutamate toxicity.

In another embodiment of the invention, the agent that suppresses Treg activity may be used for the treatment of a peripheral neuropathy. Peripheral neuropathy, a general term referring to disorders of the peripheral nerve system, can
be associated with poor nutrition, a number of diseases, and pressure or trauma. Known etiologies include complications of other diseases, mainly diabetes. Nearly 60% of all people with diabetes suffer from peripheral neuropathy. Peripheral neuropathy can be classified by where it occurs in the body: nerve damage that occurs in one area of the body is called mononeuropathy, and in many areas, polyneuropathy. It can also be categorized by cause such as diabetic neuropathy and nutritional neuropathy. When a cause cannot be identified, the condition is called idiopathic neuropathy.

According to the present invention, the agent that suppresses Treg activity may be used for the treatment of peripheral neuropathies, both mononeuropathies and polyneuropathies, caused by or associated with many diseases, disorders and conditions such as, but not limited to, adrenomyeloneuropathy, alcoholic neuropathy (associated with chronic alcoholism), amyloid neuropathy or polyneuropathy (caused by amyloidosis), axonal neuropathy, chronic sensory ataxic neuropathy associated with Sjogren's syndrome, diabetic neuropathy, an entrapment neuropathy or nerve compression syndrome such as carpal tunnel syndrome or a nerve root compression that may follow cervical or lumbar intervertebral disc herniation, giant axonal neuropathy, hepatic neuropathy (associated with viral hepatitis, liver cirrhosis, or biliary cirrhosis), ischemic neuropathy, nutritional polyneuropathy (due to nutritional deficits such as vitamin, e.g. vitamin B6, B12 deficiency, malabsorption syndromes and alcoholism), porphyrinic polyneuropathy (a severe form associated with various types of porphyria), toxic neuropathy (caused by toxins such as organophosphates), uremic polyneuropathy (caused by the uremia of chronic renal failure), a neuropathy associated with a disease or disorder such as acromegaly, ataxia telangiectasia, Charcot-Marie-Tooth disease, chronic obstructive pulmonary diseases, Fabry's disease, Friedreich ataxia, Guillain-Barré syndrome (an acute inflammatory polyneuropathy), hypoglycemia, IgG or IgA monoclonal gammopathy (non-malignant or associated with multiple myeloma or with osteosclerotic myeloma), lipoproteinemia, polycythemia vera, Refsum's syndrome, Reye's syndrome, Sjogren-Larsson syndrome, or a polyneuropathy associated with
various drugs (e.g., nitrofurantoin and metronidazole), or a polyneuropathy associated with hypoglycemia, with infections such as HIV infection, or with cancer (radiation treatments, chemotherapy or the cancer can be the cause of the nerve damage).

In yet a further embodiment, the agent can be used for conferring neuroprotection and preventing or inhibiting neuronal degeneration in an individual upon exposure to a neurotoxin, e.g. nerve gases such as organophosphate nerve gases, for example, sarin. According to this embodiment, the agent will confer neuroprotection against sarin-induced brain damage and is used preferably together with an anti-convulsant, e.g. midazolam.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g., gender, weight, etc.) of the individual, as well as on various other factors, e.g., whether the individual is taking other drugs, etc., and thus suitable doses and protocols of administration will be decided by the physician taking all these factors into consideration.
The invention will now be illustrated by the following non-limiting examples and accompanying figures.

EXAMPLES

5

Materials and Methods

5

(i) Animals. Inbred adult wild-type and nu/nu Balb/c and C57Bl/6 mice were supplied by the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel). All animals were handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee).

(ii) Antibodies and reagents. Mouse recombinant IL-2, anti-mouse ζ-CD3 (clone 145-2C11), anti-mouse CTLA-4 (CD152) (clone #63828), and purified rabbit anti-mouse ERK2 antibody were purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse phycoerythrin (PE)-conjugated CD25 antibody (PC61) was purchased from Pharmingen (Becton-Dickinson, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody was purchased from Serotec (Oxford, UK). Anti dopamine receptor-1 (D1-R; Cat no 324390) was purchased from Calbiochem (Darmstadt, Germany). The compounds 3-hydroxytyramine (3,4-dihydroxyphenethylamine; dopamine) (H-8502), norepinephrine (A-7257), SKF-38393 (D-047), SCH-23390 (D-054), quinpirole (Q-111), clozapine (C-6305), genistein (G-6649), and PD98059 (P-215) were from Sigma-Aldrich (Rehovot, Israel). The phosphatidylserine detection kit, which includes FITC-labeled annexin V, was purchased from IQ Products (Houston, TX). Anti-pERK1/2 FITC-conjugated 1 & 2 phosphospecific antibody was purchased from Biosource International (Camarillo, CA). Purified anti-pERK1/2 antibody was the generous gift of Prof. R. Seger from The Weizmann Institute of Science.

(iii) Intravitreal glutamate injection. The right eyes of anesthetized mice were punctured with a 27-gauge needle in the upper part of the sclera, and a 10-μL Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. A
total volume of 1 μL of L-glutamate (400 nmol) dissolved in saline was injected into the eye.

(iv) Retrograde labeling of retinal ganglion cells. Mice were anesthetized and placed in a stereotactic device. The skull was exposed and kept dry and clean. The bregma was identified and marked. The designated point of injection was at a depth of 2 mm from the brain surface, 2.92 mm behind the bregma in the anteroposterior axis and 0.5 mm lateral to the midline. A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was applied (1 μL, at a rate of 0.5 μL/min in each hemisphere) using a Hamilton syringe, and the skin over the wound was sutured.

(v) Crush injury of the optic nerve in mice. Animals were deeply anesthetized by intraperitoneal injection of Xyl-M® 2% (xylazine, 10 mg/kg; Arendonk, Belgium) and Ketaset (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and subjected to severe crush injury of the intraorbital portion of the optic nerve. The uninjured contralateral nerve was left undisturbed. The optic nerve was crushed 3 days after retrograde labeling of retinal ganglion cells with FluoroGold, as described above (Fisher et al., 2001).

(vi) Preparation of splenocytes. Donor splenocytes from rats (aged up to 10 weeks) were obtained by rupturing the spleen and following conventional procedures. The splenocytes were washed with hypotonic buffer (ACK) to lyse red blood cells.

(vii) FACS analysis of CTLA-4-expressing CD4⁺ T cells. Cells were immunostained according to the manufacturer’s instructions, resuspended in 0.4 mL of 1% paraformaldehyde, and analyzed by FACSsort (Becton-Dickinson), with 10,000 events scored. In single-color analysis, positive cells were defined as cells with higher immunofluorescence values, on a logarithmic scale, than those of control cells incubated with isotype antibodies as a control. The cells were scored from a region defined according to physical parameters that indicate the size
(forward scatter) and granularity (side scatter) of lymphocytes. CD4⁺ lymphocytes were then gated for analysis of CTLA-4 expression.

**(viii) FACS analysis of annexin V-positive regulatory T cells.** PE-stained CD25⁺ cells were stained for annexin V-FITC according to the manufacturer’s instructions. The cells were scored as described above, and CD25⁺ lymphocytes were then gated for analysis of annexin V-positive cells.

**(ix) FACS analysis of intracellular labeling of phosphorylated form of ERK.** T-cell subpopulations were fixed in 1.5% formaldehyde for 10 min at room temperature, washed, resuspended with vortexing in cold 100% methanol, and incubated for 1 h at 4°C. The cells were then washed with PBS containing 1% bovine serum albumin (BSA) and resuspended in 100 μL of PBS/BSA, and 12.5 μL of anti-phosphoERK1 & 2 (BioSource) was added for 20 min at room temperature. The cells were then washed, resuspended in PBS, and analyzed by FACS Calibur (Becton-Dickinson). As a negative control we incubated the phospho-peptide for 20 min with its antibody and then added the mixture to T cells.

**(x) Enzyme-linked immunosorbent assay.** Treg or Teff (0.5 x 10⁶ cells/ml) were cultured for 48 h in the presence of anti-CD3 and anti-CD28. After 48 h the cells were centrifuged and their supernatants were collected and sampled. Concentrations of IL-2 in the samples were determined by the use of sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). For detection of secreted IL-10 cells were centrifuged every 24 h and replaced with a fresh medium. Supernatants obtained from cells after 24, 48 and 72 h in culture were subjected to ELISA kit (Diaclone Research, Fleming, France). The plates were developed using a 3,3',5,5'-tetramethyl-benzidine liquid substrate system (Sigma, St. Louis, MO). The reaction was stopped by adding 1M H₃PO₄. Results for each experiment were calculated as the amount of secreted cytokine per 1 ml of sample, after subtraction of the background levels of the medium.

**(xi) Depletion of CD25⁺ cells.** Splenocytes obtained from wild-type mice were prepared by the standard procedure, and incubated with rat anti-mouse PE-conjugated CD25 antibody and then with anti-PE beads (Becton-Dickinson). The
washed splenocytes were subjected to AutoMacs (Miltenyi Biotec, Gladbach, Germany) using the ‘deplete sensitive’ program. Recovered populations were analyzed by FACS sort.

(xii) Purification of murine CD4⁺CD25⁻/CD4⁺CD25⁺ T cells. Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleens were harvested and mashed. T cells were purified (enriched by negative selection) on T-cell columns (R&D Systems). The enriched T cells were incubated with anti-CD8 microbeads (Miltenyi Biotec), and negatively selected CD4⁺ T cells were incubated with PE-conjugated anti-CD25 (30 μg/10⁸ cells) in PBS/2% fetal calf serum. They were then washed and incubated with anti-PE microbeads (Miltenyi Biotec) and subjected to magnetic separation with AutoMACS. The retained cells were eluted from the column as purified CD4⁺CD25⁺ cells. The negative fraction consisted of CD4⁺CD25⁻ T cells. Purified cells were cultured in 24-well plates (1 mL) with T cell-depleted spleen cells as accessory cells (irradiated with 3000 rad) and 0.5 μg/mL anti-CD3, supplemented with 100 units of mouse recombinant IL-2 (mrIL-2; R&D Systems).

(xiii) T cell adhesion. Adhesion of activated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells to CSPG was analyzed as previously described (23). Briefly, flat-bottomed microtiter (96-well) plates were pre-coated with CSPG (1 μg/well, 40 min, 37°C). ⁵¹Cr-labeled T cells were left untreated or were pre-incubated (30 min, 37°C) with dopamine or the specified agonist or antagonist (10⁻⁵M). The cells (10⁵ cells in 100 μL of RPMI containing 0.1% BSA) were then added to the CSPG-coated wells, incubated (30 min, 37°C), and washed. Adherent cells were lysed and the resulting supernatants were removed and counted in a γ-counter. Results were expressed as the mean percentage of the total population before adhesion of bound T cells from quadruplicate wells for each experimental group.

(xiv) Chemotaxis assay. The migration of T cells across polycarbonate filters (pore size 5 μm, diameter 6.5 mm) towards SDF-1 and MDC (CCL22) was assayed in 24-well Transwell chambers (Costar, Corning, Corning, NY). T lymphocytes (1.67 × 10⁶ cells/mL) were suspended in RPMI/ 0.1% BSA, and 150
μL of the cell suspension was added to the upper chamber after incubation with or without dopamine (90 min, 37°C). Chemokines were added to the lower chamber at concentrations of 1 μg/mL SDF-1 (CytoLab, Israel) and 0.25 μg/mL MDC (R&D Systems). The plates were incubated for 90 min at 37°C in 9.5% CO₂. T cells that migrated to the lower chambers were collected and stained with anti-CD4 and anti-CD25 antibodies. The numbers of migrating T cells were measured by flow cytometer acquisition for a fixed time (60 s). To calculate specific migration, the number of cells in each subpopulation in the absence of chemokine was subtracted from the number in the corresponding cell subpopulation that migrated in the presence of chemokines. The number of migrating CD4⁺CD25⁺ T cells was calculated as a percentage of the total T cell population before migration. For migration of purified population we used a similar protocol.

(xv) Propidium iodide staining. Cells were fixed in cold ethanol 80% and treated with RNAs. Propidium iodide was then applied, and cell samples were assessed by FACSsort.

(xvi) Activation of CD4⁺CD25⁺ regulatory T cells. Purified regulatory T cells (Treg; 0.5 × 10⁶ cells/mL) were activated in RPMI medium supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5 × 10⁻⁵ M), sodium pyruvate (1 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL), nonessential amino acids (1 mL/100 mL), and autologous serum 2% (vol/vol) in the presence of mTIL-2 (5 ng/mL) and soluble anti-CD3 antibodies (1 ng/mL). Irradiated (2500 rad) splenocytes (1.5 × 10⁶ cells/mL) were added to the culture. Cells were activated for 24 or 96 h. In some of the 96-h experiments, fresh dopamine was added to the culture every 24 h during activation.

(xvii) Inhibition assay (co-culturing of Teff with Treg). Naïve effector T cells (Teff; 50 × 10³ cells/well) were co-cultured with decreasing numbers of activated Treg for 72 h in 96-well flat-bottomed plates in the presence of irradiated splenocytes (10⁶/mL) supplemented with anti-CD3 antibodies. [³H]-thymidine (1 μCi) was added for the last 16 h of culture. After the cells were harvested, their [³H]-thymidine content was analyzed by the use of a γ-counter.
(xviii) Immunocytochemistry. T cells were fixed for 10 min with a mixture (1:1) of methanol and acetone at −20°C, incubated in blocking solution (PBS containing 0.3% Triton-X100 and 1% of normal rabbit serum) for 60 min at room temperature, and then incubated overnight with a specific antibody (dilution 1:1000) in the blocking solution. The T cells were then washed and incubated with the secondary antibody (PE-labeled goat anti-rabbit IgG) for 30 min at room temperature, then washed, and analyzed by fluorescence and confocal microscopy.

(xix) Western blotting. Cells were stimulated for 20 min with anti-CD3 and anti-CD28 antibodies in the presence or absence of dopamine or SKF-38393. Cell lysates were prepared using RIPA lysis buffer (50 mM Tris, pH 8; 0.1% SDS; 0.5% deoxycholate; 1% NP40; 500 mM NaCl; 10 mM MgCl₂) and were then kept on ice for 10 min before being vortexed and centrifuged. Supernatants were collected and 5× sample buffer (containing 25 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, 0.5 M β-mercaptoethanol) was added prior to boiling. Cell extracts were separated by SDS–PAGE (10% polyacrylamide), and blotted onto nitrocellulose. Activated ERK1/2 was detected by probing blots with a 1:30,000 dilution of monoclonal antibody. Total ERK protein was detected by using a 1:10,000 dilution of a polyclonal rabbit antibody. The blots were developed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit Fab and ECL (Amersham). Signals were quantified using NIH Image 1.62.

(xx) Polymerase chain reaction (PCR). Total RNA was purified with the RNeasy Mini Kit (Qiagen, Germantown, MD) and transcribed into cDNA using poly dT primers. For PCR the following primers were used:

for D1-R: sense, 5'-GTAGCAGATTATGATCGTC-3' [SEQ ID NO: 1],
anti-sense, 5'-GATCACAGACAGTGTCTTCAG-3' [SEQ ID NO: 2];
for D2-R: sense, 5'-GCAGCAGGTCAGTCAGG-3' [SEQ ID NO: 3],
anti-sense, 5'-GGGATGTTGCAGTCACAGT-3' [SEQ ID NO: 4];
for D3-R: sense, 5'-AGGTTCGTCTGTCAGTGC-3' [SEQ ID NO: 5],
anti-sense, 5'-GTGCTGAGGTTTTCGAACC-3' [SEQ ID NO: 6];
for D4-R: sense, 5'-CACCAACTACTTCATCGTA-3' [SEQ ID NO: 7],
anti-sense, 5'-AAGGACGACGGACGAGTGA-3' [SEQ ID NO: 8]; for D5-R: sense, 5'-CTACGAGGCGAATGACC-3' [SEQ ID NO: 9];
anti-sense, 5'-CTCTGAGCATTGCTACGCT-3' [SEQ ID NO: 10]; for CCR-4: sense, 5'-GTGCAGTCTGAAGAGACTTCAAGCCACCAG-3' [SEQ ID NO: 11],
anti-sense, 5'-GGCAAGGACCCCTGACCTATGGGTCATCAC-3' [SEQ ID NO: 12]; and
for FOXP3: sense, 5'-CAG CTG CCT ACA GTG CCC CTA G-3' [SEQ ID NO: 13],
anti-sense, 5'-CAT TTG CCA GCA GTG GGT AG-3' [SEQ ID NO: 14].

Signals were quantified using a Gel-Pro analyzer 3.1 (Media Cybernetics). Real-time PCR was performed with a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using FastStart DNA Master SYBR Green 1 kit (Roche, catalog no. 3003230) as described by the manufacturer. The following primers were used for the reactions: For D1-R, the primers listed above. For D5-R: sense, 5'-CCTTTATCCCGGTCCA-3' [SEQ ID NO: 15], anti-sense, 5'-GATAACGGCGATCTGAA-3' [SEQ ID NO: 16]; for IL-10 sense, 5'-ACCTGGTAGAGTGTGCCGCA-3'[SEQ ID NO: 17], anti-sense, 5'-CTATTGCAGCTGATAGTCAAA-3' [SEQ ID NO: 18] (Pozzi et al., 2003); for Foxp3; sense, 5'-CAG CTG CCT ACA GTG CCC CTA G-3' [SEQ ID NO: 19], anti-sense, 5'-CAT TTG CCA GCA GTG GGT AG-3' [SEQ ID NO: 20].

(ii) Assessment of mouse retinal ganglion cell survival. Mice were given a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde solution. Labeled cells from 4–6 selected fields of identical size (0.7 mm²) were counted. The selected fields were located at approximately the same distance from the optic disk (0.3 mm) to overcome the variation in RGC density as a function of distance from the optic disk. Fields were counted under the fluorescence microscope (magnification x800) by observers blinded to the identity of the retinas. The average number of RGCs per field in each retina was calculated.
Example 1. Dopamine reduces the suppression imposed by Treg

We postulated that in the injured CNS, the first message to the peripheral immune system is a stress signal that acts preferentially on Treg and blocks their suppression. To test this hypothesis, we examined whether the neurotransmitter dopamine, a member of the catecholamine family, acts on Treg and alters their suppressive effect on effector T cells (Teff). Suppression of proliferation of Teff, assayed by [³H]thymidine incorporation, was used as a measure of suppressive effect of Treg (Thornton and Shevach, 1998).

Co-culturing of effector T cells (Teff) with Treg isolated from naïve mice results in suppression of Teff proliferation. The suppressive potency depends on the Treg/Teff ratio and the state of Treg activation; the suppression is significantly increased, for example, if the Treg are activated before being added to Teff (Thornton and Shevach, 1998, 2000). Inhibition of Teff proliferation, assayed by [³H]thymidine incorporation, can therefore be taken as a measure of the suppressive effect. We examined the ability of major neurotransmitters and neuropeptides (dopamine, norepinephrine, substance P, and serotonin) to alleviate the Treg-induced suppression of Teff in vitro. Each compound was tested at several concentrations. Proliferation of Teff was significantly inhibited by co-cultivation of Teff with naïve Treg or with Treg that had been activated by incubation for 24 h with anti-CD3 antibodies and interleukin (IL)-2 in the presence of antigen-presenting cells (APCs, lethally irradiated splenocytes; Fig. 1). After incubating the activated Treg for 2 h with a neurotransmitter or a neuropeptide, we washed the cells and then co-cultured them with Teff. Proliferation of Teff co-cultured with activated Treg that had been incubated with dopamine (10⁻⁵ M) was more than twofold higher than proliferation in co-culture with activated Treg not incubated with dopamine (Fig. 1a). A significant effect on Treg suppressive activity was also obtained with 10⁻⁷ M dopamine (Fig. 1a), whereas 10⁻⁹ M had no significant effect (data not shown). The inhibitory effect of dopamine at 10⁻⁵ and 10⁻⁷ M on Treg activity was reproduced when freshly isolated (nonactivated) Treg were used (Fig. 1b). At dopamine concentration of 10⁻⁹ M, the obtained effect was slight and not
statistically significant (Fig. 1b). It should be noted, however, that the effect of dopamine on Treg suppressive activity was only partial, and that complete blocking was not seen at any of the concentrations tested.

We also examined the effect of dopamine on the activity of Treg that had been activated as described above (Fig. 1a), but for 96 h, and to which dopamine (10^{-5} M) was added for 2 h at the end of the activation period, and then washed off before the activated cells were co-cultured with naïve Teff. Again, Teff proliferation was significantly higher in the presence of activated Treg treated with dopamine than in the presence of activated Treg without dopamine (Fig. 1c). A direct effect of dopamine on Teff proliferation was ruled out by incubation of Teff for 2 h with 10^{-5} M dopamine, then washing off the dopamine and adding activated Treg without dopamine. The resulting proliferation of Teff did not differ from that seen in cultures of Teff in the absence of dopamine. Moreover, the inhibitory effects of Treg on naïve Teff and on Teff exposed to dopamine were similar (Fig. 1c), indicating that dopamine did not alter the susceptibility of Teff to Treg suppression.

The uptake of thymidine by Teff and the Treg-induced inhibition of such uptake varied from one experiment to another. In all experiments, however, the effect of dopamine on Treg (tested more than 20 times) was consistent, and in most cases the proliferation of Teff co-cultured with Treg treated with dopamine was more than twofold higher than that in the absence of dopamine treatment. The Treg used in this experiment were always obtained from naïve animals, therefore, it is unlikely that they contained any activated effector T cells. The purity of the Treg population used in all experiments was high (between 92% and 98% of the total CD4^+ population). Moreover, the use of anti-CD25 antibodies to isolate Treg reportedly does not interfere with either the suppressive activity or the state of activation of Treg (Thornton and Shevach, 1998).

In contrast to the effect seen with dopamine, no effect on the ability of Treg to suppress Teff proliferation could be detected when Treg were preincubated with different concentrations of norepinephrine (another member of the catecholamine
family; Fig. 1d), substance P (a pain- and stress-related neurotransmitter; data not shown), or serotonin (data not shown).

**Example 2. The effect of dopamine on Treg is exerted via type-1 family of dopamine receptors (D1-R)**

To establish whether the observed effect of dopamine on Treg is exerted through a receptor-mediated pathway, we employed specific agonists and antagonists of dopamine receptors. Incubation of Treg with $10^{-5}$ M SKF-38393, an agonist of the type-1 family of dopamine receptors (consisting of D1-R and D5-R), reproduced the dopamine effect (Fig. 2a). The specific D1-type antagonist SCH-23390 ($10^{-5}$M), when added together with dopamine ($10^{-5}$M), prevented the dopamine effect, further substantiating the contention that the effect of dopamine on Treg is mediated through the type-1 receptor family. Also in line with this contention was the finding that incubation of Treg with $10^{-5}$ M quinpirole, an agonist of the type-2 family of dopamine receptors (comprising D2-R, D3-R, and D4-R), had no effect on the suppressive activity of Treg. However, clozapine, an antagonist of D2-R, enhanced the dopamine-induced inhibitory effect, resulting in complete blocking of suppression (Fig. 2a).

**Example 3. Dopamine does not cause Treg apoptosis**

To exclude the possibility that dopamine exerts its effect by causing the death of Treg, we examined whether dopamine at the concentrations used here cause Treg apoptosis. No signs of apoptosis were detectable in Treg, which, after being incubated with dopamine, were stained with propidium iodide and analyzed for apoptotic cells (sub-G1) by flow cytometry (Fig. 2b). To further verify the absence of apoptotic death in Treg, after incubating Treg with dopamine we stained them for phosphatidylserine with annexin V. Again, we could not detect any signs of apoptosis in Treg beyond the background levels seen in the absence of dopamine (Fig. 2c). Thus, the reduction in Treg activity after their encounter with dopamine or
a related agonist, evidently results not from the death of Treg, but rather from alteration of their behavior.

**Example 4. Expression of dopamine type-1 and type-2 receptors in Treg and in Teff**

Since dopamine reduced the suppressive activity of Treg on Teff but did not alter the susceptibility of Teff to suppression by Treg, we examined the possibility that Teff and Treg express different subtypes or different amounts of the relevant dopamine receptors. This was done by assaying the expression of the dopamine type-1 receptors, D1-R and D5-R, in Treg and Teff, in the absence and in the presence of dopamine (incubation of the cells for 2 h with $10^{-5}$ M dopamine). PCR assays showed that Treg expressed significantly more D1-R and D5-R transcripts (4-fold and 14-fold, respectively) than Teff (Figs. 2d, 2e).

Incubation of the cells with dopamine did not significantly alter the number of D1-R transcripts in either Treg or Teff. The number of D5-R transcripts in Treg were also unchanged, but in Teff they showed a 10-fold increase, reaching numbers similar to those in Treg. In contrast, within the limit of error, such incubation did not change the number of D5-R transcripts in Treg. Dopamine did not significantly alter the number of D1-R transcripts in either Treg or Teff (Fig. 2d, 2e).

Because the suppressive effect of Treg on Teff is partly due to transforming growth factor (TGF)-β1 (Nakamura et al., 2001), we measured whether exposure of Treg to dopamine affects the level of TGF-β1 expression. Transcripts encoding TGF-β1, which might contribute to the suppressive effect of Treg, were decreased in Treg after dopamine treatment (Figs. 2d, 2e), suggesting that the observed blocking of suppression results, at least partially, from a decrease in expression of TGF-β1.

To further verify the differences in expression of dopamine receptors by Teff and Treg, we carried out real-time PCR, which showed that the amounts of D1-R and D5-R in Treg were 5-fold and 13-fold higher, respectively, than in Teff (Fig. 2f).
We also used PCR to assay the expression of dopamine type-2 family receptors, namely D2-R, D3-R, and D4-R in Treg and Teff. Although the expression of D4-R was somewhat more abundant in Teff than in Treg, the difference between the expression of each of these receptors in the two T-cell subpopulations was not significant (Figs. 2g, 2h), further substantiating our finding that the preferential effect of dopamine on Treg is through the family of D1-type receptors. To verify that the difference in D1-R between Treg and Teff observed at the transcript level is manifested also at the protein level, we subjected the cells to immunocytochemical analysis. D1-R-immunoreactivity was detected in naïve Treg, but not in naïve Teff (Fig. 2i).

Example 5. Dopamine affects CTLA-4 expression and IL-10 production by Treg

To gain further insight into the mechanism whereby dopamine affects Treg activity we examined CTLA-4, a molecule characteristic of Treg (Im et al., 2001). Expression of this molecule was slightly but consistently decreased upon exposure of Treg to dopamine. A similar effect on CTLA-4 expression was obtained with the D1-type specific agonist SKF-38393 (Fig. 2j).

Another molecule that participates in the suppressive activity of Treg is IL-10 (Maloy et al., 2003). It was therefore of interest to measure the production of IL-10 by Treg after their exposure to dopamine. Media collected after incubation of Treg with dopamine (10^{-5} M) for 24 h, 48 h and 72 h showed a significant decrease in the amount of IL-10 at all time points examined (Fig. 2k).

Dopamine did not, however, alter the anergic state of Treg; production of IL-2 was not detected in Treg that had been incubated in the presence of dopamine, as verified by ELISA for a secreted cytokine in media conditioned for 48 h by activated Treg (Fig. 2l). Teff, as expected, secreted IL-2, the level of which was not affected by dopamine (Fig. 2l). It should be noted that activation of both T cell populations was carried out in the absence of mIL-2.
Example 6. Dopamine does not affect Foxp3 expression by Treg

A gene encoding the Foxp3 protein was recently found to be associated with Treg (Hori et al., 2003; Ramsdell, 2003). We therefore examined whether the dopamine-induced reduction of Treg activity alters the expression of this gene. mRNA isolated from Treg that were activated for 24 h, exposed for 2 h to dopamine, and maintained in culture for a further 30 min or 24 h was analyzed for Foxp3 expression. Foxp3, as expected, was detected in Treg, but no significant change in its expression was observed after Treg were exposed to dopamine for 30 min (Fig. 2m) or 24 h (data not shown).

Example 7. ERK1/2 is deactivated by dopamine in Treg

The finding that dopamine down-regulated Treg activity via D1-type but not D2-type receptors, taken together with the recent report that the ERK pathway can be activated by D1-R-dependent signaling (Takeuchi and Fukunaga, 2003), led us to suspect that the down-regulatory effect of dopamine on the suppressive activity of Treg might be exerted via the ERK pathway. To examine this possibility we first treated Treg with the protein tyrosine kinase inhibitor genistein (4',5,7-trihydroxy isoflavone), which inhibits ERK and MEK activation (Mocsai et al., 2000). This treatment blocked the suppressive activity of Treg on Teff (Fig. 3a). It is interesting to note that, in the presence of genistein, Treg not only lost their suppressive activity but even underwent proliferation themselves. Genistein at the same concentration had no effect on the proliferation of Teff (Fig. 3a).

In light of these results, we also examined whether Treg activity is affected by PD98059, a specific MEK inhibitor that blocks the ERK1/2 signaling pathway (Sharp et al., 1997). PD98059 significantly reduced the suppressive activity of Treg relative to that of control activated Treg (Fig. 3b).

The above findings prompted us to examine the state of ERK phosphorylation in activated Treg and in Treg that were activated in the presence of dopamine. Treg were activated for 20 min in the presence or absence of dopamine (10^{-5} M), and were then subjected to intracellular phosphoprotein staining (Perez
and Nolan, 2002) and analyzed by flow cytometry. Significantly less phosphorylated ERK was detected in Treg that were activated in the presence of dopamine than in activated Treg without dopamine (data not shown). As a measure of the background nonspecific staining of the phosphorylated ERK we used a specific peptide of phospho-ERK, which competes for binding of the antibody. The intracellular staining procedure for phospho-ERK detection by FACS was validated by the use of Teff incubated with 20 μM phorbol 12-myristate 13-acetate (PMA), known to stimulate the activity of protein kinase C (PKC) (data not shown). To further substantiate these findings, we performed Western blot analysis of phospho-ERK1/2 expression in lysates of Treg and Teff after the cells had been activated with anti-CD3 and anti-CD28 for 20 min in the presence or absence of dopamine. Significantly more phosphorylated ERK1/2 was detected in activated Treg than in activated Teff. Moreover, phospho-ERK1/2 was found to be down-regulated in Treg that had been activated in the presence of dopamine (Fig. 3c). ERK1/2 phosphorylation in Treg was also reduced by the specific D1-type receptor agonist SKF-38393 (Fig. 3d). Results of quantitative analysis of the phospho-bands are shown in Fig. 3e.

Example 8. Dopamine alters the adhesive and migratory properties of Treg

One of the main features of T cells is their ability to migrate to tissues in need of rescue or repair [such as diseased or damaged CNS (Hickey, 1999; Flugel et al., 2001). We therefore considered the possibility that dopamine reduces not only the suppressive activity but also the migratory ability of Treg. Since T cell migration and adhesion have been linked to ERK activation (Tanimura et al., 2003), this assumption appeared even more feasible in light of the above observation that dopamine reduced ERK activation in Treg. We therefore incubated Treg with dopamine for 2 h and then examined their adhesion to chondroitin sulfate proteoglycans (CSPG), extracellular matrix proteins often associated with injured tissues (Jones et al., 2003). The ability of Treg to adhere to CSPG was significantly greater than that of Teff (Fig. 4a), and was significantly decreased, in a
concentration-dependent manner (10^{-9}–10^{-5} M), by dopamine (Fig. 4a). The dopamine effect on Treg could be mimicked by the D1-type specific agonist SKF-38393 and inhibited by the D1-type antagonist SCH-23390. Dopamine had only a slight, non-significant effect on the adhesion of Teff to CSPG (Fig. 4a). The ability of Treg to adhere to fibronectin was greater than that of Teff (Fig. 4b). Exposure to dopamine resulted in no effect on adhesion of Treg to fibronectin and a slight increase in the adhesion of Teff (Fig. 4b).

To verify that the effect of dopamine on adhesion of Treg is exerted through the ERK1/2 pathway, we incubated Treg with the ERK1/2 signaling pathway inhibitor PD98059 before carrying out the adhesion assay. PD98059 significantly reduced the ability of Treg to adhere to CSPG (Fig. 4c). Since interaction of T cells with CSPG is mediated in part by the CD44 receptor (Henke et al., 1996), and in light of the known dependence of CD44 expression on the ERK signaling pathway, it was conceivable that dopamine might affect the expression of CD44 in Treg. To examine this possibility, we assayed CD44 immunoreactivity in Treg and Teff that had been activated with anti-CD3 and anti-CD28 antibodies for 24 h and then incubated for 2 h with or without dopamine. In the absence of dopamine, CD44 immunoreactivity was significantly stronger in Treg than in Teff. Dopamine decreased CD44 immunoreactivity in Treg but not in Teff (Fig. 4d). Other adhesion-molecule receptors that we tested, such as LFA-1, I-CAM, and V-CAM (Lee and Benveniste, 1999), did not show any dopamine-related changes in Treg (data not shown).

Migration of Treg in humans is dependent on the chemokine receptors CCR-4 and CCR-8, which are abundantly present on Treg (Sebastiani et al., 2001). We therefore examined whether exposure to dopamine would also affect Treg migration. For this experiment we used a normal population of CD4^+ T cells, of which Treg (CD4^+CD25^+) accounts for approximately 11% (Fig. 4e). Of the CD4^+ cells that migrated towards CCL22 (MDC; a chemokine for CCR-4), 17% were Treg (CD4^+CD25^+), pointing to the greater migratory ability of Treg than of Teff towards MDC. However, after exposure of the CD4^+ cell population to dopamine,
migrating Treg accounted for approximately 10% (the same as their percentage in the total CD4\(^+\) population at the start of the experiment), suggesting that after their exposure to dopamine Treg lost their preference for migration towards MDC.

We also examined the migration of a mixed T cell population towards SDF-1. Migration of Teff towards SDF-1 was significantly greater than that of Treg (post-migration percentage of Treg in the total CD4\(^+\) population was less than 4%), and dopamine did not alter this pattern (Fig. 4e). To examine the direct effect of dopamine on the migration of Treg, we assayed the effect of dopamine on the migration of purified Treg towards MDC. The migratory Treg were stained for CD4 to ensure that cell debris and aggregates would not be counted among them. Dopamine almost completely abolished Treg migration (Figs. 4f, 4g), but had no effect on the migration of Teff (data not shown).

In an attempt to link the changes in migration to specific receptors, we examined the expression of mRNA for CCR-4, CCR-8, and CXCR-4. Before the cells were exposed to dopamine, their CCR-4 expression—as expected from previous findings in human Treg (Sebastiani et al., 2001, 2002)—was significantly higher in Treg than in Teff, but upon exposure to dopamine the expression of CCR-4 in Treg was decreased (Figs. 4h, 4i). The expression of mRNA encoding for CXCR-4 and CCR-8 did not change in Treg after these cells were exposed to dopamine (data not shown).

Example 9. Exogenous dopamine increases the ability to fight off neurodegeneration

A previous study by our group showed that injection of activated Treg into mice (Balb/c) immediately after CNS injury significantly inhibits the spontaneous neuroprotective response, with the result that fewer neurons survive the consequences of the insult (Kipnis et al., 2002a). In the same study we showed that depletion of Treg increases the ability to withstand the insult. The present observation that Treg and Teff respond differentially to dopamine prompted us to
examine the effect of dopamine on the ability to withstand neurotoxic conditions in vivo.

**9a. Dopamine affords neuroprotection after CNS injury**

Reasoning that systemic injection of dopamine after a CNS insult would be expected to improve recovery after a mechanical CNS injury, we subjected two groups (n=12 in each group) of BALB/c mice to a severe optic nerve crush injury (a known model of secondary neuronal degeneration) and immediately thereafter injected the mice in one group with dopamine (0.4 mg/kg) and those in the other group with PBS. Two weeks later their retinas were excised and neuronal survival assessed. Significantly more viable neurons (1110 ± 56 /mm², mean ± SD) were found in the retinas of dopamine-injected mice than in the retinas of vehicle-treated mice (789 ± 23; Fig. 5). Thus systemic injection of dopamine led to an increased ability to cope with consequences of optic nerve injury.

**9b. Dopamine protects from neuronal toxicity induced by glutamate**

To assess whether the beneficial effect of systemic dopamine is a general phenomenon, rather than unique to a single animal model, we used a model of neuronal toxicity induced by glutamate, a common player in many neurodegenerative conditions (Katayama et al., 1990; Xiong et al., 2003; Jiang et al., 2001). Injection of glutamate into the eyes of adult mice causes retinal ganglion cell death that is measurable 1 week after the injection (Mizrahi et al., 2002; Schori et al., 2001a; Katayama et al., 1990). We injected Balb/c mice intraperitoneally (i.p.) with the dopamine (0.4 mg/kg) or its specific D1-type agonist SKF-38393 (3.3 mg/kg), or its specific D1-type antagonist SCH-23390 (3 mg/kg), immediately after their exposure to glutamate toxicity. We also injected scid Balb/c mice with SKF-38393 (3.3 mg/kg) immediately after glutamate intoxication. Since the glutamate toxicity model, irrespective of the treatment approach, leaves only a narrow therapeutic window, the number of protected neurons is expressed here as a percentage of the total number of neurons amenable to protection. A single systemic
injection of dopamine (0.4 mg/kg) or its D1-type agonist given immediately after intraocular injection of a toxic dose of glutamate increased neuronal survival by 18 ± 2.5 or 19 ± 3.2 %, respectively, relative to that in glutamate-injected controls treated with PBS (Table 1). Injection of the same agonist to scid mice resulted in no effect, thus supporting the assumption that systemic dopamine benefit CNS neurons via the peripheral immune system. As a corollary, injection of the D1-type antagonist resulted in a decrease in neuronal survival (11 ± 1.5%, p < 0.01; Table 1) relative to that in PBS-injected mice. The above results suggested that dopamine might be one of the endogenous signals initiating the cascade that leads to spontaneous T cell-dependent neuroprotection. Accordingly, a single injection of mice with a D1-type antagonist would be expected to exacerbate neuronal survival, as it would compete with the endogenous dopamine for reduction of the suppressive activity of Treg after an injury.
Table 1. Neuronal survival following glutamate intoxication in mice injected with dopamine or its type-1 receptor agonist and antagonist.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dopamine</th>
<th>SKF - 38393</th>
<th>SCH – 23390</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>18 ± 2.5***</td>
<td>19 ± 3.2**</td>
<td>-11 ± 1.5**</td>
</tr>
<tr>
<td>SCID</td>
<td>NT</td>
<td>3 ± 1.8 (ns)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Immediately after glutamate intoxication mice were systemically injected with the indicated drugs. Neuronal survival was determined ten days later (see Materials and Methods). The results are expressed by changes (in percentage) in neuronal survival in treated mice relative to untreated mice. Each value represents a mean ± SEM of a group of at least 5 animals, and each experiment was performed at least twice, independently. Asterisks (***, P < 0.001; **, P < 0.01) indicate statistical significance of the presented data from a single experiment using a Student’s T-test statistical analysis. (NT – not tested; ns – no statistical significance).

Example 10. Exposure of Treg to dopamine in vitro reduces their suppressive activity in vivo.

To unequivocally show the direct effect of dopamine on Treg activity in vivo, we examined whether direct exposure of Treg to dopamine can reduce their suppressive activity in a model of neuronal survival. Systemic injection of Treg after glutamate intoxication significantly reduced the ability of the mice to withstand the glutamate toxicity and resulted in a 25% increase in neuronal death. We further found, however, that incubation of Treg with dopamine prior to their systemic injection into mice abolished their suppressive effect, indicated by the lack of change in the number of surviving neurons. No effect on neuronal survival after glutamate intoxication could be detected in control mice injected with Teff (Fig. 30).
6a). Fig. 6b shows representative micrographs of fields from retinas excised from mice that were exposed to intravitreally injected glutamate and then injected with either CD4^{+}CD25^{+} or CD4^{+}CD25^{-}.

Example 11. The D1-R agonist SKF-38393 protects mice from glutamate
toxicity

Mice were injected intra-ocular with a toxic dose of glutamate followed by
an immediate injection i.v of the D1-R family agonist SKF-38393. Retinas were
excised 7 days afterwards and survived neurons were counted. The results are
depicted in Fig. 7. Mice injected with 3.3 mg/kg of SKF-38393 showed significant
increase in neuronal survival compared to vehicle-injected mice. Injection of a
lower dose of SKF-38393 (0.33 mg/kg) showed a neuroprotective trend, however
not significant.

Example 12. Clozapine alone or with dopamine protects mice from glutamate
toxicity

Mice were injected with a toxic dose of glutamate into the eyes followed by
an immediate injection i.v of the D2-R family antagonist clozapine (5 mg/kg) or
with clozapine in combination with dopamine (a mixture of 0.4 mg/kg of dopamine
with 0.6 mg/kg of clozapine). Retinas were excised 7 days afterwards and survived
neurons were counted. The results are depicted in Fig. 8. Mice injected with
clozapine alone showed a significant increase in neuronal survival compared to
vehicle-injected mice. Moreover, mice injected with clozapine in combination with
dopamine showed even higher neuronal survival.

Example 13. Effect of dopamine/clozapine in partial spinal cord injury

Adult male C57BL/6j mice were anesthetized by intraperitoneal
administration of ketamine (80 mg/kg) and xylazine (16 mg/kg) and their spinal
cords were exposed by laminectomy at the level of T8. Thereafter, we dropped a
200 DPI for 1 sec on the laminectomized cord (defined as a "partial" injury), using
the NYU impactor (first developed at New York University), a device shown to
infllicting a well calibrated contusive injury of the spinal cord (Basso et al., 1996; Hauben et al., 2000a, 2000b).

For assessment of recovery from spinal cord contusion, functional recovery was determined by locomotor hindlimb performance. This was scored with the open-field locomotor rating scale of Basso, Beattie, and Bresnahan (BBB) on a scale of 0 (complete paralysis) to 21 (normal mobility) (Basso et al., 1996; Hauben et al., 2000a, 2000b). Blind scoring ensured that observers were not aware of the treatment received by each rat. Approximately once a week we evaluated the locomotor activities of the trunk, tail, and hindlimbs in an open field by placing the animal for 4 min in the center of a circular enclosure (90 cm in diameter, 7 cm wall height) made of molded plastic with a smooth, nonslip floor. Before each evaluation the mouse was examined carefully for perineal infection, wounds in the hindlimbs, and tail and foot autophagia.

In the experiment, a mixture of 0.4 mg/kg of dopamine with 0.6 mg/kg of clozapine was injected in the mice (n=6/7) immediately after partial spinal cord contusion (200 DPI for 1 sec). Control animals (n=6/7) were injected with vehicle (PBS). The animals were evaluated neurologically over the days following contusion by assessing locomotor activity in an open field with the BBB locomotor rating scale. The results depicted in Fig. 9a show that rats injected with clozapine/dopamine (black squares) recovered significantly better than the PBS-treated control (gray squares) rats. This shows that clozapine/dopamine confers significant neuroprotection in male C57 mice. The neuroprotective effect of this treatment was statistically significant (*, p < 0.05 and **, p <0.01, two-tailed student’s t test). Fig. 9b depicts the scores recorded for individual mice at the last time point that was examined.

Example 14. Effect of dopamine, agonists and/antagonists in acute and chronic glaucoma

Glaucoma is now recognized as a chronic neurodegenerative disease, characterized by the slow, progressive degeneration of RGCs, causing a gradual loss
of visual field and leading eventually to blindness. The primary cause of the disease is not yet known and the factors contributing to its progression are not yet fully characterized. Blockage of aqueous outflow causes an increase in intraocular pressure (IOP), which results in RGC death (Schori et al., 2001a). Increased IOP is considered the major risk factor and believed to be the primary cause of neuronal death. Though IOP reduction significantly reduces the extent of neuronal loss, loss of RGCs may continue even after the IOP has been reduced. Moreover, optic nerve degeneration sometimes occurs in the absence of elevated IOP, a condition called normal tension glaucoma (occurring in approximately one third of glaucoma patients). The present inventors have attributed the ongoing loss of neurons to secondary factors that continue to cause degeneration of neurons (RGCs and their fibers) after the primary insult (e.g. increased IOP) is removed.

Besides the mouse model of intraocular glutamate toxicity described above, the neuroprotective effect of dopamine alone or with clozapine is examined in rat models of irreversible (chronic) or transient (acute) elevation of IOP.

**Materials and methods**

*Animals.* Inbred adult male Lewis and SPD rats (8 weeks; average weight 300 g) were supplied by the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel) and maintained in a light- and temperature-controlled room and were matched for age and weight before each experiment. All animals were handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee).

*Chronic glaucoma: Induction of high IOP.* Male Lewis rats are anesthetized with a mixture of ketamine (15 mg/kg), acepromazine (1.5 mg/kg), and xylazine (0.3 mg/kg). An increase in IOP is achieved by laser photocoagulation of the limbal and episcleral veins. Rats receive 2 laser treatments, 1 week apart, with a blue-green argon laser (1 watt for 0.2 s, delivering a total of 130–150 spots of 50 or 100 μm in the 2 treatments; Coherent, Palo Alto, CA). IOP is measured once a week using TONO-PEN (Mentor, Norwell, MA), after injecting the rats intramuscularly
with acepromazine (3.0 mg/kg) and applying procaine 0.5% topically on the eyes to anesthetize the cornea.

**Acute glaucoma: Induction of high IOP.** In our rat model of acute glaucoma, the IOP is transiently elevated for one hour using Ringer fluid reservoir connected via a 27-gauge needle to the anterior chamber of the deeply anesthetized rats (ketamine hydrochloride 50 mg/kg, xylazine hydrochloride 0.5 mg/kg, injected intramuscularly). The reservoir height is adjusted to give a pressure of 50 mmHg. This is maintained for one hour during which IOP measurements are taken using a tonopen (Tonopen XL). Twenty-four hours after removal of the needle from the anterior chamber, normal IOP values (lower than 20 mmHg) are measured.

**Measurement of IOP.** Most anesthetic agents cause a reduction in IOP, thus precluding reliable measurement. To obtain accurate pressure measurements while the rat is in a tranquil state, the rat is injected intraperitoneally (i.p.) with 10 mg/ml acepromazine, a sedative drug that does not reduce IOP. Five minutes later, Localin is applied to the corneas of both eyes and the pressure in both eyes is measured using a Tono-Pen XL tonometer (Automated Ophthalmics, Ellicott City, MD, USA). Ten measurements are taken from each eye and the averages are calculated. Because of the reported effect of anesthetic drugs on IOP measured by Tono-Pen, we always measure at the same time after acepromazine injection and calculate the average of the 10 values received from each eye. Measurements are performed every 2 days for 3 weeks, all at the same time of day. One week after the first laser treatment, the IOP reaches levels of about 30 mmHg and remains without any significant change until the end of the experiment (3 weeks after the first laser treatment). IOP in the untreated contralateral eye remains normal.

**Anatomical assessment of retinal damage caused by the increase in IOP:**

**RGC survival.** The hydrophilic neurotracer dye dextran tetramethylrhodamine (Rhodamine Dextran) (Molecular Probes, Oregon, USA) is applied 3 weeks after the first laser treatment directly into the intraorbital portion of the optic nerve. Only axons that survive the high IOP and remain functional, and whose cell bodies are still alive, can take up the dye and demonstrate labeled RGCs. The rats are killed
hours after dye application and their retinas are excised, whole mounted, and preserved in 4% paraformaldehyde. The labeled RGCs are counted under magnification of ×800 in a Zeiss fluorescence microscope. Four fields from each retina are counted, all with the same diameter (0.076 mm²) and located at the same distance from the optic disc. Eyes from untreated rats are used as a control. RGCs are counted by an observer blinded to the identity of the retinas.

14a. Effect of dopamine in the model of chronic glaucoma

SPD rats are injected i.v., with dopamine one hour after the first laser treatment. A control group is injected with vehicle (PBS) alone. Protection of RGC is calculated as percentage of cells survived in the treated group out of the total cell loss in the non-treated group. Rats injected with dopamine are expected to show significant increase in the number of surviving RGCs compared to the control rats (even if the IOP remains elevated throughout the experiment).

Similar results are expected with a D1-agonist (SKF-39393) or a D2-R antagonist (clozapine) or with a combination of dopamine and clozapine or of SKF-38393 and clozapine.

14b. Effect of dopamine in the model of acute glaucoma

Rats with transient IOP elevation (acute glaucoma model) are injected with dopamine i.v. immediately after removal of the needle. A control group is injected with vehicle (PBS) alone. Protection of RGC is calculated as percentage of cells survived in the treated group relative to the total cell loss in the non-treated group. Rats injected with dopamine are expected to show significant reduction of RGC loss induced by transient IOP elevation, compared to the control rats.

Similar results are expected with a D1-agonist (SKF-39393) or a D2-R antagonist (clozapine) or with a combination of dopamine and clozapine or of SKF-38393 and clozapine.
Example 15. Effect of dopamine, D1-R agonists and/or D2-R antagonists in ALS

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig’s disease, is the most common form of motor neurone diseases (MND), a group of related diseases affecting the motor neurones in the brain (upper motor neurons) and spinal cord (lower motor neurons). It is a chronic, progressive neurodegenerative disease characterized by gradual degeneration of the nerve cells in the CNS that control voluntary muscle movement, muscle weakness, stiffness and fasciculations.

Excessive glutamate rapidly kills cells in the brain and spinal cord. Cells of ALS patients and animal models have been shown to exhibit major defects in glutamate neurotransmission and it is established that glutamate-based neurotoxicity is part of ALS, part of a process leading to motor neurons’ death. The sole drug approved for treatment of ALS is Riluzole, a glutamate release inhibitor. However, its effects are clearly modest - it can prolong the life of ALS patients approximately three months, but it does not halt the degenerative disease or repair damage already incurred. Therefore, the search for additional therapies continues.

The results in the Examples 9, 11 and 12 above show that dopamine, the D1-R agonist SKF-38393, the D2-R antagonist clozapine alone or together with dopamine protect mice from glutamate toxicity and indicate that these agents are suitable candidates for treatment of ALS and other motor neurone diseases.

Protection from motor nerve degeneration in transgenic mutant SOD1 mice

To test whether the agent according to the invention can protect from the progression of motor neuron degeneration, an animal model of ALS is used, for example, transgenic mice overexpressing the defective human mutant SOD1 allele containing the Gly93→Ala (G93A) gene (B6SJL−TgN (SOD1−G93A)1Gur (herein “ALS mice”) (from The Jackson Laboratory, Bar Harbor, ME, USA). ALS mice (n=15) are injected once or more times with different doses of the different agents in PBS, at different ages (for example, when they are 45 days old). A control group (n=15) of ALS mice is injected with PBS. The mice are then tested several times
per week for muscle strength, by blindly testing the time of hanging on a rotating vertical rod. Each experiment lasts 5 min.

The onset of the decline in muscle strength varies among individual mice. To assess the effect of the immunization on the rate of decline in each mouse, the muscle strength at any given time is compared to that found one week before the decline began. The effect of the agent is assessed by the average hanging time for each animal per week. Mice injected with dopamine, a D1-R agonist, e.g. SKF-38393, a D2-R antagonist, e.g. clozapine or a combination of a D1-R agonist or dopamine with a D2-R antagonist are expected to exhibit longer hanging time than the non-immunized mice, a significantly lower rate of muscle strength decline, regardless of their strength on the day of immunization, and to retain motor power for a longer period of time as compared to non-immunized animals.

Example 16. Effect of dopamine, D1-R agonists and/or D2-R antagonists in Huntington's disease

Huntington's Disease (HD) is a degenerative disease whose symptoms are caused by the loss of cells in the basal ganglia of the brain. This damage to cells affects cognitive ability (thinking, judgment, memory), movement, and emotional control. HD is characterized by uncontrollable, dance-like movements and personality changes.

The beneficial effect of an agent of the invention is examined for exertion of neuroprotective effects using the HD R6/2 transgenic mice test system or another suitable model. R6/2 transgenic mice overexpress the mutated human huntingtin gene that includes the insertion of multiple CAG repeats (obtainable from The Jackson Laboratory). These mice show progressive behavioral-motor deficits starting as early as 5-6 weeks of age, and leading to premature death at 10-14 weeks. The symptoms include low body weight, claspings, tremor and convulsions.

Different doses of the different agents are injected to HD R6/2 transgenic mice at different ages, once or at different intervals (4 weeks or more). The control group is injected with PBS starting on the same age as the tested group and
thereafter at the same intervals. Motor neurological functions are evaluated using the rotarod performance test which assesses the capacity of the mice to stay on a rotating rod. For this test, mice are placed on a rod rotating at 2, 5, 15 and 25 rpm. The time until the mouse falls off the rotating rod (best of three attempts, up to 180 sec for each trial) is used as the measure of animal motor-function. Each mouse is tested twice weekly and the two scores averaged. Mice injected with dopamine, a D1-R agonist, e.g. SKF-38393, a D2-R antagonist, e.g. clozapine or a combination of a D1-R agonist or dopamine with a D2-R antagonist are expected to exhibit an improved rotarod performance compared to untreated (control) mice.

Discussion

The results above show that dopamine reduces the suppressive and trafficking activities of Treg through a family of type-1 dopamine receptors (D1-R and D5-R, found here to be abundantly expressed by Treg) via the ERK signaling pathway. The physiological and pharmacological effects of dopamine, as a compound capable of down-regulating Treg activity needed for fighting off neurodegeneration by T cell-dependent mechanism, is shown in models of CNS insult.

Glutamate is a common mediator of CNS neurodegenerative conditions (Urushitani et al., 1998; Rothstein, 1995-96; Newcomer et al., 1999; Lasley and Gilbert, 1996; Gunne and Andren, 1993). Recent studies strongly suggest that the ability to withstand CNS insults, including glutamate toxicity, is T-cell dependent and is amenable to boosting by self-antigens residing in the site of damage (Moalem et al., 1999; Mizrahi et al., 2002; Yoles et al., 2001; Kipnis et al., 2001; Schori et al., 2001a, 2001b; Hauben et al., 2000a, 200b; Wekerle, 2000).

An alternative way to achieve beneficial enhancement of the autoimmune response against the self-antigens needed for protection and repair after a CNS injury or for fighting off tumors is by eliminating the normally suppressive effect of Treg (Kipnis et al., 2002a; Sakaguchi et al., 2001; Shimizu et al., 1999). Physiological compound(s) that control Treg activity on a daily basis probably
underlie the mechanisms whereby the body overcomes commonly occurring adverse conditions, which in most cases resolve without development of tumors or neuronal degeneration.

The results of the present invention indicate that one such physiological compound is dopamine. In this context it is important to note that transient changes in dopamine levels in mesolimbic brain areas in rats, associated with neuronal activity, can reach concentration as high as 600 nM (Gonon, 1997; Floresco et al., 2003; Wightman and Robinson, 2002). It was also reported that blood levels of dopamine are elevated in patients with certain types of tumors (Saha et al., 2001).

According to the present invention, dopamine reduced Treg activity, and this was correlated with a decrease in ERK1/2 activation. In line with this observed correlation was the finding that adhesive and migratory abilities of Treg (Pozzi et al., 2003; Takeuchi and Fukunaga, 2003; Tanimura et al., 2003; Lohse et al., 1996; Schneider et al., 2002; Yi et al., 2002), were reduced by dopamine via the ERK pathway.

Treg might exert their suppressive activity on Teff (autoimmune T cells) either in the lymphoid organs or at the site of the threat (degeneration or tumor growth). Mediation of the suppressive activity of Treg has been attributed partially to IL-10 and CTLA-4, whereas their migration and adhesion have been attributed to the specific repertoire of chemokine receptors and adhesion molecules that they express (Kohm et al., 2002; Sebastiani et al., 2001). Reduction of the suppressive activity of Treg was correlated with a decrease in their IL-10 production and CTLA-4 expression, which might participate in the cytokine-mediated and cell–cell mediated suppression by Treg, respectively. Moreover, Treg express relatively large amounts of the CD44 receptor (needed for their adhesion to CSPG) and the chemokine receptor CCR-4 (needed for their migratory ability). Exposure of Treg to dopamine resulted in a decrease in both their adhesion to CSPG and their migration towards MDC, in correlation with their diminished expression of CD44 and CCR-4, respectively.
The ability of dopamine to affect Treg and Teff differently, as observed in the present invention, is probably related to both the unique nature of dopamine receptors and the nature of their expression on these two T-cell populations. We found that significantly more D1-R and D5-R are expressed by Treg than by Teff. The marked difference in D1-R and D5-R expression, which is hardly detectable on Teff or any other immune cells (Ricci et al., 1997), makes the D1-type receptor family a likely candidate for the dialog of dopamine with Treg, leading—via the ERK pathway—to reduction of the suppressive activity of Treg. It is interesting to note that D2-R, which antagonizes D1-R, activates ERK (Pozzi et al., 2003).

We found that the effect of dopamine on the suppressive activity of Treg was weak compared with the effect of a protein tyrosine kinase inhibitor such as genistein (Mocsai et al., 2000) or the ERK1/2 signaling pathway inhibitor PD98059, indicating that dopamine is a suitable candidate as a physiological immunomodulator mainly in the context of autoimmune activity.

Treg exist in a state of anergy, neither proliferating in response to mitogenic stimuli nor producing IL-2. Although dopamine down-regulated the suppressive activity of Treg, it did not reverse the anergic state of these T cells with respect to proliferation or IL-2 production, supporting the contention that dopamine induces changes in the activity rather than in the phenotype of Treg. Unlike dopamine, genistein not only blocked the activity of Treg but also triggered their proliferation, suggesting that under extreme conditions the phenotype of Treg might change.

The in-vivo relevance of the effect of dopamine on the suppressive activity of Treg was demonstrated according to the present invention in the experimental paradigms of glutamate intoxication in the mouse eye and mouse optic nerve mechanical crush injury. After glutamate intoxication, passive transfer of Treg suppressed the ability to resist neurodegeneration, as indicated by an increased loss of neurons. Incubation of Treg with dopamine prior to their transfer wiped out their suppressive effect on neuronal survival. The loss of Treg activity in vivo might reflect the effect of dopamine both on homing of Treg to the damaged site and on their suppression. To further investigate the potential activity of dopamine as an
immunomodulator, we injected it systemically. Significantly more neurons survived a neurotoxic insult in mice injected with dopamine or its D1-type agonist than in PBS-injected controls. A similar effect was obtained when the mouse received a systemic injection of dopamine after an optic nerve crush injury.

It is important to note that dopamine, when injected systemically, does not cross the blood–brain barrier. The observed lack of effect of the D1-type agonist SKF-38393 in nude mice (which are devoid of mature T cells) substantiated our conclusion that the effect of peripheral dopamine on neuronal survival is exerted via the immune system and not directly on neural tissue. The potential ability of endogenous dopamine to operate spontaneously *in vivo* as a stress-related signal, emitted by the CNS to the peripheral immune system after a CNS insult, was demonstrated in mice injected with SCH-23390 immediately after glutamate intoxication. Mice injected with this D1-type antagonist showed a slight (11%) but significant decrease in neuronal survival. The weak effect of SCH-23390 on neuronal survival appears to be attributable, at least in part, to the nature of the experimental model. Thus, under the experimental conditions of this study, nude mice lost approximately 30% of their neurons relative to the wild type (Kipnis et al., 2002a; Schori et al., 2002). The 10% decrease observed in the wild-type mice resulting from manipulation of Treg activity therefore represents more than 30% of the maximal possible effect. It is also possible that dopamine is a member of a family of physiological compounds capable of controlling Treg activity after a CNS insult.

Previous studies have documented the effect of dopamine on T cell adhesion (Levite et al., 2001), on activation (Ilani et al., 2001), and on T-lymphocyte suppression of IgG production by peripheral blood mononuclear cells (Kirtland et al., 1980). No attempt was made in any of those studies to attribute the dopamine effect to subpopulations of CD4+ T cells. Subsequent studies showed that dopamine exerts its effect only on activated T cells (Ilani et al., 2001). Our results suggest that dopamine has a direct and preferential effect on Treg in initiating the immune response but will not circumvent the need for the two signals known to be needed
for eliciting a T cell response [antigen recognition by T cells on class II major histo-
compatibility complex (MHC-II) proteins and co-stimulatory molecules
(Bretscher and Cohn, 1970)]. It was recently suggested that in the presence of
strong immunogens, Teff, with the aid of APCs, can overcome the suppression
imposed by Treg (Pasare and Medzhitov, 2003). This mechanism is not likely to
operate in response to self-antigens, possibly because the self-antigens are neither
present in sufficient amounts nor sufficiently potent to induce the needed response.

In light of the observed effect of dopamine on Treg in the present invention,
the uncontrolled presence of dopamine known to occur in patients with mental
disorders (such as schizophrenia) might explain the high incidence of aberrant
immune activity in these patients (Muller et al., 2000). It is also interesting to note
the relatively low incidence of cancer development observed in patients with
schizophrenia (Teunis et al., 2002), in whom dopaminergic activity is known to be
pronounced. This apparently unexplained phenomenon could be interpreted in light
of the present finding of the dopamine effect on Treg, as well as the known
participation of autoimmune T cells in fighting off cancer (Dummer et al., 2002).

The observed correlation between the state of ERK activation and the
activity of Treg opens the way, via dopamine or its related compounds, to novel
therapeutic strategies for fine-tuning of Treg activity, and hence for fighting off
conditions in which Treg activity needs to be weakened (such as neuronal
degeneration and cancer) or strengthened (autoimmune diseases).

Dopamine as well as dopamine agonists or antagonists might thus be
candidates for therapy against tumors and neurodegenerative diseases or
autoimmune diseases, respectively. In light of the observed effect of dopamine on
Treg according to the present invention, the uncontrolled presence of a stress signal
such as dopamine might explain the aberrant immunity in patients with mental
disorders (such as schizophrenia) associated with large amounts of dopamine,
whereas deficiency in dopamine (as in Parkinson’s disease) might explain
accelerated neuronal loss.
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CLAIMS:

1. A method for treating a neurodegenerative condition, disorder or disease other than Parkinson’s disease, said method comprising administering to an individual in need an agent that down-regulates the suppressive activity of CD4⁺CD25⁺ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff), in an amount effective to protect said individual from neurotoxic conditions, wherein said agent is selected from the group consisting of:
   (i) dopamine or a pharmaceutically acceptable salt thereof;
   (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof;
   (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof;
   (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof;
   (v) a combination of (i) and (ii); and
   (vi) a combination of (i), (ii) or (iii) with (iv).

2. A method according to claim 1, wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

3. A method according to claim 1, wherein said agent is a combination of dopamine and the dopamine precursor levodopa, optionally in further combination with carbidopa.

4. A method according to claim 1, wherein said agent is a dopamine D1-R agonist.

5. The method according to claim 4, wherein said dopamine D1-R agonist is selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.
6. A method according to claim 1, wherein said agent is a dopamine D2-R antagonist.

7. The method according to claim 6, wherein said dopamine D2-R antagonist is selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

8. A method according to claim 1, wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.

9. The method according to claim 8, wherein said agent is a combination of dopamine with clozapine.

10. A method according to claim 1, wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.

11. The method according to claim 10, wherein said agent is a combination of SKF-38393 and clozapine.

12. A method according to any one of claims 1 to 11 wherein said neurodegenerative condition is a neuronal degeneration caused by a primary injury, and said agent is administered in an amount effective to reduce neuronal degeneration caused by said primary injury.

13. A method according to claim 12 wherein said primary injury is spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke, cerebral ischemia, and injury caused by surgery such as tumor excision.
14. A method according to claim 1 wherein said neurodegenerative condition, disorder or disease is associated with the eye, and said agent is administered in an amount effective to reduce neuronal degeneration caused by said condition, disorder or disease associated with the eye.

15. A method according to claim 14 wherein said neurodegenerative condition, disorder or disease associated with the eye is non-arteritic optic neuropathy, age-related macular degeneration, a retinal disorder or a disease associated with elevated intraocular pressure.

16. A method according to claim 15, wherein said disease associated with abnormally elevated intraocular pressure is glaucoma.

17. A method for lessening retinal ganglion cell death and/or lessening damage to the optic nerve arising from a condition selected from the group consisting of glaucoma, increased intraocular pressure, and glutamate toxicity, comprising administering to an individual in need of such treatment an effective amount of an agent selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof; (v) a combination of (i) and (ii); and (vi) a combination of (i), (ii) or (iii) with (iv), thereby lessening RGC death and/or lessening damage to the optic nerve.

18. The method according to claim 17, wherein said agent is administered for reducing optic nerve degeneration in glaucoma patients.
19. A method according to any one of claims 1 to 11 wherein said neurodegenerative condition, disease or disorder is one caused or exacerbated by glutamate toxicity.

20. A method according to claim 19 wherein said neurodegenerative condition, disease or disorder is selected from the group consisting of a neurodegenerative disease such as a senile dementia of both Alzheimer's type and non-Alzheimer's type, facial nerve (Bell's) palsy, Huntington's chorea, amyotrophic lateral sclerosis (ALS), Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, Guillain-Barré syndrome, and a prion disease such as Creutzfeldt-Jakob disease.

21. A method according to claim 19 wherein said neurodegenerative condition, disease or disorder is a peripheral neuropathy.

22. The method according to claim 21 wherein said peripheral neuropathy is selected from the group consisting of adrenomyeloneuropathy, alcoholic neuropathy, amyloid neuropathy or polyneuropathy, axonal neuropathy, chronic sensory ataxic neuropathy associated with Sjogren's syndrome, diabetic neuropathy, an entrapment neuropathy or nerve compression syndrome such as carpal tunnel syndrome or a nerve root compression that may follow cervical or lumbar intervertebral disc herniation, giant axonal neuropathy, hepatic neuropathy, ischemic neuropathy, nutritional polyneuropathy, porphyric polyneuropathy, toxic neuropathy, uremic polyneuropathy, a neuropathy associated with a disease or disorder such as acromegaly, ataxia telangiectasia, Charcot-Marie-Tooth disease, chronic obstructive pulmonary diseases, Fabry's disease, Friedreich ataxia, Guillain-Barré syndrome, hypoglycemia, IgG or IgA monoclonal gammopathy, lipoproteinemia, polycythemia vera, Refsum's syndrome, Reye's syndrome, Sjogren-Larsson syndrome, or a polyneuropathy associated with various drugs, or a polyneuropathy associated with hypoglycemia, with infections or with cancer.
23. A pharmaceutical composition for treatment of a neurodegenerative condition, disorder or disease other than Parkinson's disease, comprising a pharmaceutically acceptable carrier and an agent that down-regulates the suppressive activity of CD4⁺CD25⁺ regulatory T cells (Treg) on CD4⁺CD25⁻ effector T cells (Teff), wherein said agent is selected from the group consisting of:
   (i) dopamine or a pharmaceutically acceptable salt thereof;
   (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof;
   (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof;
   (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof;
   (v) a combination of (i) and (ii); and
   (vi) a combination of (i), (ii) or (iii) with (iv).

24. A pharmaceutical composition according to claim 23, wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

25. A pharmaceutical composition according to claim 23, wherein said agent is a combination of dopamine and the dopamine precursor levodopa, optionally in further combination with carbidopa.

26. A pharmaceutical composition according to claim 23, wherein said agent is a dopamine D1-R agonist.

27. The pharmaceutical composition according to claim 26, wherein said dopamine D1-R agonist is selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.

28. A pharmaceutical composition according to claim 23, wherein said agent is a dopamine D2-R antagonist.
29. The pharmaceutical composition according to claim 28, wherein said dopamine D2-R antagonist is selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

30. A pharmaceutical composition according to claim 23, wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.

31. The pharmaceutical composition according to claim 30, wherein said agent is a combination of dopamine with clozapine.

32. A pharmaceutical composition according to claim 23, wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.

33. The pharmaceutical composition according to claim 32, wherein said agent is a combination of SKF-38393 and clozapine.

34. A pharmaceutical composition according to any one of claims 23 to 33 wherein said neurodegenerative condition is a neuronal degeneration caused by a primary injury.

35. A pharmaceutical composition according to claim 34, wherein said primary injury is spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke, cerebral ischemia, and injury caused by surgery such as tumor excision.

36. A pharmaceutical composition according to claim 35, wherein said neurodegenerative condition, disorder or disease is associated with the eye.
37. A pharmaceutical composition according to claim 36, wherein said neurodegenerative condition, disorder or disease associated with the eye is non-arteritic optic neuropathy, age-related macular degeneration, a retinal disorder or a disease associated with elevated intraocular pressure.

38. A pharmaceutical composition according to claim 37, wherein said disease associated with abnormally elevated intraocular pressure is glaucoma.

39. A pharmaceutical composition according to any one of claims 23 to 33 wherein said neurodegenerative condition, disease or disorder is one caused or exacerbated by glutamate toxicity.

40. A pharmaceutical composition according to claim 39 wherein said neurodegenerative condition, disease or disorder is selected from the group consisting of a neurodegenerative disease such as a senile dementia of both Alzheimer's type and non-Alzheimer's type, facial nerve (Bell's) palsy, Huntington's chorea, amyotrophic lateral sclerosis (ALS), Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, Guillain-Barré syndrome, and a prion disease such as Creutzfeldt-Jakob disease.

41. A pharmaceutical composition according to claim 23 wherein said neurodegenerative condition, disease or disorder is a peripheral neuropathy.

42. The pharmaceutical composition according to claim 41 wherein said peripheral neuropathy is selected from the group consisting of adrenomyeloneuropathy, alcoholic neuropathy, amyloid neuropathy or polyneuropathy, axonal neuropathy, chronic sensory ataxic neuropathy associated with Sjogren's syndrome, diabetic neuropathy, an entrapment neuropathy or nerve compression syndrome such as carpal tunnel syndrome or a nerve root compression that may follow cervical or lumbar intervertebral disc herniation, giant axonal
neuropathy, hepatic neuropathy, ischemic neuropathy, nutritional polyneuropathy, porphryic polyneuropathy, toxic neuropathy, uremic polyneuropathy, a neuropathy associated with a disease or disorder such as acromegaly, ataxia telangiectasia, Charcot-Marie-Tooth disease, chronic obstructive pulmonary diseases, Fabry’s disease, Friedreich ataxia, Guillain-Barré syndrome, hypoglycemia, IgG or IgA monoclonal gammopathy, lipoproteinemia, polycythemia vera, Refsum’s syndrome, Reye’s syndrome, Sjogren-Larsson syndrome, or a polyneuropathy associated with various drugs, or a polyneuropathy associated with hypoglycemia, with infections or with cancer.

43. Use of an agent that down-regulates the suppressive activity of CD4\(^+\)CD25\(^+\) regulatory T cells (Treg) on CD4\(^+\)CD25\(^-\) effector T cells (Teff), wherein said agent is selected from the group consisting of:

(i) dopamine or a pharmaceutically acceptable salt thereof;

(ii) a dopamine precursor or a pharmaceutically acceptable salt thereof;

(iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof;

(iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof;

(v) a combination of (i) and (ii); and

(vi) a combination of (i), (ii) or (iii) with (iv);

for the manufacture of a pharmaceutical composition for treatment of a neurodegenerative condition, disorder or disease other than Parkinson’s disease.

44. Use according to claim 43, wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

45. Use according to claim 43, wherein said agent is a combination of dopamine and the dopamine precursor levodopa, optionally in further combination with carbidopa.
46. Use according to claim 43, wherein said agent is a dopamine D1-R agonist.

47. The use according to claim 46, wherein said dopamine D1-R agonist is selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.

48. Use according to claim 43, wherein said agent is a dopamine D2-R antagonist.

49. The use according to claim 48, wherein said dopamine D2-R antagonist is selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, trolapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

50. Use according to claim 43, wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.

51. The use according to claim 50, wherein said agent is a combination of dopamine with clozapine.

52. Use according to claim 43, wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.

53. The use according to claim 32, wherein said agent is a combination of SKF-38393 and clozapine.

54. Use according to any one of claims 43 to 53 wherein said neurodegenerative condition is a neuronal degeneration caused by a primary injury.
55. Use according to claim 54, wherein said primary injury is spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke, cerebral ischemia, and injury caused by surgery such as tumor excision.

56. Use according to claim 55, wherein said neurodegenerative condition, disorder or disease is associated with the eye.

57. Use according to claim 56, wherein said neurodegenerative condition, disorder or disease associated with the eye is non-arteritic optic neuropathy, age-related macular degeneration, a retinal disorder or a disease associated with elevated intraocular pressure.

58. Use according to claim 57, wherein said disease associated with abnormally elevated intraocular pressure is glaucoma.

59. Use according to any one of claims 43 to 53 wherein said neurodegenerative condition, disease or disorder is one caused or exacerbated by glutamate toxicity.

60. Use according to claim 59 wherein said neurodegenerative condition, disease or disorder is selected from the group consisting of a neurodegenerative disease such as a senile dementia of both Alzheimer's type and non-Alzheimer's type, facial nerve (Bell's) palsy, Huntington's chorea, amyotrophic lateral sclerosis (ALS), Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, Guillain-Barré syndrome, and a prion disease such as Creutzfeldt-Jakob disease.

61. Use according to claim 43 wherein said neurodegenerative condition, disease or disorder is a peripheral neuropathy.
62. The use according to claim 61 wherein said peripheral neuropathy is selected from the group consisting of adrenomyeloneuropathy, alcoholic neuropathy, amyloid neuropathy or polyneuropathy, axonal neuropathy, chronic sensory ataxic neuropathy associated with Sjogren's syndrome, diabetic neuropathy, an entrapment neuropathy or nerve compression syndrome such as carpal tunnel syndrome or a nerve root compression that may follow cervical or lumbar intervertebral disc herniation, giant axonal neuropathy, hepatic neuropathy, ischemic neuropathy, nutritional polyneuropathy, porphyric polyneuropathy, toxic neuropathy, uremic polyneuropathy, a neuropathy associated with a disease or disorder such as acromegaly, ataxia telangiectasia, Charcot-Marie-Tooth disease, chronic obstructive pulmonary diseases, Fabry's disease, Friedreich ataxia, Guillain-Barré syndrome, hypoglycemia, IgG or IgA monoclonal gammopathy, lipoproteinemia, polycythemia vera, Refsum's syndrome, Reye's syndrome, Sjogren-Larsson syndrome, or a polyneuropathy associated with various drugs, or a polyneuropathy associated with hypoglycemia, with infections or with cancer.

63. Use according to claim 43, wherein said neurodegenerative condition, disorder or disease is caused by exposure to a neurotoxin

64. Use according to claim 63, wherein said neurotoxin is an organophosphate nerve gas.

65. An article of manufacture comprising packaging material and an agent selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) an antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof; (v) a combination of (i) and (ii); and (vi) a combination of (i), (ii) or (iii) with (iv) contained within the packaging material, wherein said packaging material
comprises a label which indicates that said agent can be used for treating a neurodegenerative condition, disorder or disease other than Parkinson’s disease.

66. An article of manufacture according to claim 65 wherein said agent is dopamine or a pharmaceutically acceptable thereof.

67. An article of manufacture according to claim 65 wherein said agent is the D1-R agonist SKF-38393 or a pharmaceutically acceptable thereof.

68. An article of manufacture according to claim 65 wherein said agent is the D2-R antagonist clozapine or a pharmaceutically acceptable thereof.

69. An article of manufacture according to claim 65 wherein said agent is a combination of dopamine and the D1-R agonist SKF-38393 or a pharmaceutically acceptable thereof.

70. An article of manufacture according to claim 65 wherein said agent is a combination of the D1-R agonist SKF-38393 and clozapine.
Fig. 1b
**Fig. 2a**

- Cpm (labeled thymidine incorporation)
- Treatment conditions:
  - Treg treatment
  - Treg+Treg
  - Teff alone
  - Teff+Treg

- Compound effects:
  - Clozapine+DA
  - SCH-23390+DA
  - Quinpirole
  - SKF-38393
  - DA

- Statistical significance:
  - *** indicates significant differences
Fig. 2c

SKF-38393

Dopamine

Control

Annexin V
Fig. 2d

<table>
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<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Treg</th>
<th>Teff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>1.21±0.10</td>
<td>0.30±0.05</td>
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<tr>
<td></td>
<td>+</td>
<td>1.68±0.23</td>
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<tr>
<td>D1R</td>
<td>-</td>
<td>0.55±0.20</td>
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Fig. 2e
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Fig. 2f

Fig. 2g
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<td>D4R</td>
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**Fig. 2h**

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<tr>
<td>Teff</td>
<td>Hoechst</td>
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</tbody>
</table>

**Fig. 2i**
Control

Dopamine

Control

SKF 38393

Counts

Counts

Counts

Counts

10^-1 10^-2 10^-3 10^-4

10^-1 10^-2 10^-3 10^-4

10^-1 10^-2 10^-3 10^-4

10^-1 10^-2 10^-3 10^-4

FL1-H

FL1-H

FL1-H

FL1-H

CTLA-4

Fig. 2j
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<th>Time after DA exposure (h)</th>
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<tr>
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<td>IL-10 concentration (pg/ml)</td>
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<td>24</td>
<td>523 ± 101</td>
<td>210 ± 32</td>
</tr>
<tr>
<td>48</td>
<td>1526 ± 105</td>
<td>844 ± 113</td>
</tr>
<tr>
<td>72</td>
<td>2557 ± 200</td>
<td>1951 ± 433</td>
</tr>
</tbody>
</table>

**Fig. 2k**

<table>
<thead>
<tr>
<th>DA in culture</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2 concentration (pg/ml)</td>
<td></td>
</tr>
<tr>
<td>Treg</td>
<td>1.2 ± 0.58</td>
<td>0.95 ± 0.48</td>
</tr>
<tr>
<td>Teff</td>
<td>280 ± 9.40</td>
<td>279 ± 12.6</td>
</tr>
</tbody>
</table>

**Fig. 2l**
Dopamine

-  +

Fig. 2m

Foxp3

β-actin
Fig. 3b

- cpm (³H)Thymidine incorporation

<table>
<thead>
<tr>
<th>Cell number</th>
<th>25x10³</th>
<th>50x10³</th>
<th>50x10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50x10³ cells (const.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>act. Treg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>act. Treg + PD98059</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Teff</td>
<td>Treg</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.35±0.10</td>
<td>0.48±0.10</td>
<td>3.52±0.60</td>
</tr>
<tr>
<td>SKF-38393</td>
<td>N/V</td>
<td>N/V</td>
<td>0.72±0.04</td>
</tr>
</tbody>
</table>

Fig. 3e
Fig. 4a
Fig. 4b

Fig. 4c
Control

Dopamine

Teff

2.75%
M1

2.84%
M1

Treg

19.7%
M1

9.2%
M1

CD44

Fig. 4d
Fig. 4e

Fig. 4f
Fig. 4g

Fig. 4h
<table>
<thead>
<tr>
<th>Gene</th>
<th>Treg</th>
<th>DA</th>
<th>Teff</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td>1.7±0.11</td>
<td>0.81±0.12</td>
<td>0.35±0.10</td>
</tr>
</tbody>
</table>

Fig. 4i

![Graph showing Retinal Ganglion Cell (RGC) survival/mm²](image)

**Control**<br>**DA**

Fig. 5
\[
\left( \frac{\text{survival}_{\text{treated}} - \text{survival}_{\text{control}}}{\text{normal} - \text{survival}_{\text{control}}} \right) \times 100
\]

*Number of surviving retinal ganglion cells

**Fig. 6a**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of protection from secondary degeneration</th>
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</thead>
<tbody>
<tr>
<td><strong>CD4⁺CD25⁻</strong></td>
<td>0</td>
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<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
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<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
<td>30</td>
</tr>
<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
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</tr>
<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
<td>15</td>
</tr>
<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>CD4⁺CD25⁺</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 6b**

[Image of CD4⁺CD25⁻ and CD4⁺CD25⁺ cells]
Fig. 7

Fig. 8
| 120 | DOPAMINE AND AGONISTS AND ANTAGONISTS THEREOF FOR TREATMENT OF NEURODEGENERATIVE DISEASES |
| 130 | PRON-029 PCT |
| 150 | US 60/472,410 |
| 151 | 2003-05-22 |
| 160 | 20 |

| 210 | 1 |
| 211 | 20 |
| 212 | DNA |
| 213 | Artificial Sequence |
| 220 | Synthetic |

| 400 | gtagccatta tgatgctcac | 20 |

| 210 | 2 |
| 211 | 21 |
| 212 | DNA |
| 213 | Artificial Sequence |
| 220 | Synthetic |

| 400 | gatcagac agtgctttca g | 21 |

| 210 | 3 |
| 211 | 20 |
| 212 | DNA |
| 213 | Artificial Sequence |
| 220 | Synthetic |

| 400 | gcagccgagc tttcaggcc | 20 |

| 210 | 4 |
| 211 | 20 |
| 212 | DNA |
| 213 | Artificial Sequence |
| 220 | Synthetic |

| 400 | gggatgtttgc agtcacagt | 20 |

| 210 | 5 |
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19

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2/4
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<210> 13
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<210> 15
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 15
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<210> 16
Artificial Sequence

Synthetic

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DNA

Artificial Sequence

Synthetic

acctggtaga aagtgaagccc caggca 26

DNA

Artificial Sequence

Synthetic

catgcatg gatgaagtg tcaaa 25

DNA

Artificial Sequence

Synthetic

cagctgccata cagtgccct ag 22

DNA

Artificial Sequence

Synthetic

catttgccag cagtggtag 20

4/4