Unique Properties of R-Type Calcium Currents in Neocortical and Neostriatal Neurons

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Received 19 April 2000; accepted in final form 18 July 2000

Foehring, Robert C., Paul G. Mermelstein, Wen-Jie Song, Sasha Ulrich, and D. James Surmeier. Unique properties of R-type calcium currents in neocortical and neostriatal neurons. J Neurophysiol 84: 2225–2236, 2000. Whole cell recordings from acutely dissociated neocortical pyramidal neurons and striatal medium spiny neurons exhibited a calcium-channel current resistant to known blockers of L-, N-, and P/Q-type Ca2+ channels. These R-type currents were characterized as high-voltage–activated (HVA) by their rapid deactivation kinetics, half-activation and half-inactivation voltages, and sensitivity to depolarized holding potentials. In both cell types, the R-type current activated at potentials relatively negative to other HVA currents in the same cell type and inactivated rapidly compared with the other HVA currents. The main difference between cell types was that R-type currents in neocortical pyramidal neurons inactivated at holding potentials more positive than those in striatal neurons. Ni2+ sensitivity was not diagnostic for R-type currents in either cell type. Single-cell RT-PCR revealed that both cell types expressed the α1E mRNA, consistent with this subunit being associated with the R-type current.

INTRODUCTION

Both pharmacological and molecular techniques have indicated a diversity of high-voltage–activated (HVA) Ca2+ channel types in neurons. This diversity has been associated with differences in biophysical properties, subcellular location, coupling to cellular events and to differential modulation by neurotransmitters (Hille 1994).

At least four HVA Ca2+ channel α1 subunits have been identified in CNS neurons (α1A–D) (Birnbaumer et al. 1994; Snutch and Reiner 1992; Tsien et al. 1995). The α1B subunit has been identified with N-type channels, which are blocked by ω-conotoxin GVIA (Ctx GVIA) (McCleskey et al. 1987). α1C and α1D subunits correspond to L-type channels, which are sensitive to dihydropyridines (Bean 1989; Tsien et al. 1998). The α1A subunit, associated with P- and Q-type channels (Bourinet et al. 1996; Mermelstein et al. 1999; Pidras-Renteria and Tsien 1998; Stea et al. 1994), is sensitive to ω-agonists IVA and Ctx-MVIIC (Birnbaumer et al. 1994; Mintz et al. 1992a, b; Randall and Tsien 1995). Another calcium channel α1 subunit (α1F) is only found in the retina and is presumed to form L-type channels because of sequence homology to α1C and α1D (Bech-Hansen et al. 1998; Strom et al. 1998).

There has been some uncertainty, however, as to the type of currents generated by channels possessing the α1E subunit. The α1E subunit was originally suggested to be associated with low-voltage–activated, T-type currents (Bourinet et al. 1996; Soong et al. 1993), but may be associated with HVA, R-type currents (Piedras-Renteria and Tsien 1998; Schneider et al. 1994). To further confuse the issue, there are several reported similarities between R-type and T-type currents. By definition, R-type currents are insensitive to the aforementioned organic Ca2+ channel antagonists (Birnbaumer et al. 1994; Randall and Tsien 1995); however, T-type currents are also generally not blocked by these peptides or dihydropyridines (Huguenard 1996). Further, both currents are reported to be highly sensitive to Ni2+ block and, with strong depolarizations, to inactivate rapidly (Huguenard 1996; Randall and Tsien 1995).

Some reports suggest differences between R- and T-type currents. First, in most cell types, T-type currents activate ~20–30 mV more negative than R-type currents. Second, R-type currents deactivate with time constants of a few hundred microseconds (Randall and Tsien 1995, 1997); 10 times faster than T-type currents (Huguenard 1996; Matthews and Armstrong 1986). Third, the inactivation kinetics of T-type currents are steeply voltage dependent (Carbone and Lux 1984; Lee et al. 1999; Randall and Tsien 1997), whereas those of R-type currents are less so (Randall and Tsien 1997). Last, because of the pronounced voltage-dependent inactivation of T-type currents, they are largely inactivated at holding potentials more positive than −50 mV (Huguenard 1996).

While R-type Ca2+ currents have been observed in a variety of neuronal populations, reports on their biophysical properties have varied. This may be due to channel heterogeneity or to contamination by other voltage-dependent calcium currents that are not completely blocked under standard recording conditions. To unequivocally determine the properties of R-type currents in cortical and striatal neurons, we used three distinct pharmacological regimens as well as a biophysical strategy. To provide a complementary level of analysis, neurons also were subjected to single-cell RT-PCR analysis of class E α1 subunit expression.

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Methods

Acute isolation

Four- to 6-wk-old Sprague-Dawley rats were anesthetized with methoxyflurane and then decapitated. The brains were extracted and placed in an oxygenated high sucrose solution that contained (in mM) 250 sucrose, 2.5 KCl, 1 NaH₂PO₄, 11 glucose, 4 MgSO₄, 0.1 CaCl₂, 1 kynurenic acid, 1 pyruvic acid, 0.1 nitro-arginine, 0.05 glutathione, and 15 HEPES (pH 7.3 adjusted with 1N NaOH; 300 mOsm/l). The tissue was then sliced into 400-μM sections using a vibrating tissue slicer (Cambden Instruments). The slices were held for a minimum of 1 h in a carboxygen (95% O₂-5% CO₂) bubbled artificial cerebral spinal fluid (ACSF) that contained (in mM) 125 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 glucose, 1 kynurenic acid, 1 pyruvic acid, 0.1 nitro-arginine, and 0.05 glutathione (pH 7.4 adjusted with 1N NaOH; 310 mOsm/l). The primary motor and primary somatosensory cortex (hereafter referred to as sensorimotor cortex) or the dorsal striatum (posterior to anterior commissure) was then dissected from the slices with the aid of a stereo microscope.

The dissected tissue was incubated for 20–30 min in an oxygenated ACSF-containing Pronase E (Sigma protease type XIV, 1.0 mg/ml at 32–35°C) (cf. Bargas et al. 1994; Lorenzon and Foehring 1995), 1 mM kynurenic acid, 1 mM pyruvic acid, 0.1 mM nitro-arginine, and 0.05 mM glutathione. Following the incubation period, the tissue was rinsed in a Na isethionate solution that contained (in mM) 140 Na isethionate, 2 KCl, 1 MgCl₂, 23 glucose, 15 HEPES, 1 kynurenic acid, 1 pyruvic acid, 0.1 nitro-arginine, and 0.05 glutathione (pH 7.3 adjusted with 1N NaOH; 310 mOsm/l). The tissue was then triturated in the same solution using fire-polished Pasteur pipettes. The supernatant was collected and then poured into a plastic Petri dish (Lux) positioned on the stage of an inverted microscope (Nikon Diaphot 300). The cells were allowed several minutes to adhere to the Petri dish, and then a background flow of HEPES-buffered saline solution was initiated (~1 ml/min). This solution contained (in mM) 10 HEPES, 138 NaCl, 3 KCl, 1 MgCl₂, and 2 CaCl₂, pH 7.3 adjusted with 1N NaOH, 300 mOsm/l.

Cultured cell preparation

For a few experiments, medium spiny cells from embryonic day 19 (E19) rat embryos were cultured for 2 wk according to the procedure outlined in Bargas et al. (1991). The cells were maintained in 5% CO₂ at 37°C.

Recording solutions and pharmacological agents

The external recording solution used to isolate Ca²⁺ channel currents consisted of (in mM) 125 NaCl, 20 CsCl, 1 MgCl₂, 10 HEPES, 5 BaCl₂, 0.001 TTX, and 10 glucose (pH 7.4 adjusted with 1N NaOH; 310 mOsm/l). The internal recording solution included the following (in mM): 180 N-methyl-d-glucamine (NMG), 4 MgCl₂, 40 HEPES, 5–10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or bis-(o-aminophenoxy)N,N,N',N'-tetraacetic acid (BAPTA), 0.1 leupeptin, 0.4 guanosine triphosphate (GTP), 2 ATP, and 0.01 phosphocreatine, pH 7.2 (adjusted with 0.1 N H₂SO₄), 265–275 mOsm/l. The stock solutions of the calcium channel antagonists (with the exception of nifedipine) were dissolved in water. The stock solutions of the calcium channel antagonists (except nifedipine) were dissolved in DMSO (20% final concentration). The stock solutions of nifedipine were dissolved in 95% ethanol before being added to the external solution resulting in a final ethanol concentration of <0.05%. This concentration of ethanol had no effect on these cells (Bargas et al. 1994; Lorenzon and Foehring 1995). Nifedipine was protected from ambient light. Cytochrome C was combined with solutions containing AgTX to prevent nonspecific binding of AgTX to glass and plastic (Bargas et al. 1994; Lorenzon and Foehring 1995).

Single-cell RT-PCR

The methods utilized for the single-cell RT-PCR were similar to those described previously (Surmeier et al. 1996; Yan and Surmeier 1996). Electrodes contained ~5 μl of sterile recording solution (see above). Some cells were harvested without recording, with electrodes filled with water. The capillary glass used for making electrodes had previously been heated to 200°C for 4 h. Sterile gloves were worn during the procedure to minimize RNAse contamination.

After aspiration, the electrode was broken and contents ejected into a presiliconized, 0.5-ml Eppendorf tube containing 5 μl diethyl pyrocarbone (DEPC)-treated water, 0.5 μl RNAsein (28,000 U/ml), and 0.5 μl dithiothreitol (DTT; 0.1 M). One microliter of either oligo dT (0.5 mg/ml) or random hexanucleotides (50 ng/ml) was added and mixed before the mixture was heated at 70°C for 10 min and incubated on ice for more than 1 min. Single-strand cDNA was synthesized from the cellular mRNA by adding SuperScript II RT (1 μl, 200 U/ml), ×10 PCR buffer (2 μl, 200 mM TrisCl (pH 8.4)), 500 mM KCl, MgCl₂ (2 μl, 25 mM), RNAsein (0.5 ml, 28,000 U/ml), DTT (1.5 μl, 1 M), and mixed dNTPs (1 μl, 10 mM). The reaction mixture (20 μl) was incubated at 42°C for 5 min. The reaction was terminated by heating the mixture to 75°C for 15 min and then incubating. The RNA strand in the RNA–DNA hybrid was then removed by adding 1 μl RNAse H (2 U/ml) and incubating for 20 min at 37°C. All reagents except RNAsein (Promega, Madison, WI) were obtained from GibCO BRL (Grand Island, NY). The cDNA from the reverse transcription (RT) of RNA in single cortical neurons was subjected to polymerase chain reactions (PCR) to detect the expression of mRNAs coding for Ca²⁺ channel α1 subunits.

Conventional PCR was carried out with a thermal cycler (MJ Research, Watertown, MA) and thin-walled plastic tubes (Perkin Elmer, Norwalk, CT). PCR primers were developed from GenBank sequences with the commercially available software OLIGO (National Biosciences, Plymouth, MN) and have been described previously (Mermelstein et al. 1999). To detect individual mRNAs, 2.5 μl of the single-cell cDNA was used as a template for conventional PCR amplification. Reaction mixtures contained 2–2.5 mM MgCl₂, 0.5 mM of each of the dNTPs, 1-μM primers, 2.5 U Taq DNA polymerase and buffer (Promega). The thermal cycling program was 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min for 45 cycles.

PCR products were separated by electrophoresis in 1.5–2% agarose gels and visualized by staining with ethidium bromide. In representative cases, amplicons were purified from the gel (QIAquick Gel Extraction Kit, QIAGEN, Hilden, Germany) and sequenced by the University of Tennessee Molecular Resource Center or St. Jude Children’s Research Hospital Molecular Resource Center. These sequences were found to match published sequences.

PCR reactions were carried out following procedures designed to minimize the chances of cross-contamination (Cimino et al. 1990). Negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons. To ensure that genomic DNA did not contribute to the PCR products, neurons were aspirated and processed in the normal manner except that the reverse transcriptase was omitted. Contamination from extraneous sources was checked by replacing the cellular template with water. Both controls were consistently negative in these experiments.

Whole cell recordings

Whole cell recordings were acquired using a DAGAN 8900 or an Axopatch 200B electrometer. The recordings were monitored and controlled by pCLAMP6 (Axon Instruments) installed on a 486 computer. The electrodes were pulled from 7052 glass (Garner) and fire
polished. Typically, series resistance compensation of 70–90% was employed. Cells were not included in the comparisons of biophysical properties if the estimated series resistance–related voltage error (peak $I^\ast$ uncompensated series resistance) was $>5$ mV. Voltage control was also assessed by observing tail currents after brief voltage steps (see Bargas et al. 1994; Lorenzon and Foehring 1995). Cells were discarded if tail currents were broad or unstable. The liquid junction potential was $\sim 8$ mV and was not subtracted in the presented data. A gravity-fed parallel array of glass tubes was used to apply the drugs to the cell being studied.

SYSTAT (SYSTAT, Evanston, IL) was used to carry out all statistical calculations. The population data are represented as median, mean $\pm$ SEM, or box plots (Tukey 1977). In the box plots, the internal line represents the median while the outer edges of the box represent the inner quartiles of the data set. The bars extending from the box depict the two outer quartiles of the data set. Data points greater than two times the inner quartiles were considered outliers and indicated as asterisks on the plots. Statistical differences were determined with the Kruskal-Wallis (comparisons of multiple populations) or Mann-Whitney $U$ test ($\alpha \leq 0.05$), unless otherwise noted.

**RESULTS**

**R-type currents**

We used whole cell patch-clamp recordings from acutely dissociated pyramidal cells (sensorimotor cortex) or medium spiny neurons (dorsal striatum) to test whether there was a component of the current resistant to block by organic Ca\(^{2+}\) channel antagonists. Currents were evoked by repeated (every 5 s) voltage steps (from $-80$ to 0 mV) or voltage ramps (0.3 mV/ms) from $-80$ to +60 mV (Bargas et al. 1994; Lorenzon and Foehring 1995).

In pyramidal cells, we employed three different methods to isolate the R-type current. Two protocols involved sequential addition of specific blockers of L-type (nifedipine: 5–10 $\mu$M), N-type (Ctx-GVIA: 1 $\mu$M), and P/Q-type channel blockers (Fig. 1). In the first method, AgTx (100 nM) was added (in the presence of nifedipine and Ctx-GVIA) to block P- and some Q-type current. Subsequently, Ctx-MVIIC (1–3 $\mu$M) was added to nifedipine, GVIA, and AgTx to block Q-type currents (Fig. 1, A–C). The block of Q-type current by Ctx-MVIIC was slow ($\tau \sim 106$ s) (Mermelstein et al. 1999), requiring $\sim 10$ min to reach a quasi steady-state level in most neurons (Foehring and Armstrong 1996; McDonough et al. 1996; Mermelstein et al. 1999). With this combination of blockers, 21 $\pm$ 3% of the initial current remained (median: 16%; $n = 18$ cells; Fig. 1C). In most cells, this component inactivated rapidly (Fig. 1B). Also, it was completely blocked by 400 $\mu$M Cd\(^{2+}\) (Fig. 1A), indicating that it was carried through calcium channels.

In a separate group of cells ($n = 16$), L- and N-type currents were blocked with nifedipine and Ctx-GVIA, P-type currents were blocked with 25 nM AgTx (Randall and Tsien 1995) and Q-type currents with 1 $\mu$M AgTx (Randall and Tsien 1995) (Fig. 1, D–F). The block of Q-type current with $\mu$M AgTx had

**FIG. 1.** A component of the Ba\(^{2+}\) current is insensitive to organic Ca\(^{2+}\) channel blockers in acutely dissociated neocortical pyramidal neurons. A: peak current (absolute) plotted as a function of time. The toxins were present for the times indicated by the lines above the plot. AgTx (100 nM) blocked P-type and some Q-type channels. Nifedipine (Nif: 5 $\mu$M) blocked L-type channels. Ctx GVIA (GVIA: 1 $\mu$M) blocked N-type channels. Ctx MVIIC (MVIIC: 2 $\mu$M) blocked N-, P-, and Q-type channels. A component of the current was insensitive to all of these blockers (R-type). This resistive component was completely blocked by the inorganic Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (400 $\mu$M). Currents were elicited by a 400-ms step from $-90$ to $-10$ mV, repeated every 5 s. B: representative current traces from the cell shown in A. Note rapid inactivation of toxin-insensitive current (top trace). C: box plot illustrating population data for the percent of the whole cell current insensitive to toxins (Ctx-MVIIC used to block the Q-type component). D: peak current (absolute) plotted as a function of time. In this experiment, the Q-type current was blocked with 1$\mu$M AgTx. The toxins were present for the times indicated by the lines above the plot. AgTx (25 nM) blocked P-type channels and 1 $\mu$M AgTx additionally blocked Q-type. All: 1 $\mu$M AgTx + 1 $\mu$M Ctx-GVIA + 10 $\mu$M nifedipine. An inactivating component of the current was insensitive to all of these blockers (R-type). This resistive component was completely blocked by the inorganic Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (400 $\mu$M). E: representative current traces from the cell shown in D. F: box plot illustrating population data for the percent of the whole cell current insensitive to toxins (1 $\mu$M AgTx, 1 $\mu$M Ctx-GVIA, and 10 $\mu$M nifedipine used to isolate the R component).
a τ of ~45 s (Mermelstein et al. 1999). Under these conditions, 21 ± 3% of the initial current was unblocked (median: 20%; Fig. 1, C and D). This current also inactivated rapidly (Fig. 1D) and was blocked completely with 400 μM Cd2+ (Fig. 1D). We found no significant differences in percentage of current remaining or its biophysical properties (see Properties of R-type current) using these two methods, so we combined data for further analyses.

Dissociated medium spiny neurons were also tested with both isolation protocols, with no significant differences in the results, so the data were combined. Sequential application of the organic antagonists for >10 min left 18 ± 2% of the original current (median: 17%; n = 19; Fig. 2). In 8 of 10 cells tested with voltage steps, the resistant current inactivated rapidly. In the remaining two cells, the resistant current inactivated slowly (e.g., Fig. 2C).

In a few cortical cells, we isolated the residual current by preincubating the slices for at least 2 h in Ctx-MVIIC (2 μM) and then recorded in the presence of 10 μM nifedipine, 1 μM Ctx-GVIA, 1–3 μM Ctx-MVIIC, and 100 nM AgTx (cf., Fig. 4). The remaining current was small (median = 269 pA; n = 9 cells), but comparable in amplitude to the current isolated by sequential addition of blockers (median: 201 pA; n = 31 cells). In cultured medium spiny neurons (see METHODS), a component of the current was found to be insensitive to 12-h preincubation in Ctx-MVIIC (1 μM) and recording in the presence of Ctx-MVIIC plus nifedipine (5 μM; n = 9 cells; data not shown). These data suggest that all three protocols effectively isolated R-type currents and that this current represents a substantial fraction of the somatodendritic Ca2+ current in both pyramidal neurons and medium spiny neurons (see also Churchill and MacVicar 1998; Eliot and Johnston 1994; Foehring and Armstrong 1996; Magee and Johnston 1995; Magnelli et al. 1998; McDonough et al. 1996; Mermelstein et al. 1999; Pennington and Fox 1995; Randall and Tsien 1995; Yu and Shinnick-Gallagher 1997). We next characterized the biophysical and pharmacological properties of the residual (R-type) current in both cell types.

### Properties of R-type current

**NEOCORTICAL PYRAMIDAL NEURONS.** We first measured the kinetics (time-to-peak; τactivation) and steady-state voltage dependence of current activation and the kinetics of deactivation (at ~70 mV; Fig. 3). The average time-to-peak (TTP) at 0 mV was 5.5 ± 0.3 ms (median: 5.1 ms; n = 40 cells; Fig. 3, A and B), significantly shorter than the TTP for N-, L-, or P-type currents in these cells (Lorenzon and Foehring 1995) (Table 1). The τactivation was best fit by assuming third-order kinetics and fitting the initial current trajectory with an exponential function: $I = I_n (\exp(-t/\tau) + b$, where $I$ is current, $I_n$ is the initial baseline, $t$ is time, and $b$ is the maximum current. Mean τactivation was 3.6 ± 0.4 ms at 0 mV (median: 3.2 ms; n = 15). Deactivation time constants were determined by fitting an exponential to the tail current generated by stepping from 0 to ~70 mV (Fig. 3A). The average time constant was 221 ± 66 μs (median: 241 μs; n = 24; Fig. 4B). This is similar to other HVA subtypes in these cells (e.g., L-type: 0.4 ± 0.2 ms; N-type: 0.4 ± 0.2 ms) (Lorenzon and Foehring 1995). This feature clearly distinguished this resistant current from a T-type current (Randall and Tsien 1995, 1997).

The steady-state voltage dependence of activation was assessed with voltage ramps from ~80 to +60 mV (0.3 mV/ms; Fig. 3C). A modified version of the Goldman-Hodgkin-Katz equation was then used to provide an estimate of driving force (Bargas et al. 1994; Lorenzon and Foehring 1995) and estimates of membrane permeability were calculated as follows:

$$\gamma(V_m) = \gamma(V_m) = \gamma(\frac{V_m}{RT})$$

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where

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and $I(V_m)$ is the measured current divided by whole cell capacitance (assumed to be 10 pF), $V_m$ is the membrane potential (in mV), $z$ is 2, $F$ is $9.648 \times 10^{-10}$ C mol$^{-1}$, $R$ is 8.315 V CK$^{-1}$ mol$^{-1}$, $T$ is 273.16 K, [Ca2+]i is assumed to be 10 nM, $I(V_m)$ is 5 nM, and $P(V_m)$ is membrane permeability to Ba2+ as a function of membrane potential.
Over the range where the driving force was not near zero (−20−30 mV negative to the apparent reversal potential of +45 mV), the curves were well fit by a single Boltzmann function of the form

\[ P(V) = \frac{1}{1 + \exp[-(V - V_h)/V_c]} \]

where \( V_h \) is the half-activation voltage and \( V_c \) is the slope factor. We have previously found this method to provide comparable data to activation curves constructed from multiple steps or tail current amplitudes (Bargas et al. 1994; Lorenzon and Foehring 1995). For the inactivating R-type currents in cortical neurons, this method may bias parametric estimates toward the more slowly inactivating components of the R-type current. In 33 cells tested using ramps, the half-activation voltage was −22 ± 1 mV (median = −21 mV), with a slope factor of 7.4 ± 0.3 mV (median = 7.3 mV; Fig. 3C2). To determine whether these values were similar to those obtained with conventional tail current measurements, experiments were performed in five pyramidal neurons. R-type current was isolated as described above. Tail currents were evoked repolarizing the membrane to −70 mV after stepping to potentials between −80 and +30 mV. Tail current amplitude (at 300 μs after the step to −70 mV) was measured and plotted as a function of step potential. The protocol and representative traces are shown in Fig. 4A. Note the lack of crossover of the traces. Figure 4B shows the current-voltage (I-V) relationship for the cell in Fig. 4A. The tail currents for the same traces shown at increased gain. Right: tail current following step shown at increased gain. B: box plots summarizing R-type current time-to-peak (TTP) and \( t_{\text{deactivation}} \) data. C1: the voltage dependence of activation was studied with slow (0.3 mV/mV) voltage ramps. The Goldman-Hodgkin-Katz equation was used to generate a term for relative permeability (see text). C2: this term was plotted as a function of voltage, and the data were fit with a Boltzmann function (see text). D: the box plots summarize data from 33 cells for half-activation voltage (\( V_{\text{half}} \)) and slope factor.

**TABLE 1. Comparison of activation and inactivation properties of Ca\(^{2+}\) channel subtypes in neocortical pyramidal neurons**

<table>
<thead>
<tr>
<th>Property</th>
<th>L-type*</th>
<th>N-type*</th>
<th>P-type†</th>
<th>Q-type†</th>
<th>R-type</th>
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<tr>
<td>% Inact (2 s)</td>
<td>55 ± 8 (7)‡</td>
<td>60 ± 9 (7)‡</td>
<td>63 ± 6 (6)‡</td>
<td>77 ± 3 (12)</td>
<td>84 ± 4 (15)</td>
</tr>
<tr>
<td>TTP</td>
<td>24 ± 5 (19)‡</td>
<td>23 ± 8 (12)‡</td>
<td>22 ± 8 (11)‡</td>
<td>6 ± 1 (13)</td>
<td>6 ± 2 (40)</td>
</tr>
<tr>
<td>( V_{\text{half,act}} )</td>
<td>−17 ± 7 (10)‡</td>
<td>−16 ± 7 (12)‡</td>
<td>−11 ± 3 (3)</td>
<td>−17 ± 2 (17)‡</td>
<td>−23 ± 1 (33)</td>
</tr>
</tbody>
</table>

Values are means ± SE with number of neurons in parentheses. % Inact (2 s), percent inactivation at 0 mV (2 s into step) = 1[(peak \( I \) − (I at 2 s))/peak \( I \)] × 100 (5 mM Ba\(^{2+}\) = charge carrier); TTP, time-to-peak for current in response to 30 ms step to 0 mV (in ms); \( V_{\text{half, act}} \), half-activation voltage in mV (see text). * Data from Lorenzon and Foehring (1995). † Data from Mermelstein et al. (1999). ‡ Significant difference from R-type (\( P < 0.05 \)).
The current ($I_{\text{max}}$) was plotted as a function of membrane voltage
and the data were well fit by a single Boltzmann function of the form

$$I_{\text{max}} = \frac{1}{1 + \exp[(V - V_h)/V_c]}$$

where $V$ is the prepulse voltage, $V_h$ is the half-inactivation voltage and $V_c$ is the slope factor. The average $V_h$ was $-59 \pm 4$ mV (median $= -62$ mV; $n = 7$ cells) with a $V_c$ of $12.6 \pm 1$ mV. In five cells, we compared currents elicited by a step to $0$ mV from holding potentials of $-80$ or $-50$ mV (which should completely inactivate T-type currents). In all five cells, the reduction in current amplitude was $<30\%$ at $-50$ versus $-80$ mV (data not shown), and the kinetics of inactivation were also not altered by holding at $-50$ mV (see also Yu and Shinnick-Gallagher 1997).

**Neostratial medium spiny neurons (acutely dissociated)**

As with pyramidal neurons, the current time-to-peak (at 0 mV) was short ($6.9 \pm 0.5$ ms; $n = 6$; Fig. 6A). The $\tau_{\text{activation}}$ (fit as in cortical neurons) was $5.1 \pm 0.5$ ms ($n = 6$) and $\tau_{\text{deactivation}}$ was $275 \pm 47 \mu$s ($n = 4$; Fig. 6B), suggesting that the residual current is an HVA current. The steady-state voltage dependence of activation was tested with voltage ramps (see above; Fig. 6, C and D). The mean $V_h$ was $-15 \pm 1.2$ mV ($n = 10$), and $V_c$ was $7 \pm 0.2$ ms ($n = 10$).

Most medium spiny neurons tested (8/10) had both rapid and slow components to inactivation at 0 mV (Fig. 7A). In these eight cells, the fast component had an inactivation time constant that averaged $66 \pm 7$ ms ($53 \pm 5\%$ of inactivation); the slower component had a time constant that averaged $1,404 \pm 176$ ms ($47 \pm 5\%$). During a 400-ms step to 0 mV, the R-type current declined to $53 \pm 4\%$ ($n = 8$) of its original amplitude. In a fraction of the medium spiny neurons (2/10), the R current decayed with only a single time constant of intermediate rate ($677, 899$ ms).

We tested the voltage dependence of inactivation in medium spiny neurons using a protocol similar to that described above for pyramidal cells (Fig. 7C). The half-inactivation voltage averaged $-33 \pm 4$ mV, with a slope factor of $17 \pm 1$ mV ($n = 6$).
**Ni$^{2+}$ sensitivity**

The R-type current in neurons (Churchill and MacVicar 1998; Eliot and Johnston 1994; Forti et al. 1994; Magee and Johnston 1995; Randall and Tsien 1995), as well as α1E-associated current in expression systems (Soong et al. 1993; Williams et al. 1994; Zamponi et al. 1996), have been reported to be more sensitive to block by Ni$^{2+}$ than other HVA Ca$^{2+}$ channels. To test this hypothesis, Ni$^{2+}$ dose-response relationships were constructed for neocortical pyramidal and striatal medium spiny neurons.

We first asked whether the current blocked by a low dose of Ni$^{2+}$ (10–20 μM) inactivated rapidly, like R-type current. Of five neocortical pyramidal cells tested, the percent inactivation of the Ni$^{2+}$-sensitive current at 400 ms was 23, 70, 0, 0, and 43%; these results suggest that Ni$^{2+}$ did not selectively block the R current (data not shown). Similarly, in medium spiny neurons, Ni$^{2+}$ (10 μM) blocked a current that was indistinguishable in inactivation rate from the total current (n = 5; data not shown).

We then tested the response of the whole Ba$^{2+}$ current to doses of Ni$^{2+}$ between 100 nM and 2 mM. The data were obtained with either steps to −10 mV (30 ms) or with voltage ramps. We plotted relative peak current (I/I$_{\text{max}}$) as a function of log dose of Ni$^{2+}$. For pyramidal neurons, the resulting curve was well fit by a single Langmuir isotherm of the form

\[
I = \frac{I_{\text{max}}}{1 + \exp(2.3 \times \log(\text{dose}))/EC_{50}}
\]

where the EC$_{50}$ is the concentration at which 50% of the current was blocked. The median EC$_{50}$ for the whole current was −85 μM (n = 3). In medium spiny neurons, the EC$_{50}$ for Ni$^{2+}$ block of the whole cell current was 96 μM (n = 6; data not shown). Neither dose-response plot had features indicative of more than one Ni$^{2+}$ binding site.

![FIG. 6. Activation and deactivation properties of R-type current in acutely dissociated medium spiny neurons. A: activation was rapid. The time-to-peak was ~7 ms in this cell [protocol above (not to scale)]. B: deactivation kinetics (different cell from that in A). Exponential fits were obtained from tail currents following protocol as in A. In this cell, $\tau_{\text{deact}}$ was 268 μs (at −70 mV). The box plot summarizes $\tau_{\text{deact}}$ data for 4 cells. C: the voltage dependence of R-type current was studied with a voltage ramp (0.3 mV/ms). D: the resulting current was converted to an estimate of permeability with the Goldman-Hodgkin-Katz equation (see text). The relative permeability was then plotted as a function of voltage and fit with a Boltzmann function. The box plots summarize the half activation voltage and slope factor data for 10 cells.

To test the Ni$^{2+}$ sensitivity of the R-type current directly, we preincubated neocortical slices in 2 μM Ctx-MVIIC for at least 2 h and then recorded in the presence of 100 nM AgTx, 1 μM Ctx-GVIA, 10 μM nifedipine, and 2 μM Ctx-MVIIC (see METHODS). These data were also well fit by a single Langmuir isotherm, with an EC$_{50}$ of −48 μM (not shown).

**Single-cell RT-PCR**

Single-cell RT-PCR revealed that most neocortical pyramidal neurons express detectable levels of mRNAs for all five HVA Ca$^{2+}$ channel α1 subunits tested (α1A–E; data not shown). Detectable levels of class E α1 subunit mRNA were found in six of seven identified pyramidal neurons (Fig. 8A). In addition, nearly every cell expressed detectable levels of α1A (4/5 cells) (see Mermelstein et al. 1999) and α1B mRNA (4/5). All cells expressed detectable levels of either α1C (5/5) or α1D (3/5) mRNA (data not shown). Both α1C and α1D mRNA were co-expressed in three of five of cells tested.

Detectable levels of mRNA for α1E were also found in nearly every striatal medium spiny neuron tested (14/15; Fig. 8B). The majority of medium spiny neurons express detectable levels of mRNA for α1A, α1B, and α1C, or α1D (Bargas et al. 1994; Mermelstein et al. 1999; Song and Surmeier 1996).

**DISCUSSION**

We have previously provided pharmacological evidence for the expression of L-, N-, P-, and Q-type channels in neocortical pyramidal and striatal medium spiny cells (Bargas et al. 1994; Lorenzon and Foehring 1995; Mermelstein et al. 1999). Here we demonstrate that a component of the HVA Ca$^{2+}$ current in both cell types is resistant to block by organic antagonists (R-type). We characterized the biophysical properties and Ni$^{2+}$
sensitivity of these R-type currents. Single-cell RT-PCR was used to test for the expression of α1E subunits in these cells.

The data presented here and our previous work (Bargas et al. 1994; Mermelstein et al. 1999) demonstrate that neocortical pyramidal neurons and striatal medium spiny neurons express mRNAs for at least five different α1 subunits: α1A, B, C, D, and E. The α1E mRNA was ubiquitously expressed in both pyramidal and medium spiny neurons, in agreement with previous in situ hybridization studies reporting the presence of α1E mRNA in cortex and dorsal striatum (Ludwig et al. 1997; Plant et al. 1998; Soong et al. 1993; Tanaka et al. 1995; Williams et al. 1994; Yokayama et al. 1995). Considering that α1A–D subunits are associated with P-, Q-, N-, and L-type currents (Mermelstein et al. 1999; Piedras-Renteria and Tsien 1998; Stea et al. 1994), these data are also consistent with α1E subunits being associated with R-type. The association between α1E subunits and R-type currents has been suggested on the basis of sequence homology between the α1E subunit and other HVA subunits (Williams et al. 1994). Also, macroscopic current properties are similar in α1E-associated channels expressed in oocytes (Williams et al. 1994) or cell lines (Page et al. 1997; Stephens et al. 1997; Williams et al. 1994) to R-type currents in cerebellar granule cells (Randall and Tsien 1995). More recently, antisense experiments have strengthened the link between R-type currents and α1E subunits in cerebellar granule cells (Piedras-Renteria and Tsien 1998).

**R-type versus T-type current**

R-type current was first described in cerebellar granule neurons, where relative to other HVA Ca$^{2+}$ currents, R-type currents inactivate more rapidly and at more negative potentials (Randall and Tsien 1995, 1997). In these respects, R-type currents are similar to T-type currents. Granule cell R-type currents (Randall and Tsien 1995, 1997) typically inactivate more slowly and activate and inactivate at more positive potentials than T-type currents. Typical T-type currents have half-activation voltages of approximately −50 to −60 mV and half-inactivation voltages of approximately −80 mV, although a wide range of values has been reported (Huguenard 1996; Lee et al. 1999).

R- and T-type channels can most clearly be distinguished by their deactivation kinetics. Whereas R-type currents in granule cells deactivate with time constants of a few hundred microseconds at negative potentials (Randall and Tsien 1995) (−240 μs at −80 mV), T-type currents deactivate with time constants of a few milliseconds (Huguenard 1996; Matteson and Armstrong 1986; Randall and Tsien 1997). T-type currents (but not R-type) also typically show “criss-crossing” traces in I–V protocols (Randall and Tsien 1997). T-type and α1E channels can also be distinguished by their single channel conductance (Schneider et al. 1994; Wakamori et al. 1994; but see Meir and Dolphin 1998), T-type channels are also generally more permeable to Ca$^{2+}$ than Ba$^{2+}$ (Huguenard 1996). There is some controversy as to whether R-type and α1E channels are more permeable to Ca$^{2+}$ than Ba$^{2+}$ (e.g., Bourinet et al. 1996) or vice versa (e.g., Magnelli et al. 1998; Williams et al. 1994).

**PYRAMIDAL AND MEDIUM SPINY NEURONS.** T-type α1 subunits (primarily α1G) (Talley et al. 1999) and low-voltage–activated (LVA = T-type) currents are expressed in neocortical pyramidal neurons (Sayer et al. 1990; Tarasenko et al. 1998; Ye and

![FIG. 8. Class E α1 subunit mRNA is expressed in neocortical pyramidal neurons and striatal medium spiny neurons. A: gel indicating the expression of class E mRNA in a neocortical pyramidal cell. Calcium calmodulin kinase type II (CaMKII) mRNA expression indicates that this was a pyramidal neuron—CaMKII is expressed in pyramidal neurons but not nonpyramidal neurons in cortex (Jones et al. 1993). Similar data were obtained in 6/7 cells. B: class E mRNA is expressed in medium spiny neurons. Similar data were observed in 14/15 cells tested. This particular cell expressed substance P mRNA but not enkephalin mRNA, indicating that it was a projection neuron (medium spiny) rather than a local circuit interneuron.](image-url)
Akaike 1993). The LVA current inactivated with a τ of ∼24 ms (Tarasenko et al. 1998) and the half-inactivation voltage was approximately −90 mV (Sayer et al. 1990; Tarasenko et al. 1998; Ye and Akaike 1993). T-type, but not R-type currents are completely inactivated at holding potentials of approximately −50 mV in cortical pyramidal cells (Sayer et al. 1990; Ye and Akaike 1993) and in other cell types (Huguenard 1996). When recording with Ba2+ as the charge carrier, we find T-type to be nearly undetectable in most acutely dissociated cells. When present, T-type currents are typically <50 pA in amplitude (Lorenzon and Foehring 1995; unpublished observations). This was also true for medium spiny cells (Bargas et al. 1994), although T-type currents are more prominent in embryonic or cultured medium spiny neurons (Bargas et al. 1991; Hoehn et al. 1993). The resistant current studied here (both cell types) is clearly identified as HVA on the basis of deactivation kinetics, lack of “criss-cross” in the I-V curve, and relative insensitivity to depolarized holding potentials (−50 mV). In addition, no current was observed to activate negative to approximately −50 mV. The half-activation and inactivation voltages for R-type current reported here (both cell types) were also more positive than published values for T-type currents in cortical and striatal neurons (Bargas et al. 1991; Hoehn et al. 1993; Sayer et al. 1990; Tarasenko et al. 1998; Ye and Akaike 1993).

BIOPHYSICS OF R-TYPE CURRENTS. The R-type current comprised 18% of the whole cell Ba2+ current in pyramidal neurons and 17% in medium spiny neurons. This compares to 15–20% in cerebellar granule neurons (Randall and Tsien 1995), 32% in N. accumbens (Churchill and MacVicar 1998), and 40% in embryonic motoneurons (Magnelli et al. 1998). We isolated the R-type current with combinations of organic blockers for L- (nifedipine), N- (Ctx-GVIA), and P/Q-type channels (AgTx and Ctx-MVIIC). We used three different isolation procedures, including preincubation for several hours in MVIC, to rule out the possibility that the residual current reflects insufficient time for toxins to exert their effects. The deactivation time constant was rapid in pyramidal neurons (221 μs at 0 mV) and medium spiny neurons (270 μs), similar to values reported for R-type current in cerebellar granule cells (deactivation τ was 210 μs) (Randall and Tsien 1997), spinal motoneurons (330 μs) (Magnelli et al. 1998), and CA1 pyramidal cell dendrites (200 μs) (Kavalali et al. 1997).

Relative to other HVA current types in neocortical pyramidal cells, R-type currents activated at more negative potentials and inactivated more rapidly and completely (Table 1). The half-activation voltage for R-type current in neocortical pyramidal cells was approximately −22 mV, significantly more negative than other HVA current types in these cells (Table 1) and similar to α1E currents in oocytes (Soong et al. 1993) and central amygdaloid neurons (−17 mV: Yu and Shinnick-Gallagher 1996). In medium spiny neurons, the half-activation voltage was approximately −15 mV, also more negative than other HVA currents in those cells (Bargas et al. 1994; Merfelstein et al. 1999). More positive half-activation voltages have been reported for R-type current in cerebellar granule neurons (approximately −2 mV: Randall and Tsien 1997), nucleus accumbens neurons (−0 mV: Churchill and MacVicar 1998), and spinal motoneurons (−6 mV: Magnelli et al. 1998). It is unclear to what extent procedural differences (e.g., different charge carrier or divalent concentration) contribute to this variability.

The TTP in neocortical pyramidal cells was similar to Q-type current (Mermelstein et al. 1999) and shorter than for L-, N-, or P-type (Lorenzon and Foehring 1995) (Table 1). This appears to be primarily due to more rapid inactivation, as activation and deactivation time constants did not differ between HVA current subtypes. The time constant for activation for R-type current was similar in neocortical pyramidal neurons and medium spiny neurons, as well as spinal motoneurons (Magnelli et al. 1998) and α1E currents expressed in oocytes (Soong et al. 1993).

The aggregate whole cell Ca2+ current was half-inactivated at approximately −13 mV in neocortical pyramidal neurons (Brown et al. 1993). We found that the pyramidal cell R-type current had a half-inactivation voltage of near −60 mV, considerably more hyperpolarized than the aggregate current. The half-inactivation voltage was similar to the α1E current in oocytes (−65 to −78 mV: Soong et al. 1993; −71 mV: Williams et al. 1994), and R-type currents in cerebellar granule (approximately −58 mV) (Randall and Tsien 1997) and amygdaloid neurons (approximately −58 mV) (Yu and Shinnick-Gallagher 1997). The R-like, doe-1 currents expressed in oocytes had a half-inactivation voltage of approximately −46 mV (Ellinor et al. 1993) and spinal motoneuron R-type currents of approximately −43 mV (Magnelli et al. 1998). In medium spiny neurons, the half-inactivation voltage was more depolarized than in cortical pyramidal cells (−33 mV), suggesting less inactivation at subthreshold potentials in these cells.

R-type current inactivation is generally more complete than other HVA current types in pyramidal (Table 1) or medium spiny neurons (Mermelstein et al. 1999). In both cell types, there is a rapidly inactivating component, similar to the R-type current described in cerebellar granule cells (Randall and Tsien 1995). In pyramidal cells, this rapid component accounts for 42% of inactivation and in medium spiny cells for 53%. There is also a more slowly inactivating component in both cell types, suggesting possible channel heterogeneity. In a few cells, only the slow component was expressed. When only one τ is expressed, the single τ in pyramidal cells is faster (∼200 ms) than that in medium spiny neurons (∼700 ms). Rapidly inactivating R-type currents are also reported in cerebellar granule neurons (τ 30–40 ms at 0 mV) (Randall and Tsien 1997; Zhang et al. 1993), N. accumbens neurons (Churchill and MacVicar 1998), α1E currents in oocytes (τ 74 ms: Williams et al. 1994; τ 100 ms: Soong et al. 1993), calyx terminals in rat medial trapezoid body (Wu et al. 1998) and doe-1 currents in oocytes (τ 12 ms: Ellinor et al. 1993). Slowly inactivating, resistant currents have also been reported in supraoptic magnocellular neurons (Foehring and Armstrong 1996), spinal motoneurons (Magnelli et al. 1998; Plant et al. 1998), and raphe neurons (Pennington and Fox 1995).

PHARMACOLOGY OF R-TYPE CURRENTS. There was no clear evidence for a high affinity Ni2+ block of the whole cell Ba2+ current in either pyramidal cells or medium spiny cells. The current sensitive to low doses (10–50 μM) of Ni2+ was not rapidly inactivating in these cells. In pyramidal cells, the small difference in Ni2+ affinity for the isolated R-type current versus the whole current (EC50 48 vs. 85 μM) is of limited diagnostic utility. Our data are consistent with studies on
antagonist-resistant currents in cerebellar granule cells (R: 66 μM: Zhang et al. 1993), dentate granule cells (Eliot and Johnston 1994), raphe neurons (50 μM: Pennington and Fox 1995) and expressed α1E channels (27 μM: Williams et al. 1994; 28 μM: Soong et al. 1993) or doe-1 channels (33 μM: Elinor et al. 1993). In spinal motoneurons, the R-type current was not especially Ni2+ sensitive (Magnelli et al. 1998). Zamponi et al. (1996) reported that in addition to channel block, Ni2+ causes a shift in the voltage dependence of gating that is greater in α1E channels than other channel types. In oocytes, this leads to an apparent K, at −10 mV of 21 μM, which shifted to 144 μM at +10 mV. Our data were obtained at −10 mV.

Other agents have been reported to block R-type currents. Randall and Tsien (1997) found that ω-agatoxin IIIA blocked the R-type current in cerebellar granule cells (~70% with 1–10 nM) but had no effect on T-type current in NG108-15 cells. Mibafredil also blocked the R-type current, although not selectively (Randall and Tsien 1997; Viana et al. 1997). Miljanich and collaborators (Newcomb et al. 1998) reported that SNX 482, a peptide from the venom of an African tarrantula, selectively blocked human recombinant α1E currents and an R-type current in rat neurohypophyseal nerve terminals, but had no effect on R-type Ca2+ currents in several types of rat central neurons.

DIVERSITY OF R-TYPE CURRENTS. We have discussed several examples of variability in the biophysical properties of R-type currents in various cell types. The R-type currents in neocortical pyramidal neurons and striatal medium spiny neurons were rapidly activating, deactivating, and inactivating. In both cell types, activation occurred at relatively hyperpolarized potentials. The primary difference between these two cell types was in the voltage dependence of inactivation, which occurred at much more hyperpolarized potentials in pyramidal cells than in medium spiny neurons. Variability was evident in a single cell type as well, especially in the kinetics of inactivation. In medium spiny neurons, some cells only expressed a slowly inactivating current, whereas most cells had a prominent rapidly inactivating component as well. What could be the basis for the biophysical differences between resistant currents in different cell types?

R-type currents may be attributable to activation of more than one type of channel. Pietrobon and colleagues (Forti et al. 1994; Tottene et al. 1996) have described single channel data suggesting diversity of channels in cerebellar granule cells (G2 and G3), which might be native variants of R-type channel. The G2 channel underlies currents that half-activate at approximately −22 mV (low threshold) and half-inactivate at approximately −50 mV. The G3 channel currents half-activate (approximately −4 mV) and inactivate at high threshold. Another possibility is that the inactivating and noninactivating R-type currents are attributable to splice variants of the α1E subunit. Such variants have been described for α1E in human and mouse brain (Williams et al. 1994). It is not known whether these splice variants are associated with biophysical differences. It is also possible that the biophysical differences are dependent on differential expression of β subunits. β subunits are known to alter the voltage dependence of activation and the kinetics of inactivation of channels formed by α1E and other α1 subunits (Castellano and Perez-Reyes 1994; DeWaard and Campbell 1995; Sather et al. 1993; Stea et al. 1994). Differential expression of β subunits appear to be responsible for biophysical differences in the properties of Q-type currents between pyramidal and medium spiny neurons (Mermelstein et al. 1999). We hypothesize that some R-type channels may preferentially associate with β2 subunits, which typically are associated with the slowest inactivation rates in expression systems (DeWard and Campbell 1995; Stea et al. 1994). These channels could carry the slowly inactivating component of the currents. Other R-type channels may preferentially associate with β4 subunits, which typically are associated with the fastest inactivation rates in expression systems (DeWard and Campbell 1995; Stea et al. 1994). Finally, some apparent variability may reflect differences in experimental procedure (e.g., charge carrier, divalent concentration), rather than biology.

Functional consequences

The hyperpolarized activation voltage range of R-type current in neocortical pyramidal cells and medium spiny neurons may facilitate a role in synaptic integration in the subthreshold voltage range in those cells. Consistent with a role in synaptic integration, these channels are common in the dendrites of hippocampal pyramidal neurons (Kavalali et al. 1997; Magee and Johnston 1995; Yokayama et al. 1995). R-type currents contribute to Ca2+ action potentials in cultured cerebellar granule neurons (D’Angelo et al. 1997) and have also been shown to contribute to action potential–induced Ca2+ entry in calyx type terminals in the rat medial trapezoid body (Wu et al. 1998). R-type channels may also contribute to synaptic transmission from cerebellar inhibitory interneurons to Purkinje neurons (Doroshenko et al. 1997). Because of their hyperpolarized inactivation range and rapid kinetics of inactivation, R-type currents would be more sensitive to sustained depolarizations than other HVA current subtypes in pyramidal neurons and thus may play a lesser role during sustained activity. Due to the more depolarized half-inactivation voltage in medium spiny neurons, R-type current in these cells may be less inactivated during sustained depolarizations.

In expression systems, α1E-associated channels are generally reported to be poorly modulated by a variety of signaling pathways (Page et al. 1997; Shekter et al. 1997; Simen and Miller 1998; Toth et al. 1996), although there are exceptions (Meza and Adams 1998; Yassin et al. 1996). The modulation may be sensitive to the relationship between the α1E subunit and particular β subunits (Shekter et al. 1997). In contrast, the α1E subunit appears to be a substrate for phosphorylation by several kinases (Yokayama et al. 1995). In the calyx synapse in the medial trapezoid body, metabotropic glutamate and GABAA receptors inhibited R-type current (Wu et al. 1998). As of yet, we have not found evidence for modulations of R-type current in either neocortical pyramidal or striatal medium spiny neurons. N- and P-type channels are substrates for several pathways in these cells (Foehring 1996; Howe and Surmeier 1995; Stewart et al. 1999; Surmeier et al. 1995).
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