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Research Report

Neighbor effects of neurons bearing protective transgenes

Angela L. Lee, Laura B. Campbell, Robert M. Sapolsky*

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

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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.

1. Introduction

Neurological insults such as seizures or hypoxia–ischemia, can cause devastating damage. This is principally due to excitotoxicity, the result of excessive 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, 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).

* *Corresponding author*. Fax: +1 650 725 5356.
E-mail address: angelee@stanford.edu (A.L. Lee).

Abbreviations: βgal, 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)

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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 that transgene-bearing hippocampal neurons protect their neighbors, and that this "good neighbor" effect does not require cell–cell contact.

2. Results

2.1. *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 that 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 (Fig. 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.

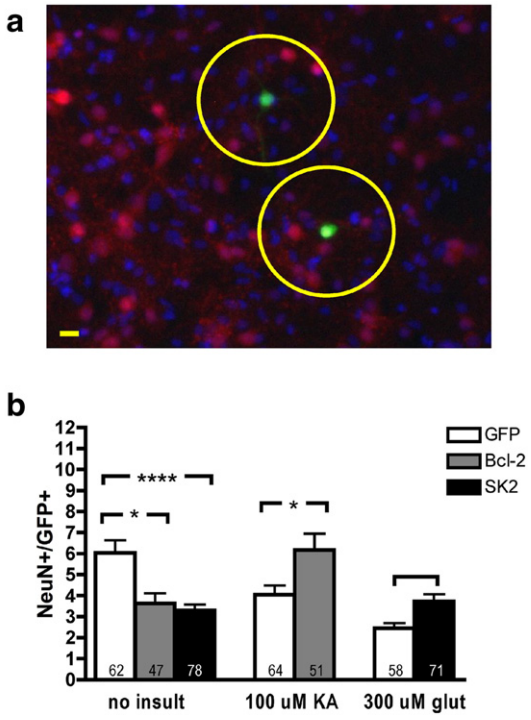

Fig. 1 – Increased number of NeuN+cells within ~5 neuronal nuclei of a transgene-expressing cell post-insult. a) Representative pseudocolor image of a NeuN-stained coverslip with 133 μM diameter regions (yellow) centered on GFP+cells. Green=GFP, red=NeuN (detected with Texas Red), blue=DAPI. Line is 10 μm. b) d12 cultures infected with vectors expressing GFP, Bcl-2, or SK2 18–22 h prior to 24 h treatment with either 100 μM KA or 300 μM 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±SEM and number in bars=n in all figures.

2.2. *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 Fig. 1.

2.3. *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 the viability of neighbors (Fig. 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