ARTICLE

Quinazoline-based tricyclic compounds that regulate programmed cell death, induce neuronal differentiation, and are curative in animal models for excitotoxicity and hereditary brain disease

A Vainshtein1,5, L Veenman1,5, A Shterenberg2, S Singh2, A Masarwa2, B Dutta2, B Island2, E Tsoglin2, E Levin1, S Leschiner1, I Maniv1, L Pe'er1, I Otradnov1, S Zubedat3, S Aga-Mizrachi3, A Weizman4, A Avital3, I Marek2 and M Gavish1

Expanding on a quinazoline scaffold, we developed tricyclic compounds with biological activity. These compounds bind to the 18 kDa translocator protein (TSPO) and protect U118MG (glioblastoma cell line of glial origin) cells from glutamate-induced cell death. Fascinating, they can induce neuronal differentiation of PC12 cells (cell line of pheochromocytoma origin with neuronal characteristics) known to display neuronal characteristics, including outgrowth of neurites, tubulin expression, and NeuN (antigen known as 'neuronal nuclei', also known as Rbfox3) expression. As part of the neurodifferentiation process, they can amplify cell death induced by glutamate. Interestingly, the compound 2-phenylquinazolin-4-yl dimethylcarbamate (MGV-1) can induce expansive neurite sprouting on its own and also in synergy with nerve growth factor and with glutamate. Glycine is not required, indicating that N-methyl-D-aspartate receptors are not involved in this activity. These diverse effects on cells of glial origin and on cells with neuronal characteristics induced in culture by this one compound, MGV-1, as reported in this article, mimic the diverse events that take place during embryonic development of the brain (maintenance of glial integrity, differentiation of progenitor cells to mature neurons, and weeding out of non-differentiating progenitor cells). Such mechanisms are also important for protective, curative, and restorative processes that occur during and after brain injury and brain disease. Indeed, we found in a rat model of systemic kainic acid injection that MGV-1 can prevent seizures, counteract the process of ongoing brain damage, including edema, and restore behavior defects to normal patterns. Furthermore, in the R6-2 (transgenic mouse model for Huntington disease; Strain name: B6CBA-Tg(HDexon1)62Gpb/3J) transgenetic mouse model for Huntington disease, derivatives of MGV-1 can increase lifespan by > 20% and reduce incidence of abnormal movements. Also in vitro, these derivatives were more effective than MGV-1.

INTRODUCTION

The 18 kDa translocator protein (TSPO) takes part in various cellular functions, including regulation of cell death and expression of numerous genes.1–6 Associated with these functions the primary locations of the TSPO include mitochondria, and nuclear and perinuclear sites.1,7,8 TSPO can be found throughout the body in various tissues.1,9 Moderate expression of TSPO also occurs in healthy central nervous system (CNS), which can increase in association with disease and injury, both in glia and in neurons.10–14 Programmed cell death and cell differentiation, separately as well as combined, constitute basic, general, and essential functions, for example, regarding embryonic development of the brain, and in adults in response to injury and disease, including cancer.15–17 The same is true for gene expression. Since a few years, TSPO function in cell differentiation is also becoming more appreciated.18

We have developed relatively small (~400 molecular weight) tricyclic compounds, based on the bicyclic quinazoline as a scaffold, to regulate the functions of the TSPO15,20 (Figure 1). In the present project, we studied their ability to modulate TSPO functions related to cell death induced by overload of glutamate, first of all in cell culture, and also in animal models (systemic injections of kainic acid in rats and the R6-2 transgenic mouse model for Huntington disease). We also studied the effects of these agents on neurodifferentiation in culture. It has been shown previously that TSPO present in progenitor cells for glia and neurons are associated with their differentiation, presenting TSPO as a promising target for research in this area.13

Both glial cells and neuronal cells are major components of embryonic development of the CNS, as well as brain damage related processes because of injury and disease.21,22 Therefore, we studied the U118MG (glioblastoma cell line of glial origin) and the PC12 cell line (cell line of pheochromocytoma origin with neuronal characteristics). PC12 cells have long been used as a model for neurodifferentiation, typically induced by application of nerve growth factor (NGF).23–25 We applied glutamate to induce cell

1Department of Neuroscience, Technion – Israel Institute of Technology, Faculty of Medicine, Rappaport Family Institute for Research in the Medical Sciences, Haifa, Israel; 2Technion – Israel Institute of Technology, Schulich Faculty of Chemistry, The Mallet Family Laboratory of Organic Chemistry, Haifa, Israel; 3Department of Physiology, Technion – Israel Institute of Technology, The Behavioral Neuroscience Laboratory, Faculty of Medicine and Emek Medical Center, Haifa, Israel and 4Tel Aviv University, Sackler Faculty of Medicine, The Felsenstein Medical Research Center, Geha Mental Health Center, Tel Aviv, Israel.

Correspondence: M Gavish (mgavish@tx.technion.ac.il or veenmanl@techunix.technion.ac.il)

© 2015 Cell Death Differentiation Association All rights reserved 2058-7716/15

OPEN

www.nature.com/cddiscovery

Cell Death Discovery (2015) 1, 15027; doi:10.1038/cddiscovery.2015.27; published online 30 November 2015
death in these cell lines. We then compared the effects of the mentioned tricyclic compounds, NGF, and glutamate on cell death and differentiation. In this context, during embryonic CNS development neuronal progenitor cells migrate to their target brain areas. There they distribute along scaffolds established by radial glia. Interactions with the glia are thought to contribute to differentiation of progenitor cells to mature neurons. The vast majority of progenitor cells do not differentiate and are weeded out by programmed cell death. Regarding trauma of healthy mature brain tissue, programmed cell death can result from various mechanistic and toxic insults, and disease, including expanding brain cancer. In adults of many species of vertebrates, migration of progenitor cells into damaged brain areas can allow for replenishment of depleted neurons.

**Figure 1.** Molecular structures of the tricyclic, quinazoline-based compounds of this study. (a) Stepwise changes in the molecular structure from MGV-1 (to 2-Cl-MGV-1, MGV-2, 2-Cl-MGV-2, MGV-3, and 2-Cl-MGV-3, respectively). Note: the stepwise changes include additions of single C atoms to the methyl side chains, and adding a Cl substituent to the third, rotatable carbocycle. Elongation of the methyl chains to ethyl (symmetric as well as asymmetric) leads to enhanced affinity. Addition of a Cl substituent to the second position in the third rotatable carbocycle does not affect affinity. Affinity is presented as Ki derived from displacement of [3H]PK 11195. (b) These compounds can counteract collapse of the ΔΨm induced by 35 mM of glutamate, for example, by 25 μM of MGV-1 as well as MGV-2, as assayed with JC-1. (c) MGV-1 and MGV-2 (25 μM) also prevent of mitochondrial ROS generation induced by glutamate (35 mM), as assayed by NAO. The results are expressed as mean ± S.D. ANOVA followed by the Bonferroni correction for multiple comparisons as post hoc: ***p < 0.001 versus control, ###p < 0.001 versus glut 35 mM (in b, n = 12; in c, n = 8).
Regarding brain cancer, glioma cells are almost by definition not differentiated and resistant to programmed cell death. At the same time, they induce cell death in surrounding healthy tissue, for example, by excessive glutamate release, as well as by mechanic compression of the brain in its enclosure of the skull.

For the present article, we studied whether the effects regarding cell death and differentiation, of our tricyclic compounds based on the bicyclic quinazoline scaffold, are associated with mitochondrial functions, including metabolic rate, maintenance or collapse of the mitochondrial membrane potential (ΔΨm), generation of reactive oxygen species (ROS), cardiolipin peroxidation, all of them functions that are known to be under the control of the TSPO and also modulated by glutamate application. Regarding neurodifferentiation, we examined and quantified the sprouting of neurites, the hallmark of neurodifferentiation. In addition, we studied expression of β-actin, tubulin-3β, and NeuN (antigen known as ‘neuronal nuclei’, also known as Rbfox3), as they present molecular biological markers for neurodifferentiation and mature neurons. We also examined whether TSPO and glial fibrillary acidic protein (GFAP) expression change in our compound-induced neurodifferentiation.

To discern whether our cell culture findings of cell death and cell differentiation may actually translate to implications for the whole organism, we applied our novel TSPO active agents to established mammalian models for progressing brain damage and neurodegeneration. These models included systemic kainic acid injections in rats to induce localized excitotoxic damage in hippocampus, amygdala, and pyriform cortex, and subsequent edemic damage spread throughout the brain. We also studied the R6-2 transgenic mouse model for the human hereditary neurodegenerative disease of Huntington, which

![Figure 2](https://example.com/fig2.png)

**Figure 2.** Protective effects of our quinazoline derivatives: MGV-1 (a); 2-Cl-MGV-1 (b); MGV-2 (c); 2-Cl-MGV-2 (d); MGV-3 (e); and 2-Cl-MGV-3 (f). In general, these compounds protect against glutamate-induced cell death of U118MG cells as measured by LDH activity. MGV-1, MGV-2, and MGV-3 protect against lethal effects of glutamate (a, c and e). MGV-2 and MGV-3 are already quite effective at 10 μM (c and e), however, MGV-2 and MGV-3 also show undesired lethal effects at high concentrations (in particular 100 μM), by themselves as well as in combination with glutamate (c and e). It appears that such undesired lethal effects can be reduced by adding a Cl substituent to the third carbocycle (b, d and f). In particular, 2-Cl-MGV-1 shows the least lethal effects when applied just by itself (b). Although 2-Cl-MGV-2 is very effective in protecting against glutamate-induced cell death at the concentrations 10, 25, 50, and 100 μM, it still has undesired lethal effect at high concentrations when given just by itself (d). 2-Cl-MGV-3 has undesired lethal effects at 50 and 100 μM both with and without glutamate exposure (f). In short, enhanced affinity for TSPO (see Figure 1) improves protective capability at 10 μM, but enhances undesired lethal effects in particular at 100 μM. Control, vehicle only (for glutamate and TSPO ligand); glu, glutamate. Further the x axis presents the treatments: control, glutamate, and application of compounds with their concentrations in μM. The y axis presents the levels of cell death according to the formula for relative LDH release given in the Materials and methods. The results are expressed as mean values ± S.D. One-way ANOVA followed by Bonferroni multiple comparison test was performed. *P < 0.05, **P < 0.01, ***P < 0.001 treatment versus control. (n = 12).
presents damage in the striatum and cortex, motor disturbances, and finally, early death. Thus, we endeavored to find out whether these tricyclic compounds based on a quinazoline scaffold could counteract the damage and behavioral deficits and potentially even restore the aberrant behavior to normal. And last but not least, whether these compounds can prolong the lifespan of the animals in question.

RESULTS

Cell culture studies

The novel tricyclic compounds based on a quinazoline scaffold of this study were tested by us, for example, to attenuate glutamate-induced cell death of the human glioblastoma cell line U118MG and to provide neuroprotective effects in animal models for brain injury and brain disease. Based on results from several

![Graph showing PC12 cell death and red/green ratio](image)
generations of drugs designed by us, this study focusses on six compounds: 2-phenylquinazolin-4-yl dimethylcarbamate (MGV-1), 2-(2-chlorophenyl)quinazolin-4-yl dimethylcarbamate (2-Cl-MGV-1), 2-phenylquinazolin-4-yl ethyl(dimethyl)carbamate (MGV-2), 2-(2-chlorophenyl)quinazolin-4-yl ethyl(methyl)carbamate (2-Cl-MGV-2), 2-phenylquinazolin-4-yl diethylcarbamate (MGV-3), and 2-(2-chlorophenyl)quinazolin-4-yl diethylcarbamate (2-Cl-MGV-3) (Figure 1). In Figure 1a, it can be seen that adding each time one C-atom to the alkyl side chains improves the affinity. Applying displacement studies of these compounds in comparison with the classical TSPO ligand PK 11195 (the classical TSPO ligand: N-butan-2-yl-1-(2-chlorophenyl)-N-methylproline-3-carboxamide), the affinities (Ki’s) presented in Figure 1a show that the affinities of MGV-3 and 2-Cl-MGV-3 are similar to PK 11195 (~2.5 nM), whereas those of MGV-2 and 2-Cl-MGV-2 are one order of magnitude lower, and those of MGV-1 and 2-Cl-MGV-1 are two orders of magnitude lower (Figure 1a), as determined in rat kidney homogenate, using [3H]PK 11195 as a radiolabeled TSPO ligand. We assumed that our compounds would prevent collapse of the ΔΨm, which is typically induced by glutamate29 and normally under the control of the TSPO. Applying JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide a cationic carbocyanine dye that accumulates in mitochondria) showed that the cell protective effects of MGV-1 and MGV-2 (25 μM) indeed prevent the collapse of the ΔΨm and generation of ROS otherwise induced by glutamate (35 mM) (Figure 1b). The fluorescent dye 10-N-nonyl-acridine orange (NAO) was applied to measure ROS generation at mitochondrial levels. This ROS generation is typically induced by glutamate28,29,30 and normally under the control of the TSPO.31 MGV-1 and MGV-2 (25 μM) prevent cardiolipin oxidation indicative of ROS generation at mitochondrial levels otherwise caused by exposure of U118MG cells to glutamate (Figure 1c). The prevention of collapse of the ΔΨm and generation of ROS show that initiation of the mitochondrial apoptosis cascade is inhibited. For several reasons, the concentration of 35 mM of glutamate was chosen: (1) as its effect should be lethal, it should be somewhat higher than can be observed in normal healthy tissue; (2) not deviate from what can be found in diseased brain tissue; (3) the level of cell death induced in our experiments should be amenable to up and down regulation by our drug treatment (for more details, see Discussion and Materials and methods section).

In Figure 2, applying lactate dehydrogenase (LDH), it is shown that at concentrations from 10 to 100 μM, all the designed compounds in question can protect well to moderately well against the lethal challenge of 35 mM of glutamate to U118MG cells. Subtle differences can be seen in the protective effects in this group of six compounds (Figures 1 and 2). For example, in vitro, 2-Cl-MGV-2 protects excellently with all of these tested concentrations of 10, 25, 50, and 100 μM, whereas the other compounds can be somewhat less effective at the relatively low concentration of 10 μM or at the relatively high concentration of 100 μM. Achieving a relatively high affinity of the compounds for TSPO, that is, MGV-3 and 2-Cl-MGV-3, does not improve the protective effects. 2-Cl-MGV-1 presenting a relatively low affinity for TSPO has the advantage of showing very little lethality by itself at any concentration (Figures 1 and 2).

Preliminary studies showed that these compounds can induce neurodifferentiation of PC12 cells. In particular, MGV-1 (50 μM) was very effective in inducing the differentiation of PC12 cells, that is, outgrowth of neurites longer than the cell bodies’ diameters. We first applied MGV-1 to a morphologically homogenous cell strain of PC12 cells, presenting a morphology of flat, polygonal, adherent cells (strain #1). A similar PC12 strain (ATCC CRL-1721.1) is also described by American Type Culture Collection (ATCC) (Manassas, VA, USA). These cell types are derived from the original PC12 source (ATCC CRL-1721), which presents clusters of floating spherical cells, but only a restricted number of flat, polygonal, adherent cells (polygonal cells). MGV-1 induced neurite outgrowth in all these three strains. Interestingly, although MGV-1 (50 μM) prevented cell death induction by glutamate exposure (35 mM) of U118MG cells, this same treatment of PC12 cells with MGV-1 enhanced cell death induction by glutamate by threefold (Figure 3a). This was associated with a threefold enhancement of the incidence of collapse of the ΔΨm (Figure 3b). Interestingly, in the days following this treatment, the surviving PC12 cells of this strain #1 started to differentiate, that is, grew ever longer neurites, generally considered the hallmark of neurodifferentiation23-25 (Figures 3c and 4). Adding NGF or glutamate to the MGV-1 treatment enhanced the differentiation induced by MGV-1 (Figure 3d). However, in contrast to MGV-1, neither NGF nor glutamate applied by themselves or combined did result in appreciable neurodifferentiation of strain #1 (Figure 3d). To our knowledge, no publications report

Figure 3. Effects of MGV-1 (and NGF and glutamate, as well as the various combinations of these three compounds) on PC12 cells regarding viability and differentiation. (a) As opposed to U118MG cells, together with 35 mM of glutamate, MGV-1 (10, 25, 50, 100 μM as indicated at the x axis) induces enhanced cell death of PC12 cells, as compared with application of 35 mM of glutamate by itself. At 100 μM, MGV-1 by itself can induce cell death of PC12 cells. (b) Separately and synergistically, MGV-1 (50 μM) and glutamate (35 mM) induce collapse of the ΔΨm in PC12 cells. (c) Representative examples of different PC12 cell strains (see Materials and methods section): strain #1 (only flat, attached polygonal cells), strain #2 (round cells and polygonal cells), and strain #3 (floating clusters of round cells and a restricted number polygonal cells). MGV-1 can induce differentiation of various strains of PC12 cells. The top row of this figure presents undifferentiated cells, and the bottom row of the figure presents typical examples of differentiated cells of the three strains. As the morphologies of the differentiated cells of the three strains are different, the morphologies of these cells strain differentiated by our applications are also distinct. Neurite sprouting from strain #1 makes the cells appear star shaped (here differentiated by MGV-1+glutamate). Strain #2 gives rise to extended thin neurites (here differentiated by MGV-1+glutamate). Strain (3) gives rise to very long thin neurites (here differentiated by MGV-1+NGF). (d) Table: MGV-1, NGF, and glutamate, separately and combined, can induce neurodifferentiation of strains of PC12 cells presenting both spherical and polygonal cells (strains 2 and 3). The lower the total number of cells, the more the cells are differentiated (differentiated cells do not proliferate). The higher the number of cells with neurite (the hallmark of differentiation), the more the cells are differentiated. The longer the average neurite length, the more the cells are differentiated. The strain presenting only polygonal cells (strain #1), can be differentiated by MGV-1 by itself, whereas NGF and glutamate by themselves do not have this effect on cells of strain #1 (as demonstrated as 0 cells presenting a neurite, that is, neurites of 0 length). The shading in the Table for all treatments is according to rank each time in one column (see ‘key’ giving the shading for each of the 8 ranks). The murkiest shading indicates the most effective differentiation. Summing up the ranks of each row (presented in the most right-hand column) it was found, looking at the individual treatments of Glu, NGF, and MGV-1, that: MGV-1 works better than NGF works better than glutamate. Interestingly, measuring the percentages of differentiated cells as part of the total cell population remaining per plate, gives the exact same rank order of effectiveness. Regarding combinations of molecules: (MGV-1+Glu+NGF) works better than (MGV-1+Glu) works better than (MGV-1+NGF) works better than (NGF+Glu). Looking at each cell type regarding capacity of differentiation (comparing each parameter for each cell strain and each treatment), the rank order of capacity to differentiate is: Strain #3 > Strain #2 > Strain #1. Statistical significance following one-way ANOVA and post hoc Mann-Whitney: *P < 0.05; **P < 0.01; ***P < 0.001. The scale bars in c are 100 μM.
Quinazolines, cell protection, and differentiation  
A Vainshtein et al

The use of double-blind studies showed that the tricyclic, animal studies of MGV-1+glutamate is very conducive for NeuN expression in increases in NeuN expression with western blot after MGV-1 strain #3 with predominantly spherical cells showed several fold changes in the polygonal type (data not shown). Interestingly, differentiated polygonal cells relative to total general protein areas: hippocampus, amygdala, pyriform cortex, as well as differentiated capabilities in these cells (Figure 3d). In applications with glutamate shown here, glycine is routinely applied. Noteworthy, however, omission of glycine does not modify the response of any of the cell strains (data not shown). Similarly, comparing the vehicle controls with and without alcohol (1% final concentration) also does not show a difference whatsoever (data not shown).

Our results further show that with virtually all parameters (Table in Figure 3d) strain #3 (compared with strains #1 and #2) shows the highest level of differentiation, that is, a larger percentage of the cells remaining in the culture dish is differentiated, and the average length of the neurites is the longest. As this cell line has the biggest proportion of spherical cells (Figure 3c), we assume that the spherical cell provide for the most extensive neurodifferentiation. MGV-1 by itself is more effective than NGF and glutamate by themselves regarding neurodifferentiation (Figure 3d). Regarding combinations of treatments to induce neurodifferentiation the rank order of effectiveness is: (MGV-1+NGF+glutamate) > (MGV-1+glutamate) > (MGV-1+NGF) > (NGF+glutamate) (Table in Figure 3d). Furthermore, each treatment that includes MGV-1 and/or NGF and glutamate is more effective than such treatments that do not include MGV-1.

To further characterize neuronal differentiation, we separately assayed tubulin-3β (Figures 4a and e) and NeuN (Figures 4f and j) expression. We used nuclear labeling with DAPI as a counter-stain to assay whether all cells would show tubulin, respectively, NeuN labeling. Immunofluorescence microscopy showed that our techniques provide intense tubulin-3β expression of cells of strain #3, both in cell bodies as well as neurites (Figures 4a and e). NeuN labeling was detected both in the nucleus and cytoplasm of cells of strain #3 (Figures 4f and j). The counterstain with DAPI showed that virtually all cells, under all conditions, show tubulin as well as NeuN labeling. The cells of strain #1 differentiated with MGV-1+glutamate typically were bigger than the non-differentiated control cells (Figure 3c), and contained six times more protein (Figure 5a). On top of this, western blots showed that tubulin expression was increased another threefold (Figures 5b and c). TSPO and β-actin expression, however, were not changed in differentiated polygonal cells relative to total general protein levels (data not shown). Also NeuN and GFAP did not show changes in the polygonal type (data not shown). Interestingly, strain #3 with predominantly spherical cells showed several fold increases in NeuN expression with western blot after MGV-1+glutamate (fivelfold) and MGV-1+NGF+glutamate exposure (sevenfold) (Figures 5d and e), suggesting that the combination of MGV-1+glutamate is very conducive for NeuN expression in differentiated cells of strain #3.

Animal studies
Applying double-blind studies showed that the tricyclic, quinazoline-based compounds had beneficial effects in animal models for various forms of brain damage, because of injury and disease. We applied MGV-1 to the rat model of systemic kainic acid injections that typically cause death of neurons and astroglia cells, associated with infiltration of microglia, in specific brain areas: hippocampus, amygdala, pyriform cortex, as well as subsequent edema throughout the brain. First of all, in comparison with vehicle control (Figure 6a), the damage typical for the hippocampus in area cornu ammonis area 1 (CA1) because of kainic acid (Figure 6b), was well attenuated by MGV-1 pretreatment (Figure 6c). Weeklong MGV-1 post-treatment also attenuated damage to area CA1, but less so than MGV-1 pretreatment (Figure 6d). The beneficial effects include prevention of cell death of neurons as determined with NeuN labeling (Figure 6). Microglia activation and cell death of astroglia was also observed (unpublished results). Comparable effects were observed in the amygdala and piriform cortex (unpublished results). The attenuation of brain damage by MGV-1 was also associated with a reduction in the emergence of seizures when MGV-1 was injected 2 h before the systemic kainic acid injections (Figure 6e). Injected 2 h after kainic injection, and subsequently once every day for 6 consecutive days, MGV-1 restores normal response to handling, that is, hyper reactivity in response to handling is returned to the level of normal responses that are typically observed in wild-type rats (Figure 6f). This may be indicative of reduction of edema.

Surprisingly, MGV-1 did not appear to have an effect in the transgenic mouse model (R6-2 mice) for the hereditary human disease of Huntington, regarding behavior and lifespan. Also the classical TSPO ligand PK 11195 did not show such effects. However, derivatives from MGV-1 considerably and consistently extended average lifespan of these mice by 20%. In particular, in Figures 7a and b the effects of 2-Cl-MGV-1 and 2-Cl-MGV-2 on R6-2 mice are shown. MGV-2 had effects reminiscent of those of 2-Cl-MGV-1 and 2-Cl-MGV-2, but less consistent (data not shown). Interestingly, behavioral data suggested a reduction of uncontrollable tremor activity of R6-2 mice treated with 2-Cl-MGV-1 and 2-Cl-MGV-2, indicative of positive effects on motor control (data not shown).

DISCUSSION
Embryonic development of brain includes maintenance of a glial scaffold to support developing neurons. The neuronal development includes migration of enormous numbers of progenitor cells into specific target areas. Subsequently, millions of these cells reaching the target area die off, whereas only a very restricted number of progenitor cells (a few ten thousands) develop into mature neurons. In this study, the presented tricyclic compounds based on a quinazoline scaffold induced effects in cell culture that mimic those of embryonic brain development. This includes: (1) protecting cells of glial origin from cell death, (2) stimulating massive cell death of neuronal progenitor cells as induced by glutamate, and (3) stimulating extensive neuronal differentiation of the surviving neuronal progenitor cells (Figure 8). To emphasize, it appears that our relatively small tricyclic molecules basically can induce overall cell processes essential for brain development, as well as protect and cure adult brain damage. The latter was indeed demonstrated in our animal models.

Specifically, the molecules of this study are expanded from a bicyclic quinazoline scaffold and were designed to bind to the TSPO and to regulate TSPO-related functions. Thus, we assume that this is at least part of the way via which they regulate neurodifferentiation. It is known that TSPO regulates several functions, including but not restricted to: (1) programmed cell death, and (2) gene expression for various proteins essential for neurodifferentiation, such as growth factors and their receptors; enzymes for glutamate metabolism, and also glutamate receptors and glutamate transporters; proteins related to adhesion, migration, cell cycle, etc.
Figure 4. Localization of tubulin 3β (a–e) and NeuN (f–j) in cell bodies and neurites of differentiated PC12 (strain #3). This figure shows that our different protocols not only result in extensive sprouting and outgrowth of neurites of PC12 cells in culture (as shown in Figure 3), but also labeling of these cells with the neuronal markers tubulin 3β (magenta in a–e) and NeuN (yellow in f–j). The cell nuclei are labeled with DAPI (cyan in a–j). (a) Tubulin 3β labeling can be detected first of all in the cell bodies of the undifferentiated vehicle control PC12 cells (control). Inducing differentiation with MGV-1 (b), MGV-1 plus glutamate (c), NGF (d), as well as MGV-1 plus NGF plus glutamate (e) enhanced tubulin 3β labeling not only of the cell body but also intensely of neurites. (f) NeuN expression is indicated with yellow fluorescent immunocytochemical labeling of the cell bodies, both in the nuclei and the cytoplasm of undifferentiated cells (control). Nuclei and cytoplasm both are typical locations for NeuN.91 NeuN labeling can also appear in the neurites of cells differentiated with MGV-1 (g), MGV-1 plus glutamate (h), NGF (i), as well as MGV-1 plus NGF plus glutamate (j). NeuN labeling can also appear in the neurites. In undifferentiated as well as differentiated cells doubly labeled for DAPI and NeuN, the cell nuclei can appear whitish, indicating the presence of NeuN in the cell nuclei. The same is true tubulin for cells doubly labeled for DAPI and tubulin. The scale bars in are 100 μM.
ligands, including our new compounds, can also regulate other cell death processes. A few studies have associated TSPO specifically with development/differentiation of progenitor cells to neuronal and glial cells.13

A classical agent to induce neuronal differentiation of PC12 cells is NGF.23–25,58 In this study, we report that apart from MGV-1 and NGF by themselves it is also possible to apply glutamate by itself for this purpose. As no difference is found with glycine added to the glutamate treatment, it seems that the N-methyl-D-aspartate (NMDA) receptor is not involved in differentiation induced by glutamate application. It is known that in healthy brain there is 5–15 mmol of glutamate per kg of tissue, depending on the region.59–61 Furthermore, in brain tissue around tumors, glutamate levels can be increased twofold.62 Intracellularly, it has been reported that glutamate concentration in synaptic vesicles can reach 60–250 mM.63–65 In synapses, glutamate levels typically are 1 mM or less.66 Keeping the physiological and pathological glutamate concentrations in cells and tissue in mind, and after generating dose-response curve from 0.1 to 100 mM of glutamate for U118MG cells, as well as PC12 cells (see Materials and methods section), we chose the concentration of 35 mM of glutamate for our study as its induction of cell death allowed for upregulation and downregulation. We then found that this concentration of 35 mM contributed substantially to neurodifferentiation typically induced by MGV-1, as well as NGF. We measured the maximal extent of neurodifferentiation (neurite length) 8 days after starting the treatments with our compounds. However, already within 3 days appreciable differentiation can be observed. Notably, our treatment protocols provide neurodifferentiation far more complete and extensive than generally presented in the research field of PC12 differentiation.23–25,58 Namely, with our most optimized methods, up to 100% of the population of cells is differentiated and average neurite length is 170 μm. Maximal neurite length we have seen in this way was 670 μm. The enhanced tubulin-3β and NeuN labeling suggests that the outgrowth of neurites indeed presents neuron-like features.54,55 For future studies, it would be interesting to test the effects of MGV-1 and related compounds on mouse progenitor cells,13 human progenitor cells,67 and primary neurons from developing brain.68 Also regarding PC12 cells, it appears to be worthwhile to apply MGV-1 and related

---

**Figure 5.** Neuroimmunochemical signs of differentiation of PC12 cells by our different exposure protocols, applied to Strain #1 (a–c) and Strain #3 (d and e) in comparison with their vehicle controls. (a) A bar graph showing protein content indicative of cell size of strain #1 differentiated by glutamate, MGV-1, and MGV-1+glutamate. MGV-1+glutamate enhances protein levels in these cells sixfold. (b) A bar graph of relative tubulin 3β expression in strain #1 cells differentiated by three different treatments (glutamate, MGV-1, and MGV-1+glutamate), compared with the vehicle control (undifferentiated cells). MGV-1+glutamate significantly enhances tubulin 3β expression in these cells. (c) Representative western blot assay of the effects on the expression levels of tubulin 3β of figures (b). (d) A bar graph showing significantly enhanced NeuN expression in cells of strain #3 differentiated by MGV-1+glutamate and by MGV-1+NGF+glutamate, compared with the vehicle control (undifferentiated cells). The other treatments shown (glutamate, MGV-1, NGF, NGF+MGV-1, NGF+glutamate) do not enhance NeuN expression significantly. (e) A representative western blot assay of NeuN expression in cells of strain #3 differentiated by our various protocols of Figure 4d. In (b) and (d), protein expression is given in arbitrary units (× 10^7) as provided the ImageQuant LAS 4010 densitometer. Data presented as means ± S.E.M. For Sa and Sb n = 4, for Sc n = 6. In all cases, statistical analysis by the Friedman ANOVA test, and Dunn’s multiple comparison test as the post hoc. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with vehicle control (control). Control, vehicle only; glu, glutamate; M, molecular weight (50 kDa MW) markers for the western blots.
compounds, as the differentiation procedure is extremely simple and productive.

As MGV-1 is able to differentiate the polygonal PC12 cells by itself (strain #1), whereas NGF and glutamate are not, it appears that in this model the MGV-1 (possibly via TSPO) is essential for differentiation in this PC12 strain #1. In summary, kainic acid causes major damage in CA1 of the hippocampus, which can be virtually prevented by pretreatment with MGV-1, and also attenuated by daily treatments with MGV-1 after kainic acid injection. CA3, cornu ammonis area 3; DG, dentate gyrus. Behavior: (e) Injections of 15 and 7.5 mg/kg of MGV-1, 2 h before kainic acid injections (pretreated), significantly prevent induction of seizures (reduction of seizure incidence by half). *p < 0.05 and **p < 0.01 versus KA. (f) Furthermore, MGV-1 treatment, 2 h before kainic acid injections (MGV-1-KA = pretreated), attenuates the incidence of the hyper reactivity in response to handling in the week after the kainic acid injections. Hyper reactivity typically is pronounced after kainic acid injections, in otherwise untreated animals (KA), likely due to the progressive effect of brain edema as a typical consequence of kainic acid injections that induce seizures.42,43,46,48 MGV-1 treatment starting 2 h after kainic acid injections that induce seizures (KA-MGV-1 = post-treatment), and subsequently given every day in the week afterward, also reduces the incidence of the hyper reactivity in response to handling in the week after the kainic acid injections. Applying ANOVA and Wilcoxon matched-pairs signed rank test regarding the number of animals presenting hyper reactivity indicates a significant difference between MGV-1-treated mice and the vehicle-treated control. **p < 0.01 both for pretreatment and posttreatment compared with vehicle control. n = 9 for the experimental groups of f.

Figure 6. Neuroprotective effects by MGV-1 treatment of kainic acid-injected rats. Histology: representative effects of kainic acid injection and MGV-1 treatment on neuronal cells in rat hippocampus. (a) NeuN labeling in hippocampus of a vehicle control rat. (b) NeuN labeling in the hippocampus of a KA+vehicle-injected rat. The arrows indicate extensive neuronal death in CA1. (c) NeuN labeling following MGV-1 pretreatment before KA injections. The hippocampus is virtually indistinguishable from vehicle control rat. (d) NeuN labeling following MGV-1 post-treatment after KA injections. The damage indicated with arrows in CA1 is considerable reduced compared with KA+vehicle-injected rat. In summary, kainic acid causes major damage in CA1 of the hippocampus, which can be virtually prevented by pretreatment with MGV-1, and also attenuated by daily treatments with MGV-1 after kainic acid injection. CA3, cornu ammonis area 3; DG, dentate gyrus. Behavior: (e) Injections of 15 and 7.5 mg/kg of MGV-1, 2 h before kainic acid injections (pretreated), significantly prevent induction of seizures (reduction of seizure incidence by half). *p < 0.05 and **p < 0.01 versus KA. (f) Furthermore, MGV-1 treatment, 2 h before kainic acid injections (MGV-1-KA = pretreated), attenuates the incidence of the hyper reactivity in response to handling in the week after the kainic acid injections. Hyper reactivity typically is pronounced after kainic acid injections, in otherwise untreated animals (KA), likely due to the progressive effect of brain edema as a typical consequence of kainic acid injections that induce seizures.42,43,46,48 MGV-1 treatment starting 2 h after kainic acid injections that induce seizures (KA-MGV-1 = post-treatment), and subsequently given every day in the week afterward, also reduces the incidence of the hyper reactivity in response to handling in the week after the kainic acid injections. Applying ANOVA and Wilcoxon matched-pairs signed rank test regarding the number of animals presenting hyper reactivity indicates a significant difference between MGV-1-treated mice and the vehicle-treated control. **p < 0.01 both for pretreatment and posttreatment compared with vehicle control. n = 9 for the experimental groups of f.
seizures, attenuates the hyper reactivity in response to handling that typically occurs in the days and weeks after the seizures, and attenuates occurrence of damage in the hippocampus, pyriform cortex, and amygdala. The MGV-1 treatments are effective when applied before kainic acid injections, indicating protective effects, and also when applied after kainic acid injections, implicating curative and restorative effects. Notably, the hyperactivity in response to handling is considered as a consequence of edema developing throughout the brain after excitotoxic hippocampal damage.\(^{43,44,46,47}\) Thus, MGV-1 appears to be able to counteract progressing brain edema, or at least its adverse effects. To summarize, MGV-1 appears to be able to counteract brain damage as well as the associated sensorimotor and behavioral deficits.\(^{68}\)

To our surprise, MGV-1 did not work in an animal model for Huntington disease (R6-2 transgenic mice). However, derivatives of MGV-1 that in cell culture do protect better than MGV-1, can reduce locomotor aberrations and also increase the lifespan of R6-2 mice. It appeared that the addition of a Cl substituent to the third carbocycle of our compounds is very advantageous for the treatment of this transgenic mouse model. The death of the R6-2 mice is reminiscent of sudden unexplained death in epilepsy in humans that may include heart failure.\(^{69}\)

To summarize, in cell culture, our new drugs regulate programmed cell death and induce neurodifferentiation (Figure 8). In the animal models for brain injury and disease, our new drugs: (1) prevent epileptic seizures, (2) improve locomotion, (3) normalize responsiveness, (4) maintain normal brain neurohistology, (5) reduce anxiety, (6) prevent heart failure associated with seizures, (7) extend lifespan, and, importantly, (8) present no adverse effects. In cell cultures, MGV-1 and related compounds prevent cell death of glial-like U118MG cells, whereas it weeds out undifferentiated PC12 cells that are not sent on a neurodifferentiation pathway.\(^{68}\) Thus, these tricyclic molecules by themselves can induce diverse processes that also occur during normal embryonic brain development and promote protective and restorative responses in adult brain tissue damage.

**Figure 7.** The MGV-1 derivatives 2-Cl-MGV-1 and 2-Cl-MGV-2 increase the lifespan of R6-2 mice, which are a transgenic animal model for Huntington disease. (A) The quinazoline-based, tricyclic compound 2-Cl-MGV-1 (which includes the Cl halogen substituent on the third rotatable carbocycle, that is, a halogenated MGV-1) increases median lifespan of R6-2 mice (\(n=5\)), compared with the vehicle DMSO-treated R6-2 mice (\(n=5\)). In more detail, 50% of untreated mice died before the 15th week, whereas 50% of the 2-Cl-MGV-1-treated R6-2 mice were still alive until the 12th week. The y axis presents the percentage of surviving animals per week. The x axis presents the number of weeks from birth. The 50% survival cut off is marked with a horizontal arrow. The week where the 50% survival cut off is reached is marked with a vertical arrow (gray for control; black for 2-Cl-MGV-1-treated mice). Applying Wilcoxon matched-pairs signed rank test regarding the number of surviving animals indicates a significant difference between the 2-Cl-MGV-1-treated R6-2 mice and the DMSO (vehicle)-injected R6-2 mice: \(P<0.01\). Applying Mann–Whitney to each week of treatment shows that at the week of 40% survival of the control mice (week 12 of age), the difference between the 2-Cl-MGV-1-treated R6-2 mice and the vehicle-injected R6-2 mice is significant: \(P<0.05\) for week 12. Linear regression applied to weeks 8–13 shows that the intercepts are not equal (\(F=11.7\), \(P<0.01\)), that is, 2-Cl-MGV-1-treated R6-2 mice start dying significantly later than vehicle-injected R6-2 mice. The slopes are very equal (\(F=3.23\), \(P<0.05\)), that is, at any given point in time fewer treated animals have died than untreated. (B) The quinazoline-based, tricyclic compound 2-Cl-MGV-2 (which in addition to the halogenation of 2-Cl-MGV-1, includes asymmetrical side chains, methyl and ethyl) increases median lifespan of the R6-2 mice (\(n=12\)), compared with the vehicle-treated R6-2 mice (\(n=10\), control). In more detail, 50% of vehicle-treated R6-2 mice died before their 10th week, whereas 50% of the 2-Cl-MGV-2-treated R6-2 mice were still alive until the 12th week. The \(y\) axis presents the percentage of surviving animals per week. The \(x\) axis presents the number of weeks from birth. The 50% survival cut off is marked with a horizontal arrow. The week where the 50% survival cut off is reached is marked with a vertical arrow (gray for control; black for 2-Cl-MGV-2-treated mice). Applying ANOVA and Wilcoxon matched-pairs signed rank test regarding the number of surviving animals indicates a significant difference between the 2-Cl-MGV-2-treated R6-2 mice and the DMSO (vehicle)-injected R6-2 mice: \(P<0.01\). Applying Mann–Whitney to each week of treatment shows that at the week of 40% survival of the control mice (week 12 of age), the difference between the 2-Cl-MGV-2-treated R6-2 mice and the vehicle-injected R6-2 mice is significant: \(P<0.05\) for week 12. Linear regression applied to weeks 8–13 shows that the intercepts are not equal (\(F=11.7\), \(P<0.01\)), that is, 2-Cl-MGV-2-treated R6-2 mice start dying significantly later than vehicle-injected R6-2 mice. The slopes are very equal (\(F=3.23\), \(P<0.05\)), that is, at any given point in time fewer treated animals have died than untreated.
Materials

**Materials**

Chemical synthesis. For synthesis of the compounds designed for this study (MGV-1, 2-Cl-MGV-1, MGV-2, 2-Cl-MGV-2, MGV-3, and 2-Cl-MGV-3), all commercially available reagents were from Sigma-Aldrich (St Louis, MO, USA) and used as received, unless otherwise stated.

Cell culture and viability assay materials. Materials for cell culture including media, serum, gentamycin, glutamine, penicillin, streptomycin, saline, Trypan blue, and trypsin were purchased from Biological Industries.

- Cytotoxicity detection kit (LDH) (Roche Pharmaceuticals, Basel, Switzerland).
- The fluorescent dye JC-1 was obtained from Calbiochem (Merck, Darmstadt, Germany).
- The NAO was obtained from Sigma.
- Cell culture vessels (Corning Life Sciences, Ramat-Gan, Israel).

Materials for binding assays and immunoblotting. Bradford solution for determination of protein concentration was obtained from Bio-Rad (Munich, Germany).

**Methods**

Compound synthesis. The compounds have been described previously,19,20 and produced by us for this study according to state of the art methods. All reactions were performed under argon atmosphere in flame-dried glassware. Progress of the reactions was monitored by analytical thin layer chromatography (TLC) using glass plates precoated with silica gel with F254 indicator to measure absorbance at 254 nm wavelength. 1H and 13C NMR were recorded on 300 (75) MHz or 400 (100) MHz spectrometers. Chemical shifts are reported in p.p.m. (δ).

Binding assay materials: [3H]PK 11195 (85.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). 1-(2-Chlorophenyl-N-methyl-1-methylpropyl)-3-isooquinolinocarboxamide (PK 11195) was purchased from Sigma-Aldrich (Rehovot, Israel). CytoScintTM was obtained from MP Biomedicals (Costa Mesa, CA, USA); Whatman GF/C filters were obtained from Tamar (Mevaseret Zion, Israel).

- Protease inhibitor Complete was purchased from Roche Diagnostics (Mannheim, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker (Phillipsburg, NJ, USA).
- Ponceau staining was obtained from Sigma-Aldrich (Rehovot, Israel).
- Antibodies: anti-tubulin-3B antibody from mouse was obtained from Sigma-Aldrich (Rehovot, Israel); and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) polyclonal anti-rat TSPO antisera (from rabbit) was purchased in our laboratory, as previously described.23 Anti-human TSPO antisera (from rabbit) was purchased in our laboratory,23 and also purchased from Abcam (from rabbit) (Zotaf, Tel Aviv, Israel); and anti-neuronal nuclei (NeuN) (from rabbit) was purchased from Cell Signalling, Petach-Tikva, Israel; secondary antibodies (anti-rabbit and anti-mouse – IgG linked to horseradish peroxidase) were obtained from Amersham GE Healthcare (Buckinghamshire, England).

- Precision pre-stained standards (10–250 kDa) were from Bio-Rad Laboratories (Hercules, CA, USA). Keyhole limpet hemocyanin (maleimide-activated KLH) was obtained from Pierce (Rockford, IL, USA).

- The dried milk was obtained from Carnation (Glendale, CA, USA).

- Nylon-reinforced nitrocellulose membranes Nytran, S & S were obtained from Schleicher & Schuell, Keene, NH, USA, unless stated otherwise.

Standard chemicals

- Standard chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless stated otherwise.

Equipment

- The Spectrophotometer Zenith 200 (ELISA reader) was from Anthos (Eugendorf, Austria).
- The Kinematica Polytron was from Brinkmann Instruments (Westbury, NY, USA).
- The flow cytometer used was the FACSCalibur, including its software, from Becton Dickinson (Mountain View, CA, USA).
- The Elisa reader Ceres UV 900 was from Bio-Tek (Burlington, VT, USA).
- The β-counter, a 1600 CA Tri-Carb liquid scintillation analyzer, was from Packard (Meriden, CT, USA).
- Surgery equipment was from Bar Naor (Ramat-Gan, Israel).
- The densitometric apparatus was ImageQuant LAS 4010 (GE Health Care Life Sciences, Rehovot, Israel).
- The thermal cycler (MJ Mini, Bio-Rad Laboratories, Rishon Le Zion, Israel). E-Gel PowerBase apparatus (Invitrogen, Life Technologies, Rhenium, Modi’in, Israel).
- LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).
- Hamilton–Kinder sensor in a soundproof ventilated apparatus (Kinder Scientific, Poway, CA, USA).
- Z1 inverted microscope (Zeiss, Oberkochen, Germany).

Quinazolines, cell protection, and differentiation

**A Vainshtein et al.**

**Figure 8.** Summary of the effects of MGV-1 in cell cultures of U118MG and PC12 cells. Glutamate (35 mM) induces cell death of U118MG cells as well PC12 cells (skulls in top boxes). MGV-1 protects U118MG cells from glutamate-induced cell death (crossed out skulls in left-hand bottom box). In contrast, MGV-1 together with glutamate induces pronounced neuronal differentiation of PC12 cells (image of a mature neuron in most right-hand bottom box). As shown in Figure 3, MGV-1 also enhances cell death induction of glutamate of PC12 cells. Thus, MGV-1 appears to be able to regulate astrocytic integrity, neuronal differentiation, and weeding out of non-differentiating progenitor cells.21,22
t = triplet, q = quartet, m = multiplet. All first-order splitting patterns were assigned on the basis of the appearance of the multiplet. Splitting patterns that could not be easily interpreted are designated as multiplet (m). High resolution mass spectrum (HRMS) was obtained by Atmospheric Pressure Photoionization Source (APPI) method. Column chromatography was performed using silica gel (230–400 mesh). 2-Arylquinazolin-4-ol (A in synthesis scheme below) was synthesized from o-aminobenzoate and corresponding aromatic aldehyde as described.19,20,74

General method for synthesis of 2-arylquinazolin-4-yl alkylcarbamate:

To a suspension of potassium hydride (6.75 mmol) under argon in 30 ml of dry 1,2-dimethoxyethane (DME) or dry tetrahydrofuran (THF) was added 2-arylquinazolin-4-ol (A in the synthesis scheme) (4.5 mmol). 2-Cl-MGV-1 was obtained as white solid in 60% of yield.1H NMR (300 MHz, CDCl3) δ 1.19–1.42 (m, 6H), 3.16 (s, 3H), 3.29–3.40 (m, 2H), 4.23 (s, 2H), 7.31–7.40 (m, 7H), 7.49–7.76 (m, 3H), 7.56 (t, J = 7.8 Hz, 1H), 7.86 (t, J = 6.9 Hz, 1H), 8.07 (d, J = 7.8 Hz, 2H), 8.53–8.58 (m, 2H). 13C NMR (75 MHz, CDCl3) δ (p.p.m.): 38.9, 117.9, 125.2, 129.0, 130.2, 130.3, 130.5, 132.5, 136.1, 151.1, 162.2, 165.9. HRMS (APPI): mass calcd for C19H19N3O2 [M+H]+: 322.1550; found: 322.1585.

MGV-1:

The title product 2-(2-chlorophenyl)quinazolin-4-yl dimethylcarbamate (2-Cl-MGV-1) was obtained as white solid in 47% of yield. 1H NMR (400 MHz, CDCl3) δ 7.51–7.59 (m, 1H), 7.63 (m, 4H), 7.85 (t, J = 8.6 and 7.2 Hz, 1H), 7.80–7.87 (m, 1H), 7.92 (t, J = 7.8 Hz, 1H), 8.05–8.21 (m, 2H). 13C NMR (100 MHz, CDCl3) δ 37.34, 116.20, 123.61, 127.00, 130.60, 130.77, 132.26, 133.26, 134.78, 137.73, 152.11, 153.04, 161.17, 164.11. HRMS (APPI): mass calcd for C12H10ClN2O2 [M+H]+: 232.0847; found: 232.0864.

2-Cl-MGV-2:

The title product 2-phenylquinazolin-4-yl diethylcarbamate (2-Cl-MGV-2) was obtained as white solid in 65% of yield. 1H NMR (400 MHz, CDCl3) δ 7.54–7.59 (m, 1H), 7.63 (s, 3H), 7.85–7.90 (m, 1H), 8.04–8.09 (m, 2H), 8.51–8.55 (m, 2H). 13C NMR (75 MHz, CDCl3) δ (p.p.m.): 12.23 and 13.40, 34.40 and 34.46, 44.55 and 44.84, 115.81 and 116.19, 123.25 and 123.29, 127.11 and 127.15, 128.28 and 123.32, 128.36, 128.56 and 128.59, 130.63, 134.21, 137.28, 153.12 and 153.20, 160.26 and 160.34, 164.05 and 164.14, 179.10. HRMS (APPI): mass calcd for C19H15N3O2 [M+H]+: 308.1394; found: 308.1397.

MGV-2:

The title compound 2-phenylquinazolin-4-yl ethyl(methyl)carbamate (MGV-3) was obtained as white solid in 60% of yield. 1H NMR (400 MHz, CDCl3) δ 1.17–1.32 (m, 3H), 3.04 and 3.08 (2 s, 3H), 3.38–3.58 (m, 2H), 7.33 (dd, J = 7.0 and 3.2 Hz, 2H), 7.40–7.50 (m, 1H), 7.61 (dd, J = 8.6 and 7.4 Hz, 1H), 7.78–7.84 (m, 1H), 7.88 (dd, J = 9.4 and 7.2 Hz, 2H), 8.08 (dd, J = 9.5 and 7.2 Hz, 2H). 13C NMR (100 MHz, CDCl3) δ (p.p.m.): 12.43 and 13.65, 34.64 and 34.74, 44.82 and 45.12, 116.07 and 116.36, 123.52 and 123.57, 126.92, 128.13 and 128.19, 128.60, 130.52, 130.71, 132.23, 133.23, 134.68, 137.70 and 137.72, 151.71 and 151.92, 152.95 and 152.99, 161.09 and 161.15, 164.11 and 164.19. HRMS (APPI): mass calcd for C19H15N3O2 [M+H]+: 342.1004; found: 342.1010.
The title product 2-(2-chlorophenyl) quinazolin-4-yl diethylylcarbamate (2-Cl-MGV-3) was obtained as white solid in 69% of yield. $^1$H NMR (400 MHz, CDCl3) δ (p.p.m.): 1.02–1.55 (m, 6H), 3.23–3.76 (m, 4H), 7.26–7.40 (m, 2H), 7.41–7.53 (m, 1H), 7.57–7.72 (m, 1H), 7.75–7.97 (m, 2H), 8.01–8.27 (m, 2H). $^{13}$C NMR (100 MHz, CDCl3), rotamers observed in spectrum, δ (p.p.m.): 13.25 and 14.55, 42.85, 116.27, 123.50, 126.86, 128.10, 128.55, 130.47, 130.68, 132.23, 133.21, 137.66, 151.55, 152.92, 161.08, 164.20. HRMS (APPI): mass calcd for C$_{19}$H$_{18}$ClN$_3$O$_2$ [M+H]$^+$: 356.1160, found: 356.1167.

Protein level measurements. Protein levels of cell homogenates for binding studies and western blot assays were measured by the Bradford method$^{26}$ using bovine serum albumin (BSA) as a standard. Also the amount of protein per cell was calculated.

TSPO binding for quality control of novel compounds.$^{6}$ Binding assays of $[^3$H]PK 11195, including displacement studies were done as previously described.$^{7}$ The reaction mixtures for the binding assays contained 400 nM of homogenized kidney membranes (5 mg of homogenate/ml) and 25 µl of $[^3$H]PK 11195 (2 nM final concentration)$^{76}$ in the absence (total binding) or presence of various concentrations (10$^{-10}$ – 10$^{-5}$ M) of the synthesized compounds described in this study. After incubation for 60 min at 4°C, samples were filtered under vacuum over Whatman GF/C filters and washed three times with 5 ml of 0.05 M Tris-HCl buffer, pH 7.4. The filters were incubated in CytoScint (MP Biomedicals) and radioactivity was measured after 12h with a 1600 CA Tri-Carb liquid scintillation analyzer (Packard). Inhibitory constant (Ki) values were calculated by the equation Ki = IC50/(1+C/Kd), where C = $[^3$H]PK 11195 concentration, IC50 = concentration causing 50% inhibition of $[^3$H]PK 11195 binding and Kd = 2 nM (from Scatchard analysis of $[^3$H]PK 11195 binding to kidney membranes).

Maintenance and use of research subjects

Cell cultures. Cell cultures were done under sterile conditions at 37°C under air with 5% CO2 using a humidified incubator. U118MG cells: Cells of the human glioblastoma cell line, U118MG, were incubated in MEM EAGLE medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 0.5 µl/ml gentamicin, and 2% L-glutamine.$^{57}$

Undifferentiated PC12 cells: PC12 cells (cell line from rat adrenal gland, Phaeochromocytoma) were cultured with complete DMEM medium containing 8% heat-inactivated FBS, 8% horse serum, 1 µg/ml penicillin–streptomycin, and 1% L-glutamine.

Exposure to glutamate to induce cell death and its modulation by MGV-1. (1) For U118MG cells, glutamate at a concentration of 35 mM was applied with or without MGV-1. As discussed, glutamate levels in normal healthy brain tissue can average 15 mM, and these levels can be doubled in diseased brain tissue, whereas in cell organelles glutamate levels can be as high as 250 mM (as discussed). The concentration of 35 mM of glutamate in vitro provides a level of cell death that can be upregulated as well as downregulated by our application of TSPO ligands, as was determined a priori by dose-response analysis. In more detail, U118MG cells were seeded, 2 × 10$^5$ cells per well of 6-well plates and 6 × 10$^5$ cells per well of 96-well plates. Twenty-four hours after seeding, U118MG cells were pretreated with MGV-1 (or one of the other compounds) at various concentrations from 0.1 nM–100 µM. Twenty-four hours after the pretreatment, the cells were exposed to 35 mM of glutamate. In all, 25 µM as well as 50 µM of MGV-1 were found to be most effective for protection against cell death (Figure 2), and were applied for subsequent experiments. The negative control was vehicle, that is, ethanol with a final concentration of 1%. The application of ethanol of this concentration was compared a priori with no ethanol application, and showed no effect for the various assays used. The positive control was exposure to 35 mM of glutamate alone (with 200 µM of glycine).

(2) For PC12 cells (as for U118MG cells), dose-response assays of the lethal effects of glutamate on PC12 cells established 35 mM as an optimal concentration for glutamate for induction of LDH activity in the medium in the range of 20–40% as compared with the effect of the positive control, 1% Triton X-100, the latter which is defined as causing 100% of LDH release in the medium. As we did not want to work with marginal effects, but effects that can be up or down regulated by various treatments, we applied 35 mM of glutamate.

(3) Dose-response curves for MGV-1 as a modulator of cell death of PC12 cells were established using 0.1 nM–100 µM of MGV-1 (50 µM was found to be an optimal enhancer of cell death induced by glutamate). Importantly, this same treatment induced also differentiation of PC12 cells.

Differentiation of PC12 cells. For our newly developed differentiation method by MGV-1 in combination with glutamate, PC12 cells were plated onto poly-L-lysine coated culture plates and Petri dishes at a relatively low density (5.2 × 10$^4$ cells/cm$^2$ for well-plates and 3.6 × 10$^4$ cells/cm$^2$ for Petri dishes), in DMEM medium containing 8% heat-inactivated FBS and 8% horse serum. Twenty-four hours after plating, MGV-1 was added (50 µM of MGV-1 was found to be very effective). Twenty-four hours after this pretreatment, the medium was replaced with DMEM medium containing 0.5% heat-inactivated FBS and 0.5% horse serum (starvation medium) supplemented with MGV-1 together with glutamate (35 mM of glutamate was proved to be effective, see further below). Exposure to vehicle alone (culture medium with 1% of ethanol) was used as the control. (1% of ethanol by itself did not induce differentiation or cell death.) Six to 7 days after treatment, cells were collected for assays (see diagram below).

Basic diagram of temporal sequence of procedures applied to induce neurite sprouting of PC12 cells

During these 6–7 days, medium was not replaced. For differentiation by MGV-1 by itself, glutamate was omitted from the procedure. For differentiation by glutamate by itself, MGV-1 was omitted from the procedure. Glycine (200 µM) was typically added to glutamate exposure, but could also be omitted as it had no effect for differentiation. With all treatments, cell cultures were subjected to daily microscopic evaluation and verification.

For differentiation of PC12 cells by NGF,$^{23}$ applied for comparisons with differentiation by glutamate and MGV-1, PC12 cells were plated in DMEM medium containing 8% FBS and 8% horse serum. Twenty-four hours after plating, the medium was replaced with starvation medium containing 100 ng/ml of NGF. Twenty-four hours after adding NGF, the medium was replaced again with starvation medium containing 100 ng/ml of NGF. Six to 7 days after treatment, cells were collected for assays. During these 6–7 days, medium was not replaced. For additional assays, NGF treatment was combined with MGV-1 and/or glutamate. With all treatments, cell cultures were subjected to daily microscopic evaluation and verification.

Assays for cell culture studies

Viability assays: These assays were performed to learn the effects of our various treatments (see above) on cell viability and cell death of U118MG cells and PC12 cells.

Microscopic analysis: Before other assays, cell morphology, including rounding and blebbing, cell fragmentation, sprouting of neurites, etc. was analyzed with an inverted microscope to recognize apoptosis, necrosis, neurodifferentiation, etc.

Trypan blue exclusion assay: This traditional assay includes cell counting. Viable cells exclude Trypan blue, whereas nonviable cells absorb the dye because of impaired plasma membranes and appear blue.

LDH cytotoxicity assay: Wells of 96-well plates were with 6000 cells per well and treated according the protocol presented above. We used the Cytotoxicity Detection Kit (LDH) (Roche Pharmaceuticals) according to the manufacturer’s instructions. When cell plasma membranes are damaged, the cytoplasmic enzyme LDH is released into the medium. The amount of formazan formed is proportional to the amount of LDH released.$^{27}$
Mitochondrial assays

As mitochondria are important for virtually all life essential cell functions, including cell death as well as differentiation, and even regulation of gene expression, related events taking place at mitochondrial levels were assayed.

Metabolism assay with XTT: We used the Cell proliferation-XTT based assay kit (Biological Industries), as described previously.72 The 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (MTS) assay is based on reduction of XTT by mitochondrial dehydrogenases of viable cells yielding an orange formazan product. Absorbance at 490 nm with reference at 680 nm was measured with the Spectrophotometer Zenyth 200 (Anthis) and the results were calculated and normalized according to the formula presented by the manufacturer.

Neuronal differentiation assays

Neurite length: The major and determining indication of neural development, including cell death as well as differentiation, and even regulation of gene expression, related events taking place at mitochondrial levels were assayed. Absorbance at 492 nm with reference at 690 nm was measured with the Spectrophotometer Zenyth 200 (Anthis) and the results were calculated and normalized according to the formula presented by the manufacturer.

Animals

All use and treatment of the rats and mice was approved by the local Institutional Review Committee of the Technion - Israel Institute of Technology (Haifa, Israel) following government guidelines. This included kainic acid injections of Sprague-Dawley rats, and the behavioral and histological assays regarding the treatments to attenuate the injurious effects of kainic acid and their consequences. It also included the breeding of R6-2 mice, their genotyping, the behavioral assays, the observations of their well-being and lifespan, in particular the treatments to attenuate the negative effects of this hereditary disease. In particular, all behavioral observations were done double blind: first of all – the technician/observer did not know which treatments applied to which rats, and the experimenter was not informed of the results until the assay in question was concluded. We chose these animal models because various compounds binding to the TSPO (e.g., PK 11195, Ro5 4864) were shown previously to have protective effects in excitotoxic models for temporal lobe epilepsy and striatal damage as part of Huntington disease.48,61,62

Seizure induction in rats by kainic acid

Rats were obtained at an age of 7 weeks (Harlan). They were then kept individually in sterile cages at the animal facilities at the Rappaport Institute of the Medical Faculty of the Technion. The cages were kept in air-conditioned rooms, at a temperature of around 24 °C with a 12-h light–dark cycle. The rats were acclimatized for a week before the experiments.

Behavioral seizures in rats including gnawing, wet dog shakes, head bobbing, forepaw abduction, rearing, forelimb clonus, loss of balance, and convulsions were induced by systemic injections of kainic acid and scored as previously described.48,63,65 Such induced seizures typically are accompanied by neurodegeneration in the hippocampus, amygdala, pyriform cortex, followed by edema in the diencephalon, and mesencephalon.48,64,65 To test the effects of pretreatment with MGV-1 on the consequences of kainic acid injections, first of all MGV-1 was dissolved in phosphate-buffered saline obtained from Sigma-Aldrich (Rehovot, Israel), pH 7.4 (PBS), and injected 1.5 h later. Injection volumes did not exceed 1 ml/kg per animal. After the kainic acid injections, the rats were monitored continuously for 2 h for the development of epileptic symptoms.48,66

For MGV-1 post-treatment, rats first received a KA injection (9 mg/kg/rat) and then a single injection of 15 mg/kg MGV-1 2 h later, that is, after the typical period for full blown seizure activity. In a single experiment, each experimental group and the vehicle control group consisted of nine rats. Then, for 1 week, daily injections of MGV-1 were given, and the rats were tested daily for hyper reactivity in response to handling.51 Histological assays were applied 2 weeks after kainic acid injections.
Neurohistological analyses of kainic acid-injected rats
For histological analyses, rats that had developed seizures and had subsequently received treatment or control vehicle were sedated with ketamine (75 mg/kg) and xylazine (10 mg/kg) injections until no reflexes were seen and were then perfused with 4% paraformaldehyde. Rats that had been pretreated with MGV-1 before kainic acid injections, and showed no seizures, were sedated and perfused in the same way. The brains were taken and then stored in paraformaldehyde 4% solution at 4 °C. After 48 h, the solution was changed to 20% sucrose solution at 4 °C. Brains of rats that were post treated with MGV-1 were prepared in the same way. After the brains had sunk they were embedded in optimal cutting temperature compound, frozen with liquid nitrogen, and stored at −70 °C. The brains were cut at −18 °C on a Cryostat Leica (Wetzlar, Germany) CM3050S microtome in 40 μm sections from Bregma −2.8 to −5.8. The sections were kept in PBS without Ca2+ and Mg2+ with 0.5% sodium azide at 4 °C. Sections were incubated with primary antibodies against anti-NeuN to label neurons. The appropriate secondary antibodies were applied with VECTASTAIN Peroxidase ABC Kits (Vector Laboratories, Burlingame, CA, USA) based on avidin–biotin, according to the manufacturer’s instructions. The labeled, floating sections were mounted on microscope slides and coverslipped applying glycerol vinyl alcohol aqueous mounting solution. For observations and micrographs, a BH2 Olympus (Tokyo, Japan) upright microscope outfitted with a Nikon (Tokyo, Japan) DSFi1 digital camera was used.

The R6-2 transgene mouse model for Huntington disease
Huntington disease in humans is a hereditary disease including neurodegeneration leading to degradation in motor performance, continual involuntary movements, tremors, cardiovascular and respiratory impairments, and finally leading to death of the patients having the disease. For this study, R6-2 mice (~120 CAG repeats B6CBA-Tg(HD exon1)62 Gpb 62 Gpb/3J) mice were used as a transgenic mouse model for Huntington disease. For this purpose, mice obtained from the Jackson Laboratory were bred. In particular, males hemizygous for Tg(HD exon1)62 Gpb, and females from the wild-type background strain B6CBAF1/J, were bred according to the protocol of Jackson Laboratory.

Genotyping
With genotyping, we determined the presence of the Tg(HD exon1)62 Gpb gene in the parents and the offspring, according to standard methods as recommended by Jackson Laboratory and performed by Amnevet Preclinical Applications for Laboratory Animals, 2amnevet@gmail.com, Halfa, Israel). Tail tips were taken and kept at temperatures of ≤4 °C until the procedures for analysis performed at the same day. For DNA extraction, the tail tips were lysed in 75 μl of 25 mM NaOH solution at 98 °C for 0.5 h. Then, the lysates were cooled to 4 °C and 75 μl of 40 mM of Tris-HCl solution (pH 5.5) was added. To ensure high purity and quality of the DNA obtained we applied the QIAGEN DNA EXTRACTION KIT. PCR was performed using a DNA polymerase kit (DreamTag PCR Master Mix (2X), of Thermo Scientific, Tamar Laboratories Supplies) and a thermal cycler (MJ Mini, Bio-Rad Laboratories, Rishon Le Zion, Israel).
To distinguish the mice carrying the Tg(HD exon1)62 Gpb mutation from the wild type, we used the following primers for the mutated alleles:
1. For the Tg(HD exon1)62 Gpb mutation:
   - oIMR2093 – forward primer. Sequence: 5′-AAGCTAGCTGAGTAA CGCCATT-3′.
   - oIMR2095 – reverse primer. Sequence: 5′-CTACACGCCCTCTCCAAAGGT TATAG-3′.
   The expected result is a 170-bp product (Jackson Laboratory).
2. For the number of CAG repeats in the Tg(HD exon1)62 Gpb mutation:
   - oIMR6533 – forward primer. Sequence: 5′-GGCTACGAGGAAGGCTGGAGG-3′.
   - Tm0IMR1954 – reverse primer. Sequence: 5′-CCGCTACGGTCTCGCTTTA-3′.
   Expected results are 566–596-bp products depending on the numbers of CAG repeats (120 ± 5 CAG repeats).
   The thermal protocol applied was:
   - Initial denaturation: 2 min at 94 °C

- 35 cycles of:
  - Denaturation: 30 s at 94 °C.
  - Annealing: 30 s at 65 °C.
  - Extension: 2 min at 72 °C.
- Final extension: 10 min at 72 °C.

Gel electrophoresis was done with the aid of an E-Gel PowerBase apparatus (Invitrogen, Life Technologies, RHENIUM, Modi’in, Israel) using E-Gel 2% Double Comb gels applying the DNA Loading Dye #80611 (Thermo Scientific, Waltham, MA, USA) and a 100-bp ladder. The gels were imaged under UV lighting using an LAS-3000 luminescent image analyzer (Fujiﬁlm). CAG repeat evaluation was done against Invitrogen’s TrackIt ladder bands by AUTOCAD 2014 software (http://www.autodesk.com). For the male R6-2 breeders obtained from Jackson Laboratories, the numbers of CAG repeats were between 115 and 120, and this was true also for the two generations of their offspring we used to test the effects of 2-CI-MGV-1 and 2-CI-MGV-2, that is, within the 120 ± 5 CAG range required for constancy in phenotype.

Drug efficacy assays
For tests of drug efficacy, male offspring hemizygous for Tg(HD exon1) 62 Gpb were used to determine drug effect on lifespan, motor activity, and general well-being of the animals. Drug treatment for this study consisted of daily (5 continuous days per week) subcutaneous injections in the neck area of mice with 20 μl of: the drug vehicle DMSO (vehicle control), 2-CI-MGV-1 (17.5 mg/kg) and 2-CI-MGV-2 (7.5 mg/kg). Pilot studies also included application of saline (sham control), DMSO plus sesame oil (1:9), classic TSPO ligand PK 11195 (15 mg/kg), MGV-1 (15 mg/kg), and MGV-2 (15 mg/kg and 7.5 mg/kg). Quantities were used according to previous studies regarding TSPO ligands48 and adapted depending on the findings in the course of the pilot studies. The treatments were given until the animals died a natural death. Behavioral experiments included distance covered in an open field apparatus,89 freezing,90 and balance on a rotor-rod.89 Spontaneous tremor activity was evaluated using a Hamilton–Kinder sensor in a soundproof ventilated apparatus (Kinder Scientiﬁc). The assays for locomotor activity, tremors, and body weight were performed on a weekly basis, together with evaluation of the well-being of the mice. Finally, the day of death was noted. As lifespan data were most pronounced, these are presented in the present description of the results. No adverse effects of any of the drug treatments were noted, neither for specific tests, nor for general observations. Importantly, DMSO had no effect by itself as compared with naive mice or saline-injected mice with various behavioral assays.20

Statistical analysis
Data are expressed as mean ± S.E.M. or S.D., as stated for each experiment. The programs used for statistical analysis were Instat version-2 and graphpad prism (GraphPad Software, San Diego, CA, USA). When comparisons of more than two groups were needed we used one-way analysis of variance (ANOVA) was performed with post hoc: Bonferroni’s multiple comparisons test, Dunnett’s multiple comparisons test, or Wilcoxon matched-pairs signed rank test, as appropriate. When required, the non-parametric Friedman test for repeated measures was used with Dunn’s as a post hoc. When comparing only between two groups, Mann-Whitney’s or Student’s t-test for dependent or independent samples was performed as appropriate. The criterion for statistical significance was P < 0.05.

AABBREVIATIONS
2-CI-MGV-1, 2-(2-chlorophenyl)quinazolin-4-yl dimethylcarbamate; 2-CI-MGV-2, 2-(2-chlorophenyl)quinazolin-4-yl ethyl(methyl)carbamate; 2-CI-MGV-3, 2-(2-chlorophenyl)quinazolin-4-yl diethylcarbamate; ΔΨm, mitochondrial membrane potential; ATCC, American Type Culture Collection; CA1, cornu ammonis area 1; CNS, central nervous system; GFAP, glial fibrillary acidic protein; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetratetraethylbenzimidazolycarbocyanine iodide a cationic carbocyanine dye that accumulates in neuronal nuclei, also

© 2015 Cell Death Differentiation Association Cell Death Discovery (2015) 15027
known as Rbfox3; NGF, nerve growth factor; NMDA receptors, N-methyl-D-aspartate receptors; PC12, cell line of pheochromocytoma origin with neuronal characteristics; PK 11195, the classical TSPO ligand; N-butyl-2-yl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide; R-6-2, transgenic mouse model for Huntington disease; Strain name: B6CBA-Tg(HD exon1)62Gpb/3J; ROS, reactive oxygen species; TSPO, 18 kDa translocator protein; U118MG, glioblastoma cell line of glial origin.

ACKNOWLEDGEMENTS

Research support for this project was provided by: Niedersachsen – Israel Project, IDF, Johnson & Johnson, KAMIN, TEVA, Israel Science Foundation (LV, MG).

COMPETING INTERESTS

The authors declare no conflict of interest.

REFERENCES

1. Veerman L, Garvis M. The peripheral-type benzodiazepine receptor and the cardiovascular system. Implications for drug development. Pharmacol Ther 2006; 110: 503–524.

2. Veerman L, Garvis M. The role of 18 kDa mitochondrial translocator protein (TSPO) in programmed cell death, and effects of steroids on TSPO expression. Curr Mol Med 2012; 12: 398–412.

3. Caballero B, Veerman L, Garvis M. Role of mitochondrial protein (18 kDa) on mitochondrial-related cell death processes. Recent Pat Endoc Metab Immune Drug Discov 2013; 7: 86–101.

4. Veerman L, Bode J, Gaittner M, Caballero B, Pe'er Y, Zeno S et al. Effects of 18-kDa translocator protein knockdown on gene expression of glutamate receptors, transporters, and metabolism, and on cell viability affected by glutamate. Pharmacogenet Genomics 2012; 22: 606–619.

5. Veerman L, Garvis M, Kugler W. Apoptosis induction by erucylphosphocholesterol via the 18 kDa mitochondrial translocator protein: implications for cancer treatment. Anticancer Agents Med Chem 2014; 14: 559–577.

6. Bode J, Veerman L, Caballero B, Lakomek M, Kugler W, Garvis M. The 18 kDa translocator protein influences angiogenesis, as well as aggressiveness, adhesion, migration, and proliferation of glioblastoma cells. Pharmacogenet Genomics 2012; 22: 538–550.

7. Papadopoulou V, Baraldi M, Guilarte TR, Knudsen TB, Lacapère JJ, Lindemann P et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006; 27: 402–409.

8. Ruksha T, Aksenenko M, Papadopoulos V. Role of translocator protein in melanoma growth and progression. Arch Dermatol Res 2012; 304: 839–845.

9. Veerman L, Bachman I, Shoukrun R, Katz Y, Veerman L, Weisinger G et al. Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev 1999; 51: 629–650.

10. Veerman L, Garvis M. Peripheral-type benzodiazepine receptors: their implication in brain disease. Drug Res Rev 2000; 50: 355–370.

11. Girard C, Liu S, Adams D, Lacroix C, Sinès M, Boucher C et al. Axonal regeneration and neuroinflammation: roles for the translocator protein 18 kDa. J Neuroendocrinol 2012; 24: 71–81.

12. Wei XH, Wei X, Chen FY, Yang A, Xue HJ, Peng RA et al. The upregulation of translocator protein (18 kDa) promotes recovery from neuropathic pain in rats. J Neurosci 2013; 33: 1540–1551.

13. Sund的实际 role in neurovascular inflammation involved in neuron-astrocyte adhesion and migration. Curr Mol Med 2014; 14: 275–290.

14. Sild M, Rutherazer ES. Radial glia: progenitor, pathway, and partner. Neuroscientist 2011; 17: 288–302.

15. Oppenheim RW. Cell death during development of the nervous system. Annu Rev Neurosci 1991; 14: 453–501.

16. Roth KA, DSA C. Apoptosis and brain development. Nat Rev Neurosci 2001; 2: 261–266.

17. Jansson LC, Akerman KE. The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells. J Neuro Transm 2014; 121: 819–836.

18. Rivera-Oliver M, Diaz-Rios M. Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review. Life Sci 2014; 101: 1–9.

19. Abdul-Muneer PM, Chandra N, Haorah J. Interactions of oxidative stress and neurovascular inflammation in the pathogenesis of traumatic brain injury. Mol Neurobiol 2014; 51: 966–979.

20. Nordeen KW, Nordeen EJ. Projection neurons within a vocal motor pathway are born during song learning in zebra finches. Nature 1988; 334: 149–151.

21. Font E, Desflis E, Pérez-Cañellas MM, García-Verdugo JM. Neurogenesis and neuronal regeneration in the adult reptilian brain. Brain Behav Evol 2001; 58: 276–295.

22. Zupancic G, Sirbulescu RF. Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. Eur J Neurosci 2011; 34: 917–929.

23. Scott DE, Hansen SL. Post-traumatic regeneration, neurogenesis and neuronal migration in the adult mammalian brain. Va Med Q 1997; 124: 249–261.

24. Wechsler-Reya R, Scott MP. The developmental biology of brain tumors. Annu Rev Neurosci 2001; 24: 385–428.

25. Ramaswamy P, Aditi Devi N, Hurmuth Fathima K, Dalavakodihalli Nanjaiah N. Activation of NMDA receptor of glutamate influences MMP-72 activity and proliferation of glioma cells. Neurol Sci 2014; 35: 823–829.

26. Hardingham GE. Coupling of the NMDA receptor to neuroprotective and neurodestuctive events. Biochem Soc Trans 2009; 37: 1147–1160.

27. Waldbaum S, Patel M. Mitochondria, oxidative stress, and temporal lobe epilepsy. Epilepsy Rev 2010; 88: 23–45.

28. Kumar BM, Maeng GH, Lee YM, Kim TH, Lee JH, Jeon BG et al. Neurogenic and cardiomyogenic differentiation of mesenchymal stem cells isolated from minimip bone marrow. Res Vet Sci 2012; 93: 749–757.

29. Lassmann H, Petsche U, Kitz K, Baran H, Sperk G, Seifertlager F et al. The role of brain edema in epileptic brain damage induced by systemic kainic acid injection. Neuroscience 1984; 13: 691–704.

30. Lassman HP, Buri SK, Ho I, Sado R, Rosenkilde HC. The pharmacokinetics and bioavailability of nonfinsme maleate in healthy men. J Clin Psychiatry 1984; 45: 26–32.

31. Sperk G, Lassmann H, Baran H, Seifertlager F, Hornykiewicz O. Kainic acid-induced seizures: dose-relationship of behavioural, neurochemical and histopathological changes. Brain Res 1985; 338: 289–295.
46 Sztiria L, Joó F, Dux L, Bött Z. Effects of systemic kainic acid administration on regional Na+, K+-ATPase activity in rat brain. J Neurochem 1987; 49: 83–87.

47 Milgram NW, Izen DA, Mandel D, Palantzas H, Pepkowski MJ. Deficits in spontaneous behavior and cognitive function following systemic administration of kainic acid. Neurotoxicology 1988; 9: 611–624.

48 Veenman L, Leschiner S, Spanier I, Wessinger G, Weizman A, Gavish M. PK 11195 attenuates kainic acid-induced seizures and alterations in peripheral-type benzodiazepine receptor (PBR) protein components in the rat brain. J Neurochem 2002; 80: 917–927.

49 Acevedo-Torres K, Berrios L, Rosario N, Dufault V, Sketchov S, Eaton MJ et al. Rab-mediated trafficking of the neurotoxic protein riboflavin 3 oxidoreductase in mammalian cell lines and rat brain. J Neurochem 2004; 89: 1754–1763.

50 Weng Y, Wang T, Brickner-Anthony C, Deng B, Reiner A. Loss of corticostriatal and thalamostriatal synaptic terminals precedes striatal projection neuron pathology in heterozygous Q140 Huntington’s disease mice. Neurobiol Dis 2013; 60: 89–107.

51 Assis LC, Straliotto MR, Engel D, Hort MA, Dutra RC, de Bem AF. β-Caryophyllene protects the C6 glioma cells against glutamate-induced excitotoxicity through the Nrf2 pathway. Neuroscience 2014; 279: 220–231.

52 Legrand C, Bour JM, Jacob C, Caspi-Asou M, Martial A, Marc A et al. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. J Biotechnol 1999; 25: 231–243.

53 Chen H, Aten A, Arav-Gad R, Lee I, Zheng J, Sepedi TE et al. Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. Biochim Biophys Acta 2004; 1648–1649.

54 Suter DM, Miller KE. The emerging role of forces in axonal elongation. Prog Neurobiol 2011; 94: 91–101.

55 Kim KK, Yang Y, Zhu J, Adelstein RS, Kawamoto S. Rbfox3 controls the biogenesis of a subset of microRNAs. Nat Struct Mol Biol 2014; 21: 901–910.

56 Zhu X, Hao X, Luo J, Hu X, Zhao S et al. Morphological changes of radial glial cells during mouse embryonic development. Brain Res 2014; 50006-8993: 01746–01746.

57 Weng Y, Veenman L, Shandlovay L, Leschiner S, Spanier I, Lakomek M et al. Ligands of the mitochondrial 18 kDa translocator protein attenuate apoptosis of human glioblastoma cells exposed to erucylphosphohomocholine. Brain Res 2010; 1317: 435–450.

58 Vaudry D, Stork PJ, Lazarovic P, Eden LE. Signaling pathways for PC12 cell differentiation: making the right connections. Science 2002; 296: 1648–1649.

59 Schousboe A. Transport and metabolism of glutamate and GABA in neurons and glial cells. Int Rev Neurobiol 1981; 22: 1–45.

60 Zhou Y, Danbolt NC. Glutamate as a neurotransmitter in the healthy brain. J Neural Transm 2014; 121: 799–817.

61 Zhu H, Xiao X, Luo J, Min S, Xie F, Zhang F. Propofol inhibits inflammatory cytokine-mediated glutamate uptake dysfunction by alleviating learning/memory impairment in depressed rats undergoing electroconvulsive shock. Brain Res 2015; 15955C: 101–109.

62 Behrens PF, Langemann H, Strohschein R, Draeger J, Hennig J. Extracellular glutamate receptor desensitization and its role in synaptic transmission. Neurol 1989; 3: 209–218.

63 Brandt L, Izen DA, Mandel D, Palantzas H, Pepkowski MJ. Deficits in spontaneous behavior and cognitive function following systemic administration of kainic acid. Neurotoxicology 1988; 9: 611–624.

64 Veenman L, Vainshtein A, Gavish M. TSPO as a target for treatments of diseases, including neuropathological disorders. Cell Death Dis 2013; 4: e1911.

65 Gross D, Benhardt G, Buschauer A. Platelet-derived growth factor receptor independent proliferation of human glioblastoma cells: selective tyrosine kinase inhibitors lack antiproliferative activity. J Cancer Res Clin Oncol 2006; 132: 589–599.

66 Trussell LO, Fischbach GD. Glutamate receptor desensitization and its role in neurological processes induced by ammonium chloride: potential implications for neuro-pathological effects due to hyperammonemia. CNS Neural Disord Drug Targets 2014; 13: 574–592.

67 Golani I, Weizman A, Leschiner S, Spanier I, Eckstein N, Limor R et al. Hormonal regulation of peripheral benzodiazepine receptor binding properties is mediated by subunit interaction. Biochemistry 2001; 40: 10213–10222.

68 Kuo S-C, Hour M-J, Huang L-J, Lee K-F. Preparation of 2-phenyl-4-quinazolines and 2-phenyl-4-alkoxy-quinazolines as anti-cancer and antiplatelet drugs. [ Patent number: US 6479499 B1, 12 November 2002 ]

69 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.

70 Fares F, Gavish M. Characterization of peripheral benzodiazepine binding sites in human term placenta. Biochem Pharmacol 1986; 35: 227–230.

71 Zeno S, Zaaroor M, Lescherin S, Veenman L, Gavish M. CoCl2 induces apoptosis via the 18 kDa translocator protein in U118MG human glioblastoma cells. Biochemistry 2009; 48: 4652–4661.

72 Caballero B, Veenman L, Bode J, Lescherin S, Gavish M. Concentration-dependent bimodal effect of specific 18 kDa translocator protein (TSPO) ligands on cell death processes induced by ammonium chloride: potential implications for neuro-pathological effects due to hyperammonemia. CNS Neural Disord Drug Targets 2014; 13: 574–592.

73 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.

74 Fares F, Gavish M. Characterization of peripheral benzodiazepine binding sites in human term placenta. Biochem Pharmacol 1986; 35: 227–230.

75 Laferrière NB, Brown DL. Expression and posttranslational modification of a subset of microRNAs. Nat Struct Mol Biol 2014; 21: 901–910.

76 Zhu X, Hao X, Luo J, Hu X, Zhao S et al. Morphological changes of radial glial cells during mouse embryonic development. Brain Res 2014; 50006-8993: 01746–01746.

77 Legrand C, Bour JM, Jacob C, Caspi-Asou M, Martial A, Marc A et al. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. J Biotechnol 1999; 25: 231–243.

78 Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardioprotective gene. Gene 2002; 286: 135–141.

79 Chelli B, Lena A, Vanacore R, De Pauro E, Costa B, Rossi L et al. Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. Biochem Pharmacol 2004; 68: 125–134.

80 Suter DM, Miller KE. The emerging role of forces in axonal elongation. Prog Neurobiol 2011; 94: 91–101.

81 Dredgem BK, Jensen KB. NeuN/Rbfox3 nuclear and cytoplasmic isoforms other acidic amino acids toward rat hippocampal neurons. J Neurochem 2004; 89: 1733–1743.

82 Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. The time course of glutamate in the synaptic cleft. Science 1992; 258: 1498–1501.

83 Danbolt NC. Glutamate uptake. Prog Neurobiol 2001; 65: 1–105.

84 Trussell LO, Fischbach GD. Glutamate receptor desensitization and its role in synaptic transmission. Neurol 1989; 3: 209–218.

85 Bracco V, Giannelli SG, Mazzocchi PG. Modeling physiological and pathological human neurogenesis in the dish. Front Neurosci 2014; 8: 183.

86 Villarroya-Camps D, Gastaldi L, Conde C, Caceres A, Gonzalez-Billault C. Rab-mediated trafficking role in neurite formation. J Neurochem 2014; 129: 240–248.

87 Veenman L, Vainshtein A, Gavish M. TSPO as a target for treatments of diseases, including neuropathological disorders. Cell Death Dis 2013; 4: e1911.