Published in final edited form as:

Brain Res. 2010 June 21; 1339: 70–75. doi:10.1016/j.brainres.2010.04.037.

Neighbor effects of neurons bearing protective transgenes

Angela L Lee^{*}, Laura B Campbell, and Robert M Sapolsky

Department of Biology, Stanford University, Stanford, CA 94305-5020

Abstract

Viral vectors bearing protective transgenes can decrease neurotoxicity after varied necrotic insults. A neuron that dies necrotically releases glutamate, calcium and reactive oxygen species, thereby potentially damaging neighboring neurons. This raises the possibility that preventing such neuron death via gene therapy can secondarily protect neighboring neurons that, themselves, do not express a protective transgene. We determined whether such “good neighbor” effects occur, by characterizing neurons that, while uninfected themselves, are in close proximity to a transgene-bearing neuron. We tested two genes whose overexpression protects against excitotoxicity: anti-apoptotic Bcl-2, and a calcium-activated K⁺ channel, SK2. Using herpes simplex virus type 2-mediated transgene delivery to hippocampal cultures, we observed “good neighbor” effects on neuronal survival following an excitotoxic insult. However, in the absence of insult, “bad neighbor effects” could also occur (i.e., where being in proximity to a neuron constitutively expressing one of those transgenes is deleterious). We also characterized the necessity for cell-cell contact for these effects. These phenomena may have broad implications for the efficacy of gene overexpression strategies in the CNS.

Keywords

ischemia; excitotoxicity; gene therapy; glutamate; kainic acid; HSV; hippocampus

INTRODUCTION

Neurological insults such as seizures or hypoxia-ischemia, can cause devastating damage. This is principally due to excitotoxicity, the result of excess levels of the major excitatory neurotransmitter in the brain, glutamate, which leads to pathological overactivation of glutamate receptors. Gene therapy in the CNS can prevent neuron death from excitotoxic insults (Sapolsky, 2003) initiated by hypoxia-ischemia or occurring during seizures. Different transgenes have targeted various cellular processes, including metabolism, oxidation, calcium and glutamate trafficking, neural repair, inflammation and apoptosis. As neurons dying necrotically can damage neighbors (e.g., by releasing neurotoxic quantities of glutamate, calcium and reactive oxygen species), saving a neuron from death with gene therapy might aid transgene-negative neighbors. We tested the hypothesis that such transgene-bearing cells would, in effect, constitute “good neighbors.”

We used HSV amplicon vectors to overexpress genes that lessen excitotoxicity by blunting two facets of neuron death (Lawrence et al., 1996; Lee et al., 2003). The first coded for Bcl-2,

© 2010 Elsevier B.V. All rights reserved.

^{*}Corresponding author: tel 650-723-3260, fax 650-725-5356, angelee@stanford.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the anti-apoptotic mitochondrial membrane protein (Hockenbery et al., 1990). The second was a calcium-activated potassium channel, SK2, a transmembrane protein that mediates the I_{medium} after hyperpolarization of the action potential (Hammond et al., 2006). Overexpression prolongs the refractory period, thus damping excitation. SK2 overexpression has also been shown to limit glutamatergic EPSP responses in hippocampal neurons (Hammond et al., 2006).

We determined whether, following an excitotoxic insult, being in close proximity to an infected neuron increases survival of uninfected neurons, and if neurons overexpressing a protective transgene release fewer neurotoxic soluble factors that would otherwise damage neighbors. Our data suggest transgene-bearing hippocampal neurons protect their neighbors, and that this “good neighbor” effect does not require cell-cell contact.

RESULTS

Increased neuron survival after excitotoxicity within a defined distance from a transgene-positive neuron

Following excitotoxic insults, we quantified the number of NeuN⁺ (a neuronal marker) cells within a radius of 5 neuronal nuclei or a generous maximum of 2 cell diameters (~67 μm) of a neuron expressing a protective transgene (identified by GFP expression) (Fig. 1a; henceforth, we will refer to being within that radius as being within the “neighborhood” of the infected neuron). This distance is an estimation of the maximum distance reactive oxygen species (ROS) may travel after release from a dying neuron (J. Beckman, personal communication, and (Pacher et al., 2007). Neurons overexpressing Bcl-2 or SK2 had “good neighbor” effects in that there were more NeuN⁺ cells in their neighborhood than in the neighborhood of neurons expressing only the reporter gene green fluorescent protein (GFP), following excitotoxin exposure (Figure 1b). Interestingly, in the absence of insult, there were fewer NeuN⁺ cells in the neighborhood of a Bcl-2 or SK2-expressing neuron, suggesting “bad neighbor” effects under non-neurotoxic conditions. We attempted to quantify the number of dead cells (with propidium iodide staining) with and without excitotoxin as well, but saw no trends in the number of dead cells. This is most likely because dead cells do not adhere to the coverslip, and thus cannot be quantified by microscopy.

Fewer soluble neurotoxic factors are released by transgene-positive neurons

In a soluble transfer assay, we tested whether these effects required cell-cell contact (e.g., decreased excitability in an SK2-expressing neuron causing the same in networked neighboring cells) or were mediated by soluble factors. We transferred tissue culture inserts, overexpressing a protective transgene and then exposed transiently to an excitotoxic insult, atop a second monolayer, whose viability was measured. Survival was increased in co-cultures exposed to conditioned medium transferred with Bcl-2 or SK2-overexpressing cultures (Fig. 2). Thus, soluble factors can mediate these good neighbor effects. However, there was no difference in survival of neighboring neurons in the absence of insult, suggesting cell-cell contact is necessary for the “bad neighbor” effects shown in Figure 1.

During an insult, a transgene can potentially protect a neuron at the cost of its neighbors

We next explored whether “bad neighbor” effects of an additional type could be produced. We overexpressed the glucose transporter (GT), which protects against necrotic insults (Ho et al., 1993); insofar as GT enhances neuronal access to extracellular glucose, it could be to the detriment of uninfected neighbors competing for the same glucose. This was observed: in the absence of an insult, GT-overexpressing neurons did not affect viability of neighbors (Figure 3). However, following an excitotoxic insult, GT-overexpressing neurons decreased survival of neighbors. The fact that this occurred only after glutamate exposure (with a trend in the

opposite direction without insult) is in accord with increased energy demands during insult. Thus, in the face of an excitotoxin-induced energy crisis, detrimental soluble factors may be increased in cultures overexpressing GT. Further, the enhanced ability of glucose transporter-overexpressing neurons to access extracellular glucose may be to the detriment of neighboring cells competing for the same resource. This is even more striking, given that GT overexpression has been shown to have a “good neighbor” effect in reducing extracellular glutamate release after an excitotoxic insult (Gupta et al., 2001). Thus, whether a “good neighbor” effect occurs depends on the specific protective transgene expressed. Further experiments with limiting amounts of glucose, perhaps in conjunction with quantifying local concentrations of ROS and other damaging soluble factors, could clarify how much each mechanism may contribute to this “bad neighbor” effect.

DISCUSSION

To our knowledge, this study is the first to show that proximity to a therapeutically manipulated neuron increases survival of its neighbors in the face of excitotoxic insult. Previous gene therapy studies have focused on cell population benefits, and behavioral effects. This report presents evidence that preventing excitotoxic neuron death by inhibiting apoptosis (which has been shown for both Bcl-2 (Zhao et al., 2003; Zhao et al., 2004) and SK2 (Lee et al., 2003)) or decreasing neuronal excitability (Lee et al., 2003) can produce good neighbor effects. This is most likely due to a decrease in the release of toxic molecules, such as superoxide radicals, which are released by dying cells. Alternatively, it may be due to the induction of protective proteins, such as heat shock proteins, which are induced by neuronal injury and are generally neuroprotective (reviewed (Yenari et al., 1999)), or trophic factors.

If our in vitro experimental manipulations can be considered insults, we speculate that the “bad neighbor” effect in the absence of excitotoxin could possibly be akin to preconditioning, an in vivo phenomenon whereby brief sublethal ischemia conducted in an organ reduces ischemic damage caused by a subsequent prolonged ischemia in the same organ (Liu et al., 1992).

Neighbor effects have been reported previously. In cancer gene therapy, tumor cells transfected by a suicide gene induce the death of neighboring, untransfected tumor cells. Bystander effects are crucial for this therapeutic strategy, since transfection efficiency is unlikely to be greater than 10% (reviewed (Greco and Dachs, 2001)). An example of a “bad neighbor” effect in excitotoxicity is a model of spinocerebellar ataxia, whereby the overexpression of polyglutamine-expanded ataxin-7 in the Bergmann glia surrounding Purkinje neurons causes Purkinje cell degeneration (Custer et al., 2006). This mechanism is likely due to insufficient glutamate uptake by these glia.

As we characterized neighboring effects on neurons only, it is possible that these effects (both good and bad) are indirect: perhaps other CNS populations are the cells directly affected by a transgene-bearing neighbor. Our experiments were performed in mixed hippocampal cultures, so we cannot exclude roles for astrocytes, microglia, etc. as mediators of this effect on neuron survival. For instance, networked astrocytes clear glutamate from synaptic clefts (Drejer et al., 1983), and perhaps the changed physiology of transgene-overexpressing neurons affects the uptake ability of neighboring astrocytes. This, of course, would not require cell-cell contact, and could possibly underlie both the “good” and “bad” effects demonstrated here.

Another potential mechanism is illustrated by the lessening of the bystander effect in cancer gene therapy, whereby gap junction communication (akin to that of astrocytic networks) lowers efficacy and allows more tumor cells to survive (Elshami et al., 1996). Gap junctions have recently been reported to be critical for neuronal stem cell protection of host neurons, by

enabling transcellular delivery of beneficial molecules as well as modulating host network activity via calcium waves (Jaderstad et al., 2010).

A mechanism involving gap junction communication, however, would require cell-cell contact. Thus, while gap junctions could possibly be relevant in our “good neighbor” effect, they most likely are not in our “bad neighbor” effect. As a third possibility, microglia are neuroprotective by removing toxic cellular debris (Block et al., 2007). Furthermore, activated macrophages have been shown to cause axonal retraction in spinal cord injury, and this effect requires macrophage-neuron interaction (Horn et al., 2008). It is possible that neuronal overexpression of a transgene (in the absence of insult) may attract and activate resident microglia, leading to neurotoxicity.

In conclusion, a neuron spared from death can indirectly protect its neighbors. This is relevant to clinical circumstances where an intervention directly protects scattered subsets of neurons. It is particularly relevant to neuronal gene therapy, where low rates of infectivity by vectors could limit the utility of this approach; our data counter this concern. This “good neighbor” effect, when coupled with the fact that even small reductions in lesion size can spare function, suggest that gene therapy may well be an effective approach (Dumas and Sapolsky, 2001). However, not all protective transgene-expressing cells are good neighbors, and a good neighbor during an insult may be very different when none is present.

EXPERIMENTAL PROCEDURES

Virus preparation

Bcl-2, SK2, and glucose transporter (GT) vectors were purified as previously described (Lawrence et al., 1996). (Note: GT was previously named GLUT-1.) These were bi-promoter, constitutively active vectors expressing both the therapeutic protein along with a reporter, either eGFP or β gal. Negative control plasmid was p α 22eGFP, or for a subset of the laminar culture experiments, p α 22 β gal (β -Galactosidase); either one expresses the reporter gene only. After packaging these amplicons into modified HSV vectors and purifying the vectors, typical yields were $1\text{--}10 \times 10^6$ vectors/ml and $0.5\text{--}15 \times 10^6$ helper virus particles/ml. The preparation of viral particles was titrated on Vero cells, scoring positive for a chromogenic β -galactosidase substrate (in the case of β gal reporter constructs) or by dissociation and analysis by standard fluorescence-activated cell sorting (in the case of GFP-expressing constructs). With this bipromoter system there is greater than 98% coexpression between the reporter gene and the experimental transgene (Fink et al., 1997).

Primary hippocampal culture

Day 18 fetal hippocampal cultures (approximately 40% neurons, 60% glia) from rats were established as previously described (Brooke et al., 1997). Briefly, hippocampal tissue was dissected from day 18 fetal Sprague–Dawley rats (Charles Rivers Laboratories, Inc., Wilmington, MA), digested with papain and mechanically triturated. The dissociated cells were then plated onto poly-d-lysine treated dishes or glass coverslips and maintained in MEM-PAK media (UCSF Tissue Culture Facility, San Francisco, CA) containing 25 mM glucose with 10% horse serum (Hyclone, Logan, UT). Hippocampal cells were plated at a density of 3×10^5 cells/ml in poly-d-lysine coated 24-well culture polystyrene plates (353047, Becton Dickinson, Franklin Lakes, NJ), on 12-mm poly-d-lysine coated microscope cover glass (12-545-82, Fisher Scientific, Pittsburgh, PA) or on a membrane in a tissue culture insert (polycarbonate, 3 μ M pore size, 6.5 mm diameter, Costar #3472, Corning, NY) placed within 24-well plate not coated with poly-d-lysine. Cultures were maintained in a 37° C humidified incubator at 5% CO₂.

Quantification of neighboring neuron survival

Primary mixed hippocampal cultures were infected at MOI 0.067 (resulting in 1–3 GFP+ cells per field at 200× magnification) at day 10 after plating. A maximum of 45% of neurons can be infected with this amplicon system. For SK2, 18–22 h after viral infection, 300 μ M glutamate (glut, Sigma, St. Louis, MO) was added. For Bcl-2, 18–22 h after viral infection, 100 μ M KA was added. Different excitotoxic insults were chosen based on robustness of response (data not shown).

24 h after the excitotoxic insult was added, cultures were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) and neuron number quantified by NeuN+ staining (Ahlemeyer and Baumgart-Vogt, 2005). Briefly, coverslips were washed with phosphate buffered saline (PBS) and then fixed in 3% paraformaldehyde (PFA). They were washed again in PBS, and then permeabilized, first in PBS/1% glycine and subsequently in PBS/1% glycine/0.3% Triton X-100 (Sigma, St. Louis, MO). After another PBS wash, coverslips were blocked with PBS/1% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 1 hour at room temperature. Next, they were stained overnight in the dark at 4° C using a NeuN mouse antibody (Chemicon International, Temecula, CA) diluted 1:150 into PBS/0.1% Triton X-100. After another PBS wash, coverslips were stained for four hours in the dark at room temperature with 1:200 dilution of secondary antibody, horse anti-mouse IgG-conjugated Texas Red (Vector Laboratories, Burlingame, CA) in PBS/1% BSA/1% Triton X-100. After NeuN staining and extensive washes, each coverslip was dipped into a 1:1000 dilution of 4', 6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) before being placed onto a custom-designed microscope stage for fluorescence microscopy.

Images were taken on an Olympus IX microscope at 200× magnification with a CCD camera (Hamamatsu, Scientific Instrument Company, Sunnyvale, CA). GFP signal was read at 490 nm emission, DAPI at 405 nm, and Texas-Red at 570 nm. MetaMorph Software (Molecular Devices, Downingtown, PA) was used for image collection and analysis. For each GFP+ cell, a region with a 67 μ m radius (approximately 5 neuronal nuclei, or 2 cell diameters, see Figure 1a) centered on the GFP+ cell, and the number of NeuN+ cells within this region recorded. Data were collected only from coverslips with similar cell density, and from at least five coverslips from independent cultures with 1–3 GFP+ cells per image.

Soluble transfer (laminar culture) assay

Cells in a tissue culture insert were infected with vector on day 10 of culture at 5 000 viral particles/300 000 cells (MOI 0.017). This amount of vector typically infects at a ratio of 4:1 neurons/glia (unpublished observation) and has previously been shown to be therapeutic. Insult (300 μ M glutamate for SK2 and GT, 200 μ M KA for Bcl-2) was added 18–22 h after infection; the insert then was transferred atop a second, uninfected and non-insulted monolayer after 2 h of insult exposure. The insult doses were chosen to cause 30–50% death in the tissue culture insert monolayer, and were tested to have no effect on the co-cultured monolayer at 5–10 fold dilution without the presence of cells in the transwell. The physical distance separating the monolayers was 1 mm. Thus, cells in the top layer were exposed to insult for 2 hours prior to transfer. Neuronal survival was assayed 24 h later on the uninfected bottom layer only, whose only insult exposure was from the residual insult (approximately a 5–10 fold dilution) transferred with the insert, along with any soluble factors secreted by the insert cells themselves during the 24 h co-culture. Cultures were methanol-fixed and assayed for neuronal survival by quantifying MAP-2+ cells via an ELISA-based assay (Brooke et al., 1999), as an independent measure of neuronal survival from NeuN staining. Data are reported from *n* wells from at least 3 independent cultures.

Statistics

Statistical analyses and graphics were generated in R (Free Software Foundation, Boston, MA) and Prism (GraphPad Software, Inc., San Diego, CA). Bartlett's test for equal variances was run on all data prior to further analysis and α levels were set to 5%. Neuronal quantitative analyses were one-way ANOVA with Tukey-Kramer post-hoc tests with each experimental insult. Soluble transfer data analyses were one-way ANOVA with Tukey post-hoc tests within each experimental insult. Data from GFP and β gal control vectors were pooled after testing showed no statistically significant difference between them in the absence of insult ($p>0.05$ by ANOVA). 95% confidence intervals of differences are presented in the supplementary statistics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

β gal	β -galactosidase
GT	glucose transporter (GLUT-1 isoform)
GFP	green fluorescent protein
glut	glutamate
h	hour(s)
KA	kainic acid
min	minute(s)
ROS	reactive oxygen species
sec	second(s)

Acknowledgments

The authors would like to thank Sheila Brooke, Daniela Kaufer, and Ki Goosens for valuable advice and discussion, Ilona Zemlyak and Hui Wang for cell culture assistance, and John P. Bowman for statistical assistance.

FUNDING

This work was supported by National Research Service Award 5F32NS10879-02 (A.L.L.), Exploratory/Developmental Research Grant Award MH70815 (awarded to K.G., supported A.L.L.), and Research Project Grant Award AG020633 (R.M.S.) from the National Institutes of Health.

REFERENCES

- Ahlemeyer B, Baumgart-Vogt E. Optimized protocols for the simultaneous preparation of primary neuronal cultures of the neocortex, hippocampus and cerebellum from individual newborn (P0.5) C57Bl/6J mice. *J Neurosci Methods* 2005;149:110–120. [PubMed: 16084598]
- Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 2007;8:57–69. [PubMed: 17180163]
- Brooke S, Chan R, Howard S, Sapolsky R. Endocrine modulation of the neurotoxicity of gp120: implications for AIDS-related dementia complex. *Proc Natl Acad Sci U S A* 1997;94:9457–9462. [PubMed: 9256504]
- Brooke SM, Bliss TM, Franklin LR, Sapolsky RM. Quantification of neuron survival in monolayer cultures using an enzyme-linked immunosorbent assay approach, rather than by cell counting. *Neurosci Lett* 1999;267:21–24. [PubMed: 10400239]

- Custer SK, Garden GA, Gill N, Rueb U, Libby RT, Schultz C, Guyenet SJ, Deller T, Westrum LE, Sopher BL, La Spada AR. Bergmann glia expression of polyglutamine-expanded ataxin-7 produces neurodegeneration by impairing glutamate transport. *Nat Neurosci* 2006;9:1302–1311. [PubMed: 16936724]
- Drejer J, Larsson OM, Schousboe A. Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochem Res* 1983;8:231–243. [PubMed: 6856028]
- Dumas TC, Sapolsky RM. Gene therapy against neurological insults: sparing neurons versus sparing function. *Trends Neurosci* 2001;24:695–700. [PubMed: 11718873]
- Elshami AA, Saavedra A, Zhang H, Kucharczuk JC, Spray DC, Fishman GI, Amin KM, Kaiser LR, Albelda SM. Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. *Gene Ther* 1996;3:85–92. [PubMed: 8929915]
- Fink SL, Chang LK, Ho DY, Sapolsky RM. Defective herpes simplex virus vectors expressing the rat brain stress-inducible heat shock protein 72 protect cultured neurons from severe heat shock. *J Neurochem* 1997;68:961–969. [PubMed: 9048741]
- Greco O, Dachs GU. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives. *J Cell Physiol* 2001;187:22–36. [PubMed: 11241346]
- Gupta A, Ho DY, Brooke S, Franklin L, Roy M, McLaughlin J, Fink SL, Sapolsky RM. Neuroprotective effects of an adenoviral vector expressing the glucose transporter: a detailed description of the mediating cellular events. *Brain Research* 2001;908:49–57. [PubMed: 11457430]
- Hammond RS, Bond CT, Strassmaier T, Ngo-Anh TJ, Adelman JP, Maylie J, Stackman RW. Small-conductance Ca²⁺-activated K⁺ channel type 2 (SK2) modulates hippocampal learning, memory, and synaptic plasticity. *J Neurosci* 2006;26:1844–1853. [PubMed: 16467533]
- Ho DY, Mocarski ES, Sapolsky RM. Altering central nervous system physiology with a defective herpes simplex virus vector expressing the glucose transporter gene. *Proc Natl Acad Sci U S A* 1993;90:3655–3659. [PubMed: 8386379]
- Hockenbery D, Nunez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348:334–336. [PubMed: 2250705]
- Horn KP, Busch SA, Hawthorne AL, van Rooijen N, Silver J. Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. *J Neurosci* 2008;28:9330–9341. [PubMed: 18799667]
- Jaderstad J, Jaderstad LM, Li J, Chintawar S, Salto C, Pandolfo M, Ourednik V, Teng YD, Sidman RL, Arenas E, Snyder EY, Herlenius E. Communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host. *Proc Natl Acad Sci U S A* 2010;107:5184–5189. [PubMed: 20147621]
- Lawrence MS, Ho DY, Sun GH, Steinberg GK, Sapolsky RM. Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J Neurosci* 1996;16:486–496. [PubMed: 8551333]
- Lee AL, Dumas TC, Tarapore PE, Webster BR, Ho DY, Kaufer D, Sapolsky RM. Potassium channel gene therapy can prevent neuron death resulting from necrotic and apoptotic insults. *J Neurochem* 2003;86:1079–1088. [PubMed: 12911616]
- Liu Y, Kato H, Nakata N, Kogure K. Protection of rat hippocampus against ischemic neuronal damage by pretreatment with sublethal ischemia. *Brain Res* 1992;586:121–124. [PubMed: 1380876]
- Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315–424. [PubMed: 17237348]
- Sapolsky RM. Neuroprotective gene therapy against acute neurological insults. *Nat Rev Neurosci* 2003;4:61–69. [PubMed: 12511862]
- Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK. The neuroprotective potential of heat shock protein 70 (HSP70). *Mol Med Today* 1999;5:525–531. [PubMed: 10562718]
- Zhao H, Yenari MA, Cheng D, Sapolsky RM, Steinberg GK. Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. *Journal of Neurochemistry* 2003;85:1026–1036. [PubMed: 12716434]

Zhao H, Yenari MA, Cheng D, Barreto-Chang OL, Sapolsky RM, Steinberg GK. Bcl-2 transfection via herpes simplex virus blocks apoptosis-inducing factor translocation after focal ischemia in the rat. *Journal of Cerebral Blood Flow & Metabolism* 2004;24:681–692. [PubMed: 15181376]

Figure 1. Increased number of NeuN+ cells within ~5 neuronal nuclei of a transgene-expressing cell post-insult

a) Representative pseudocolor image of a NeuN-stained coverslip with 133 μ m diameter regions (yellow) centered on GFP+ cells. Green = GFP, red = NeuN (detected with Texas Red), blue = DAPI. Line is 10 μ m. b) d12 cultures infected with vectors expressing GFP, Bcl-2, or SK2 18–22 h prior to 24 h treatment with either 100 μ M KA or 300 μ M glutamate. **Bcl-2:** $p < 0.0001$ by ANOVA followed by Tukey post-hoc tests: $**p < 0.01$ GFP vs. Bcl-2, both without KA (95% confidence interval of the difference, $CI = 0.4801$ to 4.340), $**p < 0.01$ GFP without KA vs. with ($CI = 0.5358$ to 4.092), $*p < 0.05$ GFP vs. Bcl-2, both with KA ($CI = -3.829$ to -0.06214), $p < 0.05$ Bcl-2 without KA vs. with ($CI = -4.069$ to -0.01431). **SK2:** $p < 0.0001$

by ANOVA followed by Tukey post-hoc tests. *** $p < 0.001$ GFP vs. SK2, both without glutamate ($CI = 1.387$ to 4.095), *** $p < 0.001$ GFP without glutamate vs. with ($CI = 2.272$ to 5.193), * $p < 0.05$ GFP vs. SK2, both with glutamate ($CI = -2.832$ to -0.001389), $p > 0.05$ SK2 without glutamate vs. with ($CI = -1.730$ to 0.8806). Mean $\pm$ SEM and number in bars = n in all figures.

Figure 2. Differing MAP2+ cells in cultures exposed to soluble factors emitted from transgene-expressing co-cultures

d12 cultures exposed for 24 h to culture inserts overexpressing protective transgenes, with transgene-expressing cultures exposed to 200 μ M KA (Bcl-2) or 300 μ M glutamate (SK2) for 2 h prior to co-culture. Ratios are expressed as MAP2+ cells compared to control vector without insult, mean $\pm$ SEM. **Bcl-2:** $p = 0.0126$ for Bcl-2 by ANOVA followed by Tukey post-hoc tests for pairwise comparison of the means. $*p < 0.05$ for GFP vs. Bcl-2, both with KA ($CI = -0.5905$ to -0.04940). $*p < 0.05$ for control vector without KA vs. GFP with KA ($CI = 0.02897$ to 0.4417). $p > 0.05$ for control vector vs. Bcl-2, both without KA ($CI = -0.1543$ to 0.1402). **SK2:** $p < 0.0001$ for SK2 by ANOVA followed by Tukey post-hoc tests. $***p < 0.001$ for β gal vs. SK2, both with glutamate ($CI = -0.6252$ to -0.1453), $***p < 0.001$ for control vector without glutamate vs. SK2 with glutamate. $p > 0.05$ for control vector vs. SK2, both without glutamate ($CI = -0.1010$ to 0.3383). Numbers in bars are corresponding n .

Figure 3. Decrease of MAP2+ cells in cultures exposed to soluble factors emitted from co-cultures overexpressing GT

d12 cultures exposed for 24 h to culture inserts overexpressing GT and exposed to 300 μ M glutamate for 2 h prior to co-culture. Ratios are expressed as MAP2+ cells compared to control vector without insult, mean $\pm$ SEM. $p < 0.0001$ for GT by ANOVA followed by Tukey post-hoc tests. *** $p < 0.001$ for vs. GT, both with glutamate ($CI = 0.1935$ to 0.8000). $p > 0.05$ for control vector vs. GT, both without glutamate ($CI = -0.4654$ to 0.08755). *** $p < 0.001$ for control vector without glutamate vs. with glutamate ($CI = 0.2542$ to 0.5730). Numbers below bars are corresponding n .