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26. There are intriguing parallels with single-cell electrophysiology work, where both tonic and phasic signals are seen in inferior temporal cortex cells when monkeys attend to stimuli [(*25*); B. J. Richmond and T. Sato, *Soc. Neurosci. Abstr.* **8**, 812 (1982); *J. Neurophysiol.* **58**, 1292 (1987)]. However, there are important differences with our data; in terms of modulation by attention our data suggest only a tonic modulation of inferior temporal cortical activity. There is no phasic modulation by attention (Fig. 1). Rather, we see a correlation between the rate of presentation of stimuli and inferior temporal cortex activity in addition to the attention-related modulation of this activity.
27. An alternate explanation for the modulatory effect seen in inferior temporal cortex might be that it reflects differences in the stimuli presented. Activity decreases in inferotemporal cortex units in monkey have been observed associated with target identification, and also with repetition of visual items [for example, (*29*)]. The probability of a target was kept constant at 50% in our study, and targets were physically identical in the conjunction condition but shared one feature in the feature tasks; therefore, there were more repetitions of physically identical targets per unit time in the conjunction task. However, if unequal repetition probabilities were to have an effect on our results, they should have led to increased activity with increasing rate of presentation. Such an effect would lead to a change in the slope relating rCBF to presentation rate in conjunction and feature tasks. We found no such evidence; the modulatory effect is tonic and so is task-dependent and stimulus-independent.
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Dopaminergic Neurons Protected from Degeneration by GDNF Gene Therapy

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Glial cell line–derived neurotrophic factor (GDNF) supports growth and survival of dopaminergic (DA) neurons. A replication-defective adenoviral (Ad) vector encoding human GDNF injected near the rat substantia nigra was found to protect DA neurons from the progressive degeneration induced by the neurotoxin 6-hydroxydopamine (6-OHDA) injected into the striatum. Ad GDNF gene therapy reduced loss of DA neurons approximately threefold 6 weeks after 6-OHDA lesion, as compared with no treatment or injection of Ad lacZ or Ad mGDNF (encoding a biologically inactive deletion mutant GDNF). These results suggest that Ad vector–mediated GDNF gene therapy may slow the DA neuronal cell loss in humans with Parkinson's disease.

Parkinson's disease is characterized by the progressive loss of DA neurons in the substantia nigra (SN) that project to the striatum. Current therapies do not prevent the continuing degeneration of DA neurons. GDNF, a neurotrophic factor for DA neurons (*1*), protects DA neurons in several rodent and primate models of Parkinson's disease when administered to the adult nigrostriatal system (*2*). Although these studies elucidated GDNF as a therapeutic molecule for limiting the neuronal damage caused by Parkinson's disease, single, repeated, or continuous infusions of recombinant GDNF protein in microgram quantities directly into brain parenchyma or cerebrospinal fluid were used. Continuous targeted delivery of neurotrophic factors to specific neurons in the central nervous system (CNS) in amounts that are therapeutic, but not deleterious to other cells, is a challenge that remains to be met. In vivo gene therapy has the potential to meet this challenge by delivering neurotrophic factors continuously to a focal brain area. In this study we delivered GDNF via an adenoviral (Ad) vector in a progressive degeneration rat model of Parkinson's disease.

Ad vectors were constructed for human GDNF, for a mutant form of GDNF (mGDNF) with 12 amino acids deleted, and for nuclear-localizing lacZ encoding β-galactosidase (β-Gal) (*3*). The GDNF vectors were tested in vitro for bioactivity. PC12 cells (*4*) were infected with 300 to 1000 plaque-forming units (PFU) of Ad GDNF or Ad mGDNF per cell or were mock-infected. Five days later, 24-hour conditioned medium (CM) was analyzed by enzyme-linked immunosorbent assay (ELISA) (*5*); 0.5 to 3.2 ng of human GDNF was secreted per 10^4 infected cells per day, as compared to 0 to 0.2 ng from Ad mGDNF– or mock-infected cells. DA bioactivity conferred by the vectors was assessed with embryonic day 14 (E14) ventral mesencephalon cultures, as described previously (*6*). Cultures were either maintained on 50% CM from PC12 cells infected with Ad vectors or were directly infected with 10 PFU per cell for 2 hours. Seven days later, cultures were stained for tyrosine hydroxylase immunoreactivity (TH-IR) to identify DA neurons. Ad GDNF led to a 65 to 84% increase in TH-IR neuron number, whereas Ad mGDNF did not improve survival (Fig. 1). These results confirmed that bioactive

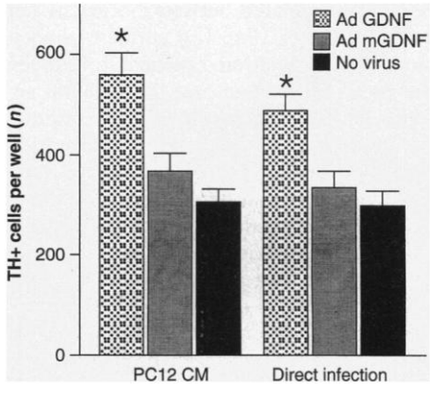

Fig. 1. Bioactive GDNF is produced after infection with Ad GDNF. The number of TH-IR (DA) neurons was counted in E14 ventral mesencephalon cultures maintained on 50% PC12 CM or directly infected with Ad GDNF or Ad mGDNF or mock-infected (mean ± SEM, $n = 6$). Ad GDNF increased survival versus Ad mGDNF and no virus in both groups [analysis of variance (ANOVA), $F = 10.49$, $P < 0.001$; asterisk indicates Tukey's post hoc pairwise comparisons at a family error rate of 0.02].

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GDNF is produced and secreted by cells infected with Ad GDNF.

The efficacy of Ad GDNF in protecting DA neurons from degeneration in vivo was assessed quantitatively with the striatal 6-hydroxydopamine (6-OHDA) progressive lesion rat model of Parkinson's disease (*7*). Male Fischer 344 rats (weighing 200 to 250 g) received bilateral striatal injections of fluorogold (FG) to retrogradely label a subpopulation of DA neurons in the SN. During the same surgery, rats were injected unilaterally immediately dorsal to the SN with Ad GDNF, Ad mGDNF, or Ad lacZ. An additional group of rats received no injection above the SN. Seven days later, 6-OHDA was injected unilaterally into the striatum at the same coordinates used for the FG injection and on the same side as the Ad injection (*8*). Because the same coordinates were used for FG and 6-OHDA injections, FG-labeled DA neurons were most susceptible to the 6-OHDA lesion. Rats were killed 42 days after 6-OHDA injection to assess the protective effects of Ad GDNF on FG-labeled neurons in the SN.

After striatal injection of FG, large numbers of FG-positive (FG+) neurons were observed in the SN, with fewer being present in A8 and the ventral tegmental area (VTA), as described previously (*7*). TH immunofluorescence revealed most large FG+ cells to be TH+, that is, dopaminergic (Fig. 2A). Forty-two days after 6-OHDA injection, a loss of TH-IR fibers was observed surrounding the striatal injection site (Fig. 2B). There were no obvious differences in the size and appearance of the striatal lesions among experimental groups. The number of FG+ cells with cross-sectional area larger than approximately 40 μm^2 was determined in every seventh section through the rostrocaudal extent of the midbrain dopaminergic cell groups (SN, VTA, and A8). Therapy with Ad GDNF significantly protected FG+ DA neurons from cell death after striatal 6-OHDA lesion (Fig. 2, C through G; Fig. 3; and Table 1). In rats treated with Ad mGDNF, Ad lacZ, or untreated (no virus), there were more small FG+ cells within and around the SN. These cells were not TH+ (*9*). Although some of these cells may be atrophied DA neurons that have lost TH expression, many have the classic morphology of microglia that have phagocytosed FG+ neuronal debris and subsequently migrated away from the site of neuronal degeneration (*7*), as has also been reported after facial and vagal nerve axotomy (*10*).

Stability of infection and transgene expression was investigated at the DNA, mRNA, and protein levels. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of rats injected with Ad lacZ revealed blue nuclei within 0.8 to 1.0 mm of the injection site, through most of the rostrocaudal extent of the SN (Fig. 2H). DA neurons with blue nuclei were occasionally observed (inset, Fig. 2H). Expression declined from 39,000 ± 5200 (n = 3) blue nuclei at 10 days to 7100 ± 1400 (n = 3) at 49 days. ELISA, reverse transcriptase polymerase chain reaction (RT-PCR), and PCR were performed at 1 and 4 weeks after Ad GDNF or Ad lacZ injection (before and 3 weeks after 6-OHDA injection) on protein, RNA, and DNA, respectively, isolated from tissue surrounding the site of Ad vector injection (*11*). At 1 week, 13 ± 1 ng of

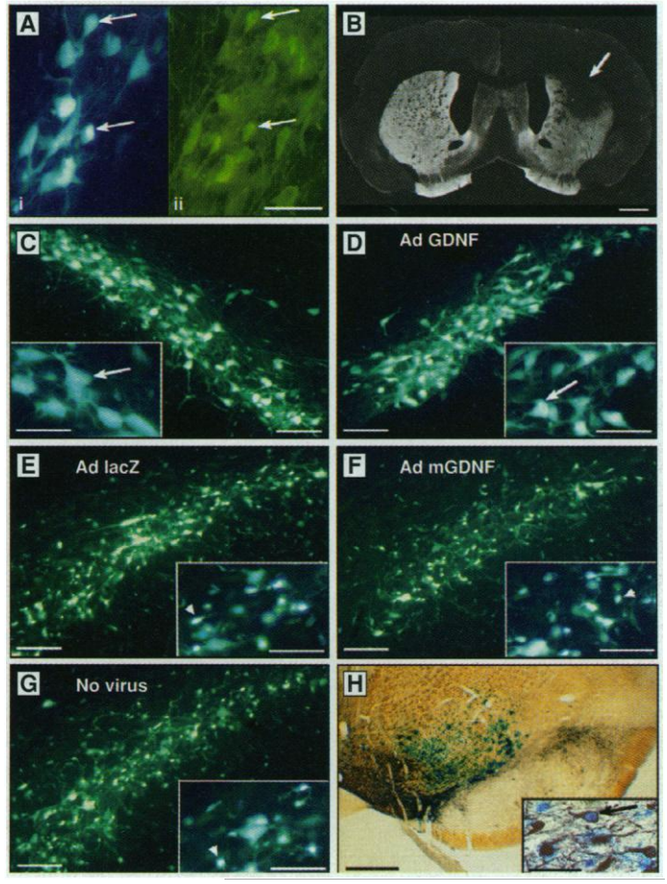

Fig. 2. Sections from the striatum and SN of rats injected with FG and 6-OHDA into the striatum, and with Ad vector into the SN or uninjected in the SN. (**A**) SN 49 days after FG. (i) FG, (ii) TH–fluorescein isothiocyanate. Most large FG+ cells are TH+ neurons (arrows). (**B**) Loss of TH-IR fibers (arrow) in the striatum 42 days after 6-OHDA injection (TH-ICC with DAB and nickel; image acquired as a negative). (**C** through **G**) FG+ cells in the anterior SN from the unlesioned (C) and lesioned side (D through G) 42 days after 6-OHDA injection. Many large FG+ cells (DA neurons; arrows) are visible on the unlesioned side (C) and lesioned side (D) of an Ad GDNF–treated rat, whereas fewer large FG+ cells but numerous small FG+ cells (atrophied neurons, microglia, or other nonneuronal cells; arrowheads) are apparent in rats treated with Ad lacZ (E) or Ad mGDNF (F) or uninjected (G). (**H**) Blue nuclei and cells dorsal to and within the medial SN and lateral VTA 10 days after injection of Ad lacZ (sections stained for β-Gal histochemistry and TH-ICC with diaminobenzidine and nickel). Inset of (H), several TH+, several blue, and one TH+/blue cell (arrow). Scale bars, 50 μm in (A) and insets of (C) through (H); 1000 μm in (B); 100 μm in (C) through (G); and 500 μm in (H).

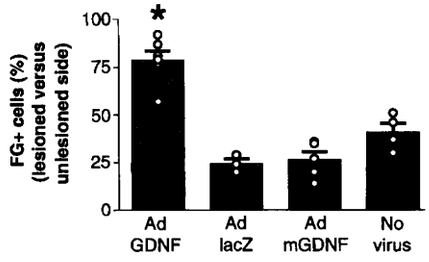

Fig. 3. Ad GDNF gene therapy prevents loss of large FG+ cells (DA neurons) in 6-OHDA–lesioned rats. Data are expressed as the percentage of FG+ neurons on the lesioned side relative to the unlesioned side. Columns and error bars represent the mean and SEM, and each circle represents the value for an individual rat. Ad GDNF (*) increased survival as compared with that of Ad mGDNF–, Ad lacZ–, and uninjected (no virus) rats (ANOVA, $F = 32.77$, $P < 0.001$; Tukey's post hoc pairwise comparisons at a family error rate of 0.001). There was no significant difference among Ad mGDNF–, Ad lacZ–, and uninjected rats.

Table 1. The mean ± SEM of large FG+ cells on the lesioned (L) and unlesioned (U) side of each group of rats 42 days after 6-OHDA. Lesioned side, ANOVA, $F = 20.28$, $P < 0.001$; unlesioned side, ANOVA, $F = 0.16$, $P > 0.9$.

	Ad GDNF	Ad lacZ	Ad mGDNF	No virus
L	620 ± 55*	209 ± 28	208 ± 40	327 ± 25
U	799 ± 76	852 ± 32	779 ± 56	822 ± 86

*Ad GDNF > Ad lacZ, Ad mGDNF, and no virus; Tukey's post hoc pairwise comparisons at a family error rate of 0.003.

GDNF and 57 ± 22 ng of β-Gal, and at 4 weeks, 4.7 ± 0.9 ng and 20 ± 0.3 ng, respectively, were present in the ventral mesencephalon. Similar decreases were observed in transgene mRNA, whereas transgene DNA levels were unchanged (Table 2), which suggests down-regulation of the Rous sarcoma virus (RSV) promoter but not loss of Ad vector–infected cells. The comparable changes in GDNF and β-Gal expression suggest that host responses to Ad GDNF and Ad lacZ were similar.

To control for the possibility that Ad vectors might have affected initial retrograde FG labeling or caused acute toxicity to DA neurons of the SN, additional rats were injected with FG and Ad vectors and killed after 7 or 10 days. The ratio of FG+ neurons on the Ad-injected side to those on the uninjected side was 0.97 ± 0.04 (n = 5) for Ad GDNF, 1.04 ± 0.06 for Ad mGDNF (n = 4), and 0.99 ± 0.11 for Ad lacZ (n = 3) (mean ± SEM; no ratios were different from the expected ratio of 1.0. Nissl-stained sections were also scored for the degree of cellular reaction around the needle tract and SN 49 days after injection of Ad vector. All rats had mild reactions around the needle tract (Fig. 4, A through C). Near the SN, no host reaction was observed in 1 of 3 (1/3) Ad lacZ, 1/6 Ad GDNF, and 0/5 Ad mGDNF; there was a mild reaction in 2/3 Ad lacZ, 3/6 Ad GDNF, and 3/5 Ad mGDNF; and a moderate reaction in 0/3 Ad lacZ, 2/6 Ad GDNF, and 2/5 Ad mGDNF rats (Fig. 4, D through F). Host reactions to the vectors were similar regardless of the encoded transgene.

These results demonstrate that an increased level of biosynthesized GDNF resulting from an Ad vector injected immediately dorsal to the SN protects the majority of DA neurons from degeneration after exposure of their terminals to 6-OHDA. 69 ± 3% of FG-labeled DA neurons degenerated in the Ad lacZ, Ad mGDNF, and uninjected groups, as compared with only 21 ± 5% in Ad GDNF–treated animals. Ad GDNF and Ad mGDNF vectors differ only by a 36-nucleotide deletion that renders mGDNF biologically inactive, and Ad GDNF and Ad lacZ differ only by the encoded transgene. The Ad vectors also had similar titers and particle ratios, and host responses to the vectors were similar. Thus, the success of Ad GDNF and failure of Ad mGDNF and Ad lacZ to protect DA neurons can be attributed to production of GDNF protein by host tissue modified by Ad GDNF. The dissociation constants (K_d) for GDNF binding to its receptors, GDNFR-α and c-Ret, which are expressed in the adult rat SN, are in the range of 2 to 300 pM (*12, 13*), and the half-maximal effective concentration (EC_{50}) of GDNF in embryonic DA cultures is 1 pM (40 pg/ml) (*1*). In this experiment, 13 ng of human GDNF was present in the vicinity of the SN at the time of 6-OHDA lesion, and 4.7 ng was present 3 weeks after the lesion, amounts that are more than adequate to activate GDNF receptors on DA neurons.

Delivery of GDNF by in vivo gene therapy rather than repeated injection or infusion has several advantages. Gene therapy is less invasive and can continuously deliver a neurotrophic factor that is biologically synthesized, processed, and secreted. Gene delivery can be located near degenerating neuronal soma or target neurons. Further specificity in delivery could be effected through the use of a cell type–specific promoter, such as the tyrosine hydroxylase promoter, which would selectively produce neurotrophic support in DA neurons in an autocrine or paracrine manner, or of a promoter specific to DA target neurons in the striatum, such as enkephalin or substance P. Alternatively, an astrocyte-specific promoter might be used to increase neurotrophic factor expression in the vicinity of DA neurons. GDNF and its receptors are expressed throughout the CNS and periphery (*13–15*); therefore, localized gene therapy is likely to minimize deleterious side effects that may result from exposure of other cells to excess GDNF.

Declining transgene expression as reported here and by others (*16, 17*) may be due to host inflammatory and immune responses to the vector, infected cells (*17*), and the transgene (*18*), as well as to down-regulation of viral promoters. Here, the decline in transgene expression was shown to be primarily a consequence of down-regulation rather than loss of Ad-infected cells, because the amount of vector DNA did not change significantly over time. Although the mechanisms underlying this down-regulation are unknown, host responses to degenerating neurons, including inflammation and activation of microglia and astrocytes, may have contributed. Similar down-regulation of transgene expression in the human Parkinsonian brain where DA neurons are degenerating may be a concern if the vectors presently available are used. However, stable transgene expression in the Parkinsonian brain may be achieved in the future through the use of new generation Ad vectors, other classes of vectors, or nonviral gene transfer methods for the CNS that will eliminate or minimize the expression of viral genes (*19*) and host responses to vectors.

It is not known how closely neurotoxin-induced lesions mimic the state of diseased neurons in humans with Parkinson's disease. The mechanism of DA neuronal loss in Parkinson's disease is unknown, although several mechanisms have been suggested, including the production of oxidative free

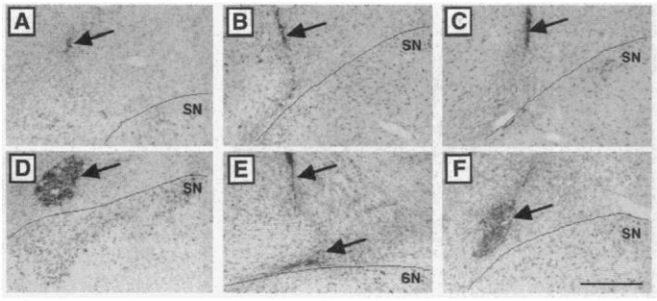

Fig. 4. Similar host reactions around the needle tract and near the SN in Ad GDNF–, Ad lacZ–, and Ad mGDNF–injected rats. Mild host responses were observed around the needle tract in all rats [representative sections from Ad GDNF (**A**), Ad lacZ (**B**), and Ad mGDNF (**C**) injections]. Near the SN, host responses varied from none to moderate. Sections representing the most severe responses observed in each group are shown; mild for Ad lacZ (**E**) and moderate for Ad GDNF (**D**) and Ad mGDNF (**F**). Arrows point to areas of host reactions; the dorsal border of the SN is indicated by a black line. Scale bar, 500 μm for (A) through (F).

Table 2. Protein, RNA, and DNA levels at 1 and 4 weeks after Ad GDNF or Ad lacZ injection. Data are listed as mean ± SEM, with each value expressed as the percentage of the corresponding 1-week value. Amounts of protein in nanograms are also shown (n = six rats at 1 week and four rats at 4 weeks).

Vector	Weeks after Ad injection	Protein		RNA (%)	DNA (%)
		Percentage	Nanograms		
Ad GDNF	1	100 ± 7	13 ± 1	100 ± 7	100 ± 4
	4	36 ± 7*	4.7 ± 0.9*	54 ± 4*	82 ± 8
Ad lacZ	1	100 ± 38	57 ± 22	100 ± 17	100 ± 14
	4	35 ± 1	20 ± 0.3	40 ± 2*	126 ± 5

*P < 0.025, 4 weeks versus 1 week, Student's t test.

radicals generated from the enzymatic or auto-oxidation of dopamine and other sources, compromised mitochondrial energy metabolism resulting from an environmental molecule such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or oxidative stress, excitatory amino acid toxicity, and calcium toxicity (*20*). Because GDNF is able to protect DA neurons against several different types of injury in animal models of Parkinson's disease (*2*), GDNF gene therapy is likely to protect diseased human neurons, regardless of the mechanism of degeneration involved.

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3. DNA encoding human GDNF preproprotein (GenBank accession numbers L19062 and L19063) was synthesized by PCR with overlapping oligonucleotides. mGDNF, with deletion of amino acids 74 through 85 of mature GDNF, corresponding to nucleotides 300 through 335 of exon 2, was generated by PCR error. A black beetle virus translation enhancer element [Y. N. Chang, L. Y. Dong, G. S. Hayward, S. D. Hayward, *J. Virol.* **54**, 3358 (1990)] and Not I, Xba I, and Nde I restriction sites were included at the 5′ end; Sal I, Bgl II and Cla I sites were included at the 3′ end. Human GDNF and mGDNF DNAs were ligated into Xba I and Cla I sites in the Ad shuttle plasmid pAvS6 [T. A. G. Smith *et al.*, *Nature Genet.* **5**, 397 (1993)]. lacZ DNA with an NH_2-terminal SV40 large T antigen nuclear localization signal was also cloned into pAVS6. Transgenes were under the control of the RSV long terminal repeat promoter and were followed by an SV40 polyadenylation signal. Plasmids were cotransfected into 293 cells with the large fragment of Cla I–digested adenovirus serotype 5 genome dl327, and homologous recombination yielded Ad vectors with E1a and E3 (1.9-kb) deletions. Positive plaques were plaque-purified twice, cesium chloride banded, and characterized by PCR and Xba I digestion. Infectious particle concentrations (or PFU) were determined by infection of 293 cells, and total particle concentrations were determined by 260-nm absorbance. Viral stock titers (PFU per milliliter) and particle ratios (total particles per PFU) were as follows: Ad GDNF, 4×10^{10}, 30; Ad mGDNF, 4×10^{10}, 35; Ad lacZ, 2×10^{10}, 50.
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11. Rats were injected with FG, Ad vector, and 6-OHDA (*8*), then killed by CO_2 suffocation. The ventral mesencephalon was dissected and frozen in liquid N_2. Tissue was sonicated in nine volumes of PBS, 0.1% Tween-20, 0.5% BSA, 2 mM EDTA, and protease inhibitors, then centrifuged at 40,000*g* for 12 min. The supernatant was used for GDNF (*5*) and β-Gal ELISA (5 Prime → 3 Prime, Boulder, CO), and RNA and DNA were isolated from the pellet with Tri Reagent (Molecular Research Center, Cincinnati, OH). RT-PCR and PCR with β-actin primers were performed as described (*15*), except that PCR conditions were 94°C for 30 s; 52°C for 45 s, and 72°C for 45 s. GDNF primers that recognize human, but not rat, GDNF (5′-GATAAACAAATGGCAGTGCT and 5′-AGCCTTCTATTTCTGGATAA) yielded a 269–base pair product with PCR conditions of 94°C for 30 s, 56°C for 45 s, and 72°C for 30 s. lacZ primers were as described [B. Lu, S. Gupta, H. Federoff, *Hepatology* **21**, 752 (1995)]. PCR products from the linear range of amplification were quantified from ethidium bromide–stained gels with NIH Image.
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Paradoxical Improvement of Impulse Conduction in Cardiac Tissue by Partial Cellular Uncoupling

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Generally, impulse propagation in cardiac tissue is assumed to be impaired by a reduction of intercellular electrical coupling or by the presence of structural discontinuities. Contrary to this notion, the spatially uniform reduction of electrical coupling induced successful conduction in discontinuous cardiac tissue structures exhibiting unidirectional conduction block. This seemingly paradoxical finding can be explained by a nonsymmetric effect of uncoupling on the current source and the current sink in the preparations used. It suggests that partial cellular uncoupling might prevent the initiation of cardiac arrhythmias that are dependent on the presence of unidirectional conduction block.

Cardiac tissue belongs to the broad class of reaction-diffusion systems that support excitation waves (*1*). In these systems, the reduction of diffusion, which corresponds to uncoupling of gap junctions in the myocardium, is generally believed to impair wave propagation, promoting slow conduction, conduction block, and initiation of spiral waves (*2*). Disturbances of propagation can also be caused by structural discontinuities of the excitable medium, which correspond

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