Abstract—Calcium channel blockers are among the most commonly used therapeutic drugs. Nevertheless, the utility of calcium channel blockers for heart disease is limited because of the potent vasodilatory effect that causes hypotension, and other side effects attributable to blockade of noncardiac channels. Therefore, focal calcium channel blockade by gene transfer is highly desirable. With a view to creating a focally applicable genetic calcium channel blocker, we overexpressed the ras-related small G-protein Gem in the heart by somatic gene transfer. Adenovirus-mediated delivery of Gem markedly decreased L-type calcium current density in ventricular myocytes, resulting in the abbreviation of action potential duration. Furthermore, transduction of Gem resulted in a significant shortening of the electrocardiographic QTc interval and reduction of left ventricular systolic function. Focal delivery of Gem to the atrioventricular (AV) node significantly slowed AV nodal conduction (prolongation of PR and AH intervals), which was effective in the reduction of heart rate during atrial fibrillation. Thus, these results indicate that gene transfer of Gem functions as a genetic calcium channel blocker, the local application of which can effectively modulate cardiac electrical and contractile function. (Circ Res. 2004;95:398-405.)

Key Words: calcium channel blocker • gene therapy

Intracellular Ca\(^{2+}\) plays a pivotal role in diverse biological processes, including gene regulation, memory, and cell death.\(^1,2\) In the heart, Ca\(^{2+}\) is essential as the activator of muscle contraction.\(^3\) Although Ca\(^{2+}\) is vital for normal function, excessive increases in intracellular Ca\(^{2+}\) foster arrhythmias, hypertrophy, apoptosis, and cardiac remodeling. Modulation of Ca\(^{2+}\) homeostasis could be useful for the therapeutic manipulation of such pathophysiological processes.\(^4-6\) Among the cell’s many Ca\(^{2+}\) handling proteins, the L-type calcium channel, initiating excitation-contraction coupling, represents a logical target for intervention. Indeed, in animal experiments, calcium channel blockers reduce cardiac hypertrophy in spontaneously hypertensive rats,\(^7\) and inhibit the electrical remodeling induced by rapid atrial pacing.\(^8\) Unfortunately, the use of calcium channel blockers is often accompanied by side effects (eg, hypotension, heart block, constipation),\(^6\) which limits their utility for the treatment of heart disease. It would be highly desirable to be able to achieve calcium channel blockade in the heart (or part of the heart) without affecting other tissues.

Such a motivation prompted us to establish a novel method to modulate calcium channel activity focally. This was accomplished by viral gene transfer of Gem into the heart in vivo. Gem is a member of a small GTP-binding family of proteins within the Ras superfamily,\(^9,10\) and was recently found to suppress L-type calcium currents in PC12 cells by inhibiting the trafficking of calcium channel \(\alpha\) subunits to the plasma membrane.\(^11\) Reasoning that a similar effect might be recruitable in native heart cells, we transduced an adenovirus encoding Gem into the heart, resulting in the suppression of L-type calcium currents and a concomitant reduction of cardiac contractility, as expected with cardiac-specific calcium channel blockade. As proof of principle for the value of focal Ca\(^{2+}\) channel blockade in vivo, we demonstrated that Gem was effective in slowing atrioventricular (AV) conduction when delivered into the porcine AV node, resulting in the reduction of heart rate during atrial fibrillation.

Materials and Methods

Plasmid Construction and Adenovirus Preparation

The full-length coding sequences of Gem (kindly supplied by Dr Katherine Kelly, NIH, Bethesda, Md, and independently by Dr Susumu Seino, Chiba, Japan) was cloned into the multiple cloning site of the adenovirus shuttle vector pAdCIG to generate pAdCIG-Gem. This construct is bicistronic (through an internal ribosome entry site) driven by a cytomegalovirus promoter and carrying green fluorescent protein (GFP) as a reporter. The point mutation W269G
was introduced into Gem by site-directed mutagenesis, creating the vector pAdCIG-Gem W269G. Detailed methods of adenovirus vector construction have been described. In vivo adenoviral transduction into guinea-pig hearts was performed as described. Adenoviruses (160 μL, equivalent to ~3×10⁸ plaque-forming units, pfu) were injected in the left ventricular (LV) cavity of guinea pigs (280 to 340 g), whereas the aorta and pulmonary artery were clamped for 50 to 60 seconds.

**Myocyte Isolation and Electrophysiology**

Seventy-two hours after gene delivery, myocytes were isolated from the left ventricles of guinea pigs, using enzymatic digestions as previously described. Membrane currents and action potentials were recorded using whole-cell patch clamp with an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif). All myocyte recordings were performed at 37°C. The micropipette electrode tip resistances of 1 to 3 MΩ when filled with internal recording solution. Uncompensated capacitance currents in response to small hyperpolarizing voltage steps were recorded for off-line integration to measure cell capacitance. Cells were allowed >5 minutes to equilibrate after whole-cell access was obtained. Action potentials were initiated by short depolarizing current pulses (2 ms, 100 to 300 pA, 10% to 15% over the threshold) at every 3 seconds. APD was measured as the time from the overshoot to the indicated percentage of repolarization. A xenon arc lamp was used to view green fluorescent protein (GFP) fluorescence at 488/530 nm (excitation/emission). Transduced cells were recognized by their obvious green fluorescence.

To measure gating currents, ionic currents were blocked by adding 2 mmol/L CdCl₂ and 0.1 mmol/L LaCl₃ to the bath solution. Leaks and capacitative transients were subtracted by a P⁴ protocol from a −100 mV holding potential. Charge movement was quantified by calculating the area under the curve for each trace, using the steady-state level of the current as a baseline.

**Electrocardiograms**

Body surface electrocardiograms (ECGs) were recorded within 2 hours after operation (baseline) and 72 hours after adenovirus injection, as previously described. Guinea pigs were anesthetized with isoflurane, and needle electrodes were placed under skin. Needle electrode positions were marked postoperatively on the skin to ensure exactly the same electrode position for 72-hour recordings. Measured QT intervals were corrected (QTC) for heart rate as previously described.

**Cardiac Hemodynamic Studies**

Seventy-two hours after LV injection, guinea-pigs were anesthetized with isoflurane. The right carotid artery was isolated and cannulated. The right carotid artery, right internal jugular vein, and right femoral vein were inserted via the right carotid artery and passed retrogradely into the left ventricle. Position was confirmed by the characteristic decrease in diastolic pressure that occurred with passage of the catheter across the aortic valve into the LV cavity, after which LV pressure and the first derivative of LV pressure (dP/dt) were recorded.

**Focal Gene Transfer Into AV Node**

Adenoviral gene transfer into AV node was performed as described. Briefly, immediately before catheterization, domestic swine (25 to 30 kg) received 25 mg sildenafil orally. The right carotid artery, right internal jugular vein, and right femoral vein were accessed by sterile surgical technique, and introducer sheaths were
Figure 2. Effect of Gem on gating properties in guinea pig ventricular cardiomyocytes. a, Representative recordings of calcium channel gating current in an AdCIG-WT Gem-transduced (control), an AdCIG-WT Gem-transduced, and an AdCIG-W269G mutant-transduced cell. Vertical scale bar = 2 pA/pF; horizontal scale bar = 10 ms; dash marks designate zero current. b, Pooled data for calcium channel gating charge in control, AdCIG-WT Gem-transduced, and AdCIG-W269G mutant-transduced cells. Q vs V data were fit to a Boltzmann distribution using the following equation: \( Q = Q_{\text{max}} / (1 + \exp(V - V_{1/2})/k) \), where \( V_{1/2} \) is the half maximum potential, \( k \) is the slope factor. Calcium channel gating charge was significantly reduced in AdCIG-WT Gem-transduced cells compared with control cells, whereas restored in AdCIG-W269G mutant-transduced cells. \#<0.05 vs control, *P<0.01 vs control.

**Results**

**Overexpression of Gem in Guinea Pig Ventricular Cardiomyocytes**

We first investigated whether Gem overexpression could inhibit L-type calcium current (\( I_{\text{Ca,L}} \)) in guinea-pig ventricular cardiomyocytes. Adenoviruses were injected into the left ventricular (LV) cavity of guinea-pig hearts, and 3 days later, LV cells were isolated. Overexpression of AdCIG-wild-type (WT) Gem resulted in a dramatic decrease of \( I_{\text{Ca,L}} \) from a peak density of 4.7±0.5 pA/pF at 10 mV (\( n=11 \)) in AdCIG-transduced (control) cells to 0.5±0.2 pA/pF at 10 mV (\( n=8 \)) in AdCIG-WT Gem-transduced cells (Figure 1a and 1b). The inhibitory effect of Gem on \( I_{\text{Ca,L}} \) attributable to the prevention of interaction between \( \alpha \) and \( \beta \) subunits of L-type calcium channels by scavenging \( \beta \) subunits.\(^{11}\) Based on this, we examined the effect of the less-effective AdCIG-W269G mutant.\(^{11}\) Overexpression of the AdCIG-W269G mutant reduced \( I_{\text{Ca,L}} \) modestly, but significantly (30% inhibition versus control, 3.3±0.2 pA/pF at 10 mV, \( n=10 \)).

Because Gem is a GTP binding protein possibly involved in many signal transduction pathways, it is possible that Gem might have effects such on other ion currents. We therefore investigated the effects on other ion currents such as \( I_{\text{K}}, \) \( I_{\text{Na}}, \) and \( I_{\text{Ko}}, \) before and after Gem gene transfer. No changes in \( I_{\text{Ko}} \) were observed (at −50 mV, 3.5±0.2 pA/pF, \( n=5 \) versus 3.4±0.9 pA/pF, \( n=5 \), in AdCIG-WT Gem-transduced, and AdCIG-transduced cells, respectively; Figure 1c and 1d). Additionally, neither \( I_{\text{Na}} \) (at −40 mV, 25.8±2.5 pA/pF, \( n=5 \) versus 26.5±3.2 pA/pF, \( n=6 \), in AdCIG-WT Gem-transduced, and AdCIG-transduced cells, respectively), nor \( I_{\text{Ko}} \) (at −20 mV, 0.43±0.13 pA/pF, \( n=4 \) versus 0.42±0.17 pA/pF, \( n=5 \), in AdCIG-WT Gem-transduced, and AdCIG-transduced cells, respectively) were affected; thus, transduction of Gem appears to specifically affect L-type \( \text{Ca}^{2+} \) channels.

If, as previously proposed, Gem binds to \( \beta \)-subunits of calcium channels and thereby inhibits the trafficking of \( \alpha \)-subunits to the plasma membrane,\(^{11}\) a decrease in the number of functional channels would be predicted. To assess channel number electrophysiologically, we measured the gating charge attributable to L-type calcium channels and isolated the calcium channel–specific component using 10 μmol/L nitrendipine, a pharmacological calcium channel blocker.\(^{10,19}\) Overexpression of AdCIG-WT Gem resulted in a marked reduction of nitrendipine-sensitive gating currents compared with control (Figure 2a and 2b). The gating currents during depolarization were integrated to calculate charge movements during depolarization (\( Q_{\text{on}} \)). Control \( Q_{\text{on}} \) was significantly greater than that in AdCIG-WT Gem-transduced myocytes (2.2±0.2 fC/pF at +30 mV, \( n=6 \), versus 0.67±0.1 fC/pF at +30 mV, \( n=6 \); \( P=0.001 \)). The voltage dependence of \( Q_{\text{on}} \) was comparable in AdCIG-transduced (control) and AdCIG-WT Gem-transduced cells, as demonstrated by simultaneously fit Boltzmann distributions to the mean data sets, with \( V_{1/2} \) =0.5 mV, \( k=13.3 \) mV in control, and \( V_{1/2} =-0.3 \) mV, \( k=9.8 \) mV in AdCIG-WT Gem-transduced cells. AdCIG-W269G mutant only modestly affected these parameters (1.8±0.1 fC/pF at +30 mV, \( V_{1/2}=5.5 \) mV, \( k=15.5 \) mV, \( n=6 \)). These results indicate that the number of functional calcium channels in the cell membrane is indeed decreased in Gem-transduced cells, compared with AdCIG-transduced (control) cells. These channels that do make it to the surface, however, appear to have normal gating properties.

Next, we investigated the effects of Gem overexpression on action potentials in ventricular cardiomyocytes. Overexpression of AdCIG-WT Gem resulted in the abbreviation of action potential duration (APD) without any change in resting membrane potential (−84.6±0.4 mV versus −84.9±0.4 mV) or
phase 0 depolarization (dV/dt max) (−86.1±3.5 V/s versus −82.2±3.9 V/s) (Figure 3a). Both APD90 and APD90 were significantly shortened in AdCIG-WT Gem-transduced cells compared with control cells, whereas overexpression of AdCIG-W269G mutant had little effect (Figure 3a and 3b). Notably, the robust plateau phase was blunted in WT Gem-transduced myocytes.

The modification of action potentials became more pronounced as $I_{Ca,L}$ decreased. There was a clear correlation between $I_{Ca,L}$ density, calculated as the nitrendipine-sensitive ionic current, and APD90; both were reduced in AdCIG-WT Gem-transduced myocytes compared with AdCIG-transduced (control) cells (n=21, r=0.87, $P<0.0001$) (Figure 3c).

**In Vivo Phenotype of Cardiac Calcium Channel Blockade**

We previously reported that the LV cavity injection method resulted in a transduction efficiency of 15% to 25% in the heart. Western blot analysis (Figure 4a) confirmed Gem overexpression in all cardiac chambers, amounting to a 300±60% increase in the AdCIG-WT Gem-transduced animals relative to control levels ($P=0.002$). The presence of endogenous Gem in heart was unexpected, and gives reason to wonder whether it may play a physiological role in myocardial Ca$^{2+}$ homeostasis; however, this possibility was not explored in the present study.

To assess the electrophysiological phenotype in intact animals, electrocardiograms were performed 3 to 4 days after injection of adenoviruses into the LV cavity. The QT interval was shortened in AdCIG-WT Gem-transduced animals compared with AdCIG-transduced (control) animals (eg, Figure 4b). Consistent with action potential recordings in isolated AdCIG-WT Gem-transduced myocytes, the QTc intervals of the ECG measured 3 days after transduction were abbreviated in AdCIG-WT Gem-transduced animals compared with the same animals immediately after surgery (165±3.5 ms versus 148±2.3 ms, n=9; $P<0.05$). In contrast, no change in the QTc interval was observed in the animals transduced with AdCIG (165±3.3 ms versus 166±1.8 ms, n=6) or AdCIG-W269G mutant (166±1.8 ms versus 163±1.1 ms, n=7). Interestingly, we observed PQ interval prolongation in one of the AdCIG-WT Gem-transduced animals (central panel, Figure 4b), which was presumably induced by fortuitously intense expression of Gem in the AV node.

**Effects of Gem on Cardiac Hemodynamics**

Next, we examined the effect of WT Gem transduction on cardiac hemodynamics. Heart rate did not change (Figure 5a),
but transduction of AdCIG-WT Gem resulted in the reduction of contractile activity in guinea pig hearts. The peak LV systolic pressure (LVSP) was reduced 3 days after gene delivery in AdCIG-WT Gem-transduced animals compared with AdCIG-transduced (control) animals (65.8 ± 5.2 mm Hg, n = 5, versus 85.8 ± 2.7 mm Hg, n = 4; P < 0.05) (Figure 5b). Furthermore, the maximum first derivative of LV pressure (dP/dt_max) was reduced as well (4028 ± 150 mm Hg/s, n = 5, versus 4842 ± 158 mm Hg/s, n = 4; P = 0.01) (Figure 5c). These data indicate that transduction of Gem produced a significant negative inotropic effect, as expected with myocardial calcium channel blockade.

**Focal Modification of AV Nodal Conduction by Gem Gene Transfer**

Atrial fibrillation is a disturbance of cardiac rhythm in which a rapid heart rate produces breathlessness and decreased exercise tolerance. Inhibition of AV nodal conduction, by calcium channel blockade, is the mainstay of drug therapy, but such therapy is fraught with side effects that are attributable to calcium channel blockade outside the AV node. We previously developed an intracoronary perfusion model for adenoviral gene delivery in pigs, and succeeded in the modification of AV nodal conduction by overexpression of the inhibitory G protein, Gαi2.17 We reasoned that overexpression of Gem in the AV node would likewise slow AV nodal conduction, with possible benefit for rate control in atrial fibrillation. Seven days after gene transfer in the same swine model, AdCIG-WT Gem-transduced animals revealed prolongation of the PR interval on the surface ECG and the AH interval (but not the HV interval) on the intracardiac electrogram, confirming slowed conduction in the AV node (Figure 6b through 6e). During acute episodes of atrial fibrillation (Figure 6a), overexpression of AdCIG-WT Gem in the AV node caused a 20% reduction in the ventricular rate during atrial fibrillation (Figure 6f). This effect persisted in the setting of β-adrenergic stimulation as well as cholinergic inhibition (Figure 6g and the Table). Given the previous demonstration17 that adenoviral transduction per se does not affect AV nodal conduction, we conclude that focal calcium channel blockade induced by Gem gene transfer into the AV node effectively reduces the heart rate in atrial fibrillation.

**Discussion**

The utility of calcium channel blockers for heart disease has been limited by potent vasodilatation and hypotension attributable to blockade of noncardiac channels. In this regard, gene therapy would be a feasible strategy for organ-specific or regionally specific treatment within an organ. In this study, we reported the novel finding that gene transfer of a ras-
related small G-protein, Gem could be useful as a genetic calcium channel blocker in the heart, expressed globally for the depression of contractility, or in part of the heart (the AV node), delivered focally to alter conduction.

Overexpression of AdCIG-WT Gem prominently inhibited $I_{Ca,L}$ in guinea-pig ventricular cardiomyocytes, resulting in marked abbreviation of APD, whereas W269G mutant had directionally similar but weaker effects. Furthermore, in vivo delivery of AdCIG-WT Gem shortened the QT interval, consistent with the abbreviation of APD seen in isolated cardiomyocytes. The prominent inhibitory effect of Gem WT on $I_{Ca,L}$ was effective in genetic modification of AV nodal conduction to treat atrial fibrillation, a common arrhythmia that afflicts >2 million Americans. Once AF becomes chronic, therapy is directed at achieving rate control with the use of AV nodal blocking agents.21 Because $I_{Ca,L}$ underlies impulse conduction in the AV node, calcium channel blockers are preferred agents for rate control during AF, but often are not tolerated because of contractile depression from block of non-AV nodal calcium channels in the heart, or hypotension from block of noncardiac channels. In this study, we have achieved focal modification of AV nodal conduction by gene transfer of Gem WT via the AV nodal artery; the resultant regionally selective $I_{Ca,L}$ blockade is effective at rate control during AF, without undermining calcium channel function in the pumping chambers of the heart.

Based on the marked inhibitory effect of WT Gem on $I_{Ca,L}$ complete AV block might have been a potential consequence of WT Gem gene transduction. Anticipating this possibility, we introduced electronic pacemakers into all animals for backup purposes before gene transfer.

Figure 5. Effect of Gem on cardiac hemodynamics. Heart rate (HR) was not changed (a), and LV contractility (LVSP, $dP/dt_{max}$) (b and c) and relaxation ($dP/dt_{min}$) (d) were significantly reduced in AdCIG-WT Gem-transduced animals, compared with AdCIG- and AdCIG-W269G mutant-transduced animals.

Figure 6. Focal modification of AV nodal conduction by WT Gem gene transfer in swine hearts. a, Representative ECG recordings during sinus rhythm and atrial fibrillation before gene transfer of WT Gem. Scale bar=200 ms. b, Representative ECG recordings during sinus rhythm and atrial fibrillation 7 days after gene transfer of WT Gem. Scale bar=200 ms. c, PR interval on the surface ECG before (day 0) and 7 days after transduction (day 7). $P<0.05$ vs PR interval at day 0 ($n=4$). d, AH interval on the intracardiac electrogram before (day 0) and 7 days after transduction (day 7). $P<0.05$ vs AH interval at day 0 ($n=4$). e, HV interval on the intracardiac electrogram before (day 0) and 7 days after transduction (day 7). $P<0.05$ vs AH interval at day 0 ($n=4$). f, Heart rate during sinus rhythm and atrial fibrillation before (day 0) and 7 days after transduction (day 7). $P<0.05$ vs heart rate at day 0 ($n=4$). g, Heart rate during atrial fibrillation stimulated by isoproterenol (ISP) or atropine before (day 0) and 7 days after transduction (day 7). $P<0.05$ vs heart rate at day 0 ($n=4$).
EP Parameters Before and 7 Days After Gene Transfer

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 7</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Heart rate during sinus rhythm, bpm</td>
<td>112±12</td>
<td>102±7</td>
<td>NS</td>
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<td>ECG</td>
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<tr>
<td>PR interval, ms</td>
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<td>61±4</td>
<td>75±5</td>
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<td>HV interval, ms</td>
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<td>NS</td>
</tr>
<tr>
<td>AVNERP, ms</td>
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<td>NS</td>
</tr>
<tr>
<td>Heart rate during AF, bpm</td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>170±10</td>
<td>137±8</td>
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<tr>
<td>Isoproterenol</td>
<td>371±15</td>
<td>272±30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Atropine</td>
<td>318±48</td>
<td>252±7</td>
<td>&lt;0.05</td>
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Mean±SE, n=4 in each group

Nevertheless, AV conduction was significantly slowed without high-grade AV block. This effect is not inconsistent with the observed efficacy of gene transfer to the AV node (=40% of AV nodal cells are transduced by our delivery method, which might not suffice for complete AV block to develop.

A second application is for modulation of cardiac contractility. Hemodynamic studies showed that there was a significant negative inotropy in AdCIG-WT Gem-transduced hearts compared with AdCIG-transduced (control) hearts in guinea pigs, indicating that gene transfer of Gem could be useful to reduce cardiac contractility. Negative inotropic drugs are first-line treatment for patients with hypertrophic obstructive cardiomyopathy (HOCM), to reduce contractile activity in the hypertrophic heart as a means of improving overall pumping efficacy. In accordance with this idea, iatrogenic myocardial infarction has been developed as another means of treating severe HOCM.

KIF9 and Rho kinase, resulting in changes in cell morphology and excitable. More work will be necessary to reduce this approach to HOCM, in that part of the myocardium would be rendered regionally passive whereas remaining alive and excitable. More work will be necessary to reduce this idea to practice.

Although Gem is weakly expressed in the heart, its function has never been elucidated. In other cell types such as fibroblast and neurons, Gem was reported to interact with KIF9 and Rho kinase, resulting in changes in cell morphology and cytoskeletal organization. We are now investigating Gem-mediated signal transduction pathways in cardiomyocytes, with a view to determining its potential utility in the suppression of hypertrophy. However, such data are beyond the scope of the present study.

The present study was designed for proof of concept. For such a purpose, adenoviruses are well-suited; they can be readily made and grown to high titers, they are highly effective at transducing cardiac myocytes, and they lead to intense expression of the transgene for days to weeks. Longer-term expression is limited with adenoviruses, and adverse effects have occurred clinically with their systemic use. Although our adenoviral transduction strategy should lead to higher expression in the heart, there could inevitably be some contaminated infection in other organs such as liver and kidney. For these reasons, other vectors such as aden-associated virus or usage of a cardiac-specific promotor would be better-suited for chronic experiments, including those which will be required before the present approach can be translated to patients.

With the appropriate vector and delivery method, the present strategy is generalizable. If we can selectively block cardiac calcium channels, why not target arterioles to control hypertension as a durable surrogate for lifetime antihypertensive therapy? Might specific regions of the brain involved in memory benefit from local suppression of L-type channel activity? These and many other intriguing possibilities are now amenable to study by straightforward modifications of the novel approach described here.

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References


Creation of a Genetic Calcium Channel Blocker by Targeted Gem Gene Transfer in the Heart
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