Voltage-dependent Calcium Channel Abnormalities in Hippocampal CA3 Neurons of Spontaneously Epileptic Rats

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Running title: Ca^{2+} channel abnormalities in SER

Abstract

Purpose Hippocampal CA3 neurons of spontaneously epileptic rats (SER; *zi/zi, tm/tm*), which show both absence-like seizures and tonic convulsions, exhibit a long-lasting depolarization shift with repetitive firing by a single stimulation of mossy fibers. Therefore, a whole cell patch clamp study using acutely dissociated hippocampal CA3 neurons from SER was performed to elucidate whether or not such abnormal excitability was due to abnormalities in voltage-dependent Ca^{2+} channels (VDCCs).

Methods Hippocampal CA3 neurons were acutely dissociated with enzymatic and mechanical treatments. In a voltage-clamp mode with whole-cell recording, depolarizing step pulses were applied to induce Ca^{2+} currents in the presence of tetrodotoxin and tetraethylammonium.

Results The threshold level of the Ca^{2+} current induced by depolarizing pulses was found to be lower in hippocampal CA3 neurons of SER compared with that of control Wistar rats. In addition, the Ca^{2+} current peak amplitude was greater, and decay of the current was weaker in CA3 neurons of SER than those of normal Wistar rats.

Conclusion These findings suggest that enhancements of Ca^{2+} influx into hippocampal CA3 neurons due to the easier activation properties of VDCCs, as well as a decrease in decay, are involved in SER epileptic seizures.

Key Words: Spontaneously epileptic rats (SER), Hippocampal CA3 neuron,

Voltage-dependent Ca^{2+} channel (VDCCs), Ca^{2+} current, Patch-clamp.

Introduction

The spontaneously epileptic rat (SER; *zi/zi, tm/tm*) is a double mutant obtained by mating the heterozygote tremor rat (*tm*/+), a mutant found in an inbred colony of Kyoto-Wistar rats [1], with a homozygote zitter rat (*zi/zi*) found in a Sprague-Dawley colony [2]. After eight weeks of age, SER spontaneously **show** both tonic convulsions and absence-like seizures concomitantly with 5-7 Hz spike-wave complexes in cortical and hippocampal EEG [3, 4]. The SER are a useful model animal for evaluation of the acute and chronic effects of novel antiepileptic drugs [3, 5], since the profiles of antiepileptics in this animal parallel those in human absence and convulsive seizures.

This animal lacks a gene encoding aspartoacylase, which converts N-acetyl-L-aspartate (NAA) into acetic acid and aspartate, resulting in accumulation of NAA in the brain and peripheral tissues [6]. However, analysis showed no alteration in genes encoding Ca^{2+} channels. Nevertheless, our previous studies have demonstrated that the hippocampal CA3 pyramidal neurons in SER show a long-lasting (100 ms) depolarization shift accompanied by repetitive firing with a single stimulus given to the mossy fibers [7], and this probably results from an increase in Ca^{2+} influxes induced by enhanced L-type Ca^{2+} channel activities since depolarization shift and excessive Ca^{2+} influx were blocked by L-type Ca^{2+} blockers [8, 9, 10]. Ca^{2+} channels play a critical role in inducing epileptic

seizures. Mutation of the high-voltage-activated (HVA) subunit genes Ca_V 2.1/α_{1A}, β_4 and γ_2 is related to the absence seizures in tottering, lethargic and stargazer mice, respectively [11, 12, 13]. Furthermore, potentiation of a low-voltage-activated (LVA) Ca^{2+} current in thalamic neurons has been found to be responsible for absence epilepsy seizures in tottering, lethargic and stargazer mice that show absence epilepsy, but have no mutant allele of the LVA Ca^{2+} channel [14]. Neuronal hyperexcitability of hippocampal pyramidal neurons has also been reported in tottering mice [15] and kindled rats [16, 17, 18]. Furthermore, enhancement of the Ca^{2+} current [19, 20, 21] and N-type Ca^{2+} channel density [22] in hippocampal neurons have been observed in kindling rats. Thus, abnormalities in Ca^{2+} channel functions are considered to contribute to the epileptogenesity of SER. Therefore, a whole cell patch clamp study using acutely dissociated hippocampal CA3 neurons was performed to clarify the abnormal characteristics of voltage-dependent Ca^{2+} channels (VDCCs) in SER.

Materials and Methods

SER were bred in a specific pathogen-free barriered facility at the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University. They received food and water ad libitum in shoe box-type cages in a room maintained at 23±2°C and 55±5% humidity with light from 8:00am to 8:00pm.

Acutely dissociated hippocampal CA3 neurons were prepared from 18 SER of both genders (10-13 weeks old) exhibiting convulsive seizures [3] , and nine age-matched control Wistar rats. SER were differentiated from tm heterozygotes (tm/+, zi/zi) according to appearance (curled whiskers and hair) [3,4]. After decapitation under anesthesia with diethyl ether, the brain was rapidly removed and placed in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 113, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 25, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 11 (pH 7.4). Transverse brain slices (500 μm in thickness) were cut along the sagittal plane using a microslicer (DTK 1500, Dosaka, Kyoto, Japan). Hippocampal slices were between L 1.5-3.5mm corresponding to L 1.4-3.4 mm in Paxinos' and Watson's brain map [23]. Thereafter, the hippocampal CA3 region dissected from brain slices with a small knife was incubated for 8-10 min at 34°C in ACSF containing trypsin 0.05% (Sigma type XI, St. Louis, MO, USA) under perfusion with 95% O_2 and 5% CO_2. After washing five times, the mini-slices were stored in ACSF, and bubbled with 95% O_2 and

5% CO_2 until use. Hippocampal CA3 neurons, dissociated by gently triturating with fire–polished Pasteur pipettes, were plated on cover glasses (12 mm in diameter), and then transferred to a recording chamber (2 ml volume). The exact cell type dissociated acutely could not be clearly identified, but most neurons were a pyramid-like shape. All experiments were performed at room temperature. Patch pipettes with 3-6 MΩ resistance were filled with a solution containing (in mM): CsCl 120, tetraethylammonium chloride (TEA-Cl) 20, $MgCl_2$ 2, $CaCl_2$ 1, Ethylene Glycol Bis (β-aminoethyl-ether)-*N,N,N',N'*-tetraacetic Acid (EGTA, Sigma, St. Louis, MO, USA) 11, HEPES 10, K_2ATP 2, and pH was adjusted to 7.3 with Tris [hydroxymethyl] aminomethane (Sigma, St. Louis, MO, USA). The recording chamber was continuously perfused with an external solution at a rate of 2-4 ml/min. The bath solution contained (in mM): NaCl 154, KCl 5, TEA-Cl 10, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, glucose10 and tetrodotoxin (TTX, Wako Pure Chemical, Osaka, Japan) 0.0003, and the pH was adjusted to 7.3 with NaOH.

Voltage-dependent Ca^{2+} currents were induced by depolarizing pulses of 100 msec-duration given to the hippocampal CA3 neurons from a holding potential of –90 mV to +50 mV with 10 mV increments. After the amplitude of Ca^{2+} currents was stabilized, external $CaCl_2$ was replaced with equimolar $BaCl_2$ as the charge carrier. The currents were measured using the conventional whole-cell patch clamp recording method described

previously [24]. Electrophysiological signals obtained using a patch-clamp amplifier (EPC-7, List-Medical Electronic, Germany) were displayed on an oscilloscope (VC-10, Nihon Kohden, Tokyo, Japan). The current signals were stored on a personal computer (FMV-Desk Power TI16, Fujitsu, Tokyo, Japan), a video cassette recorder (SLV-779 HF, Sony, Tokyo, Japan) via a DigiData interface (1200 series, Axon Instrument, USA), and a digital data recorder (VR-10B, Instrutech, Corporation, NY, USA) for subsequent analysis using pCLAMP 8.0 software (Axon Instrument, Foster, CA, USA). Depolarizing pulses to induce the current were generated using a personal computer and pCLAMP. Leak current was subtracted on-line with P/4 protocol by pCLAMP software. An approximate value of whole-cell capacitance was determined by adjusting the amplifier capacitance.

In order to characterize the Ca^{2+}channel abnormalities in SER, the activation kinetics were calculated. The voltage-dependent activation of the Ca^{2+} current was quantified as follows: the conductance (G) was calculated using the formula, $G=I/(V_m- Ex)$, where I is the amplitude of the current, V_m is the tested membrane potential, and Ex is the equilibrium potential of Ca^{2+}. The voltage-dependent activation data of Ca^{2+} currents were plotted using normalized conductance in which the conductance at the given potential was divided by the maximum conductance (Gmax). The smooth curves could be fitted by a least squares method with a Boltzmann

equation of the form: $G/Gmax=1/\{1+\exp[(V-V_{1/2})/K]\}$, where $V_{1/2}$ (half activation potential) is the test potential at which the ratio of G/Gmax was 0.5 and K is the slope factor. All data in the text and figures are expressed as mean values±S.E.M., and the statistical significance of differences was determined using the unpaired Student's *t*-test.

Results

Ca^{2+} currents

In the solution containing Ba^{2+} instead of Ca^{2+}, the inward currents were obtained by giving depolarizing pulses (100 msec duration) to acutely dissociated hippocampal CA3 neurons of Wistar rats and SER with 10 mV increments from a holding potential of –90 mV to +50 mV (Fig. 1). The inward currents were completely abolished by external application of $CdCl_2$ (1 mM) (Fig. 1B), indicating that Ba^{2+} currents flowed through VDCCs.

In the Wistar rats, the threshold potential for induction of the Ca^{2+} current was between –40 and –30 mV in all 19 neurons tested, and maximum currents were obtained between –20 and 0 mV (Fig. 1 and Table 1). In the SER, however, the maximum currents were at a wide range of potentials between –40 and 0 mV (Fig. 1 and Table 1). According to the test potentials for the maximum current, the SER hippocampal neurons were classified into two types, type 1 and type 2 neurons, of which maximum currents were obtained at values more negative than –30 mV and more positive than –10 mV in 13 and 16 neurons examined, respectively (Fig. 1, Fig. 2A and Table 1). Type 1 and 2 neurons were sometimes observed in the same SER hippocampus. Figure 1 shows examples of Ca^{2+} current traces recorded from hippocampal CA3 neurons of the normal Wistar rats, and type 1 and 2 neurons of SER, indicating that the Ca^{2+}

currents began to be induced at –30 mV, while the maximum currents were obtained at –10 mV in both Wistar rats and type 2 neurons of SER (Fig.1 type 2). In SER type 1 neurons, however, Ca^{2+} currents were initiated at about –40 mV and the peak current was observed as low as –30 mV (Fig. 1 type 1). Current-voltage relationships (I-V curve) for the averaged peak Ca^{2+} current against the test potentials in Wistar rats, and type 1 and 2 neurons of SER, are illustrated in Figure 2A. In the type 1 neurons of SER, the threshold potential for inducing the Ca^{2+} current was lower than that in Wistar rats. The current amplitude induced at –40 mV was 64.85±6.8% (n=13) of the maximum amplitude, and was significantly ($p<0.01$) greater than that in Wistar rats (17.6±2.8% of the maximum amplitude, n=19) (Fig. 2A). In the type 2 neurons of SER, the mean amplitude of the currents (5.8±1.2% of maximum amplitude, n=16) induced at –40 mV was comparable to that in Wistar rats. The respective mean capacitances of the hippocampal neurons in Wistar, type 1 and type 2 neurons of SER were 12.21±0.99 (n=19), 11.21±0.71 (n=13) and 13.48±0.57 pF (n=16), respectively, and there were no significant differences among these values. The peak current induced at –30 mV in type 1 neurons of SER was –1080.21±184.74 pA (n=13), which was significantly ($p<0.05$) greater than those induced at -10 mV in the Wistar rats and type 2 neurons of SER (Fig. 2A). The peak currents induced at –10 mV of the membrane potential in the neurons of Wistar rats and type 2

neurons of SER were –635.51±64.11 pA (n=19) and –758.73±68.76 pA (n=16), respectively, and there were no significant differences between the former and latter values (Fig. 2A).

Ca^{2+} channel kinetics

To characterize the Ca^{2+}channel abnormalities in SER, the activation kinetics were calculated (Fig. 2B). In the type 1 neurons of SER, the sigmoid activation curve was shifted by about 14 mV in the negative direction, with a steep slope (Fig. 2B). The mean values of $V_{1/2}$ and K for type 1 neurons of SER were –41.08±1.21 mV and 2.81±0.41 (n=13), respectively, and these values were significantly ($p<0.01$) greater and smaller than those in the neurons of Wistar rats (-27.41±1.90 mV and 6.34±0.65, respectively; n=19), and in type 2 neurons of SER (-23.07±0.75 mV and 5.56±0.42, respectively; n=16) (Fig. 2B, Table 2). Accordingly, the level of activated Ca^{2+} channel in SER type 1 neurons was significantly lower than SER type 2 neurons and those of Wistar rats.

Decay of the Ca^{2+} channel current

When a Ca^{2+} current was evoked by applying a 100-msec-duration pulse depolarizing from a -90 mV holding potential to –10 mV, an inward current with gradual decay was obtained in the neurons of Wistar rats (Fig. 3A-a), while SER type 1 and type 2 neurons rarely showed such decay (Fig. 3A-b and c). The peak currents induced at 10 msec and 90 msec after onset of the depolarizing pulse were measured as $I_{(1)}$ and $I_{(2)}$, respectively,

and then decaying components (DC) to total current were calculated using the following formula: $DC=([I_{(1)}-I_{(2)}]/I_{(1)})\ x100\%$ The decaying component of Wistar rats was 23.27±3.29% (n=19), while those in type 1 and type 2 neurons of SER were 10.33±1.66% (n=13) and 5.26±0.96% (n=16), respectively, which were significantly ($p<0.01$) smaller than that in Wistar rats (Fig. 3B).

Discussion

In this patch clamp study, SER hippocampal neurons were classified into two types: type 1 and 2 neurons, which showed peak current between -40 and -30mV and between -20 and 0mV, respectively. The type 1 and 2 neurons herein are considered to correspond to group 1 and 2 neurons that showed long-lasting (over 100msec) and not long-lasting (less than 100msec) depolarization shifts upon mossy fiber stimulation in the intracellular recording studies using SER hippocampal slice preparations [7,10]. It was found that the Ca^{2+} channel had easier activation properties (lower threshold and higher conductance) in approximately 40% (type 1 neurons) of the SER hippocampal CA3 neurons, although the threshold and properties of the Ca^{2+} channel for type 2 neurons (approx. 60%) were not much different from those of Wistar rats. These findings imply that the Ca^{2+} channel is easy to open for Ca^{2+} influx in type 1 neurons, thereby overexciting them. Such lowering of the threshold for the Ca^{2+} channel has also been reported in kindled animals [25]. In addition, an enhancement in the Ca^{2+} current with less decay was also observed in type 1 neurons of the SER hippocampus. These results suggest that Ca^{2+} influx is increased in the type 1 neurons of the SER hippocampal CA3 field. This conclusion is completely in accordance with our cytochemical studies showing that increases in Ca^{2+} level induced by a high K^{+} solution and mossy fiber stimulation were much greater in the hippocampal CA3 field of

SER than of Wistar rats [8, 9].

The enhancement in peak Ca^{2+} current with slower decay found herein is considered to be due to an increase in the number of Ca^{2+} channels and/or prolongation of the channel's open time. Unlike type 1 neurons, type 2 neurons did not show an enhancement in the Ca^{2+} current with lower threshold potential. However, type 2 neurons showed a decrease in the current decay, which was less than that in type 1 neurons. These findings suggest that type 2 neurons might be intermediate type neurons between normal neurons and type 1 neurons relating to epileptic seizures in the SER hippocampus, although the reason for less decay in type 2 neurons than type 1 neurons remains unknown. Even in type 2 neurons, slower decay of the Ca^{2+} current is considered to induce more excitation in hippocampal neurons, generating epileptic seizures. These enhancements in the Ca^{2+} current are also thought to contribute to inducing long-lasting depolarization with repetitive firing in SER CA3 neurons. In our previous studies, both a depolarization shift with repetitive firing, and Ca^{2+} spikes in SER CA3 neurons were completely blocked by L-type Ca^{2+} channel antagonists such as nicardipine [10]. Furthermore, enhanced Ca^{2+} levels with high K^{+} in SER CA3 neurons were also inhibited by nicardipine [8]. Therefore, enhancement of Ca^{2+} influxes is suggested to be due to the abnormalities of the L-type Ca^{2+} channel function in SER hippocampal CA3 neurons. Taken in consideration about the above mentioned findings,

the channel subtype involved in abnormal Ca^{2+} current we observed in SER hippocampal CA3 neurons is considered to be an L-type Ca^{2+} channel, although this remains to be clarified. Such abnormal hippocampal excitation due to a Ca^{2+} channel abnormality is considered to be involved in induction of epileptic seizures, since the L-type Ca^{2+} channel antagonist S-312-d inhibited tonic convulsions in SER [29], although the neuronal circuit, including the hippocampus, involved in the seizures remains to be determined. In other epileptic model animals, kindling is known to induce an increase in density of N-type Ca^{2+} channels and an alternation in low- and high-voltage activated Ca^{2+} channels in the hippocampus [19, 20, 22, 25]. Interestingly, an increase in conductance in the T-type Ca^{2+} channel has been found in reticular thalamic neurons of genetic absence epilepsy rats from Strasbourg (GAERS) that show absence seizures [26]. In addition, high-voltage activated Ca^{2+} currents in tottering and lethargic mice, which have mutations in Ca^{2+} channel subunits, were larger than those in normal mice [11].

A new question is how an abnormality in the Ca^{2+} channel in the SER hippocampus appears during development, since a linkage study has shown that loci of Ca^{2+} channel genes are different from those of abnormal genes in tremor (*tm*/*tm*) and zitter (*zi*/*zi*) rats, which are the father and mother of SER, respectively [6, 27]. Therefore, a calcium channel gene mutation did not probably contribute to the abnormal Ca^{2+} currents

observed in SER. The function of the Ca^{2+} channels in SER is suggested to be enhanced by alteration of a mechanism underlying the opening and/or closing of channels, that is, due to conformational and/or constitutional changes in the channels. Tremor rats have been found to be devoid of the aspartoacylase gene, resulting in accumulation of the NAA that is responsible for epileptic seizures, since NAA applied in the cerebroventricle induced convulsive seizures with multiple spike discharges in cortical EEG. In addition, NAA produced depolarization and repetitive firing in hippocampal slices, as well as a metabotropic glutamate receptor-mediated inward current of CA3 neurons in normal Wistar rats [6, 24, 28]. Together with these previous findings, the epileptic seizures in SER are considered to be due to the fact that NAA depolarizes the neurons, thereby resulting in activation of easy-opening high-voltage-activated Ca^{2+} channels.

In line with the observations of other epileptic model animals, the Ca^{2+} channel abnormalities of hippocampal CA3 neurons are considered to be involved in the production of convulsive and/or absence-like seizures in SER. This conclusion is further supported by the previous finding that the convulsive seizures in SER were inhibited by S-312-d, an L-type Ca^{2+} channel blocker which can pass the blood brain barrier [29].

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Figure Legends

Fig. 1.

(A) Inward current induced by different command test potentials in Wistar rat and SER. The current traces obtained by depolarizing from a holding potential of –90 mV to test potentials ranging from –60 mV to 0 mV with 10 mV increments in hippocampal CA3 neurons of Wistar rats (left traces), type 1 neuron (middle traces) and type 2 neuron (right traces) in SER.
(B) The lower graphs show that typical I-V curves for peak currents in the presence (open circles) and absence(closed circles) of $CdCl_2$ (1mM). The graphs were created from different neurons shown in (A).

Fig. 2.

I-V relationships (A) and activation curves (B) for averaged peak Ca^{2+} currents at a holding potential of –90 mV in Wistar rats and SER type 1 and 2 neurons. A: The peak Ca^{2+} currents evoked by depolarizing pulses of different potentials from the holding potential are plotted against the test potentials. The peak amplitude in type 1 neurons of SER at –30 mV is significantly greater than the peak amplitudes of Wistar rats at –10 mV (*$p<0.05$, **$p<0.01$). B: The peak amplitudes of Ca^{2+} currents evoked by depolarizing pulses of different potentials from –90 mV, where the conductance is normalized to the maximum value. Points were fitted by the Boltzmann equation (see text).

Fig. 3.

Ca^{2+} currents' decay components in hippocampal CA3 neurons of Wistar rats and SER. A: The current traces evoked by depolarizing pulses to –10 mV from holding potential of –90 mV in neurons of Wistar rats (a), SER type 1 (b) and type 2 neurons (c). $I_{(1)}$ and $I_{(2)}$ were values of measured peak amplitude at 10 ms and 90ms after command pulses, respectively. B: Decay components were $[I_{(1)}-I_{(2)}]$ x100% /$I_{(1)}$ in the neurons of Wistar rats, SER type 1 and type 2 neurons. **p<0.01.

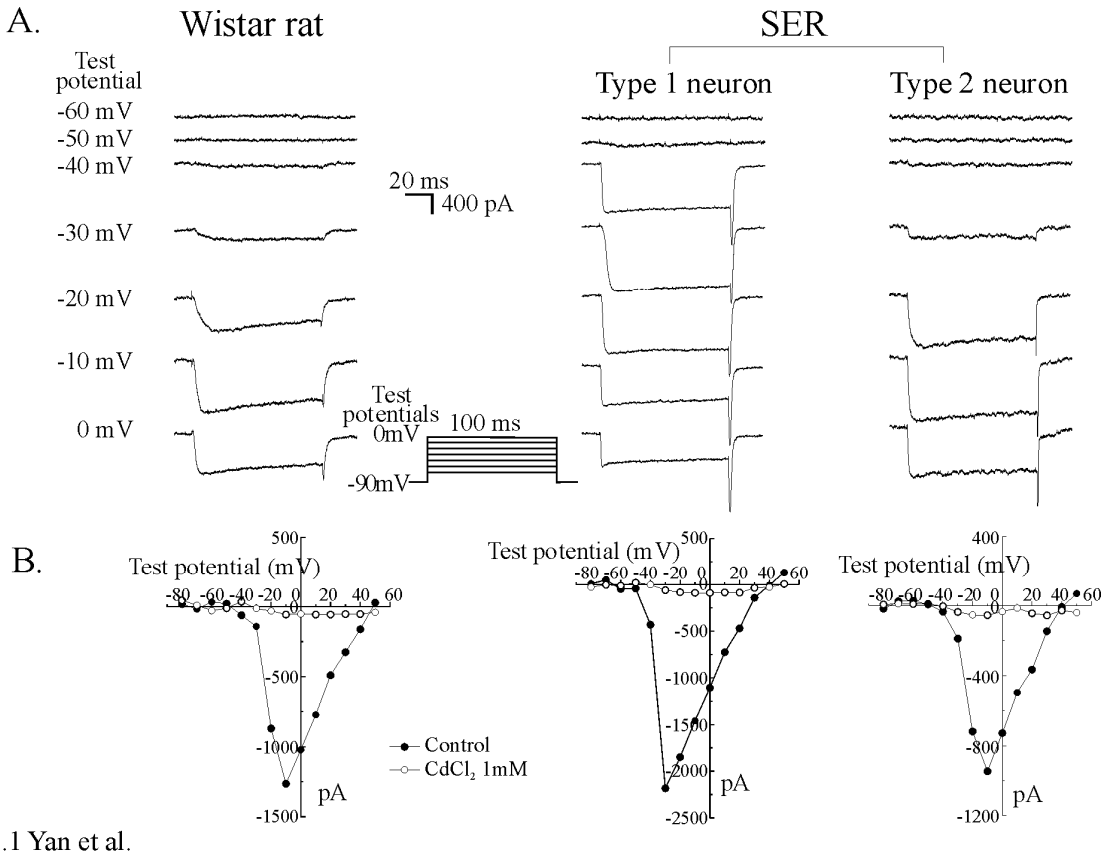

Fig.1 Yan et al.

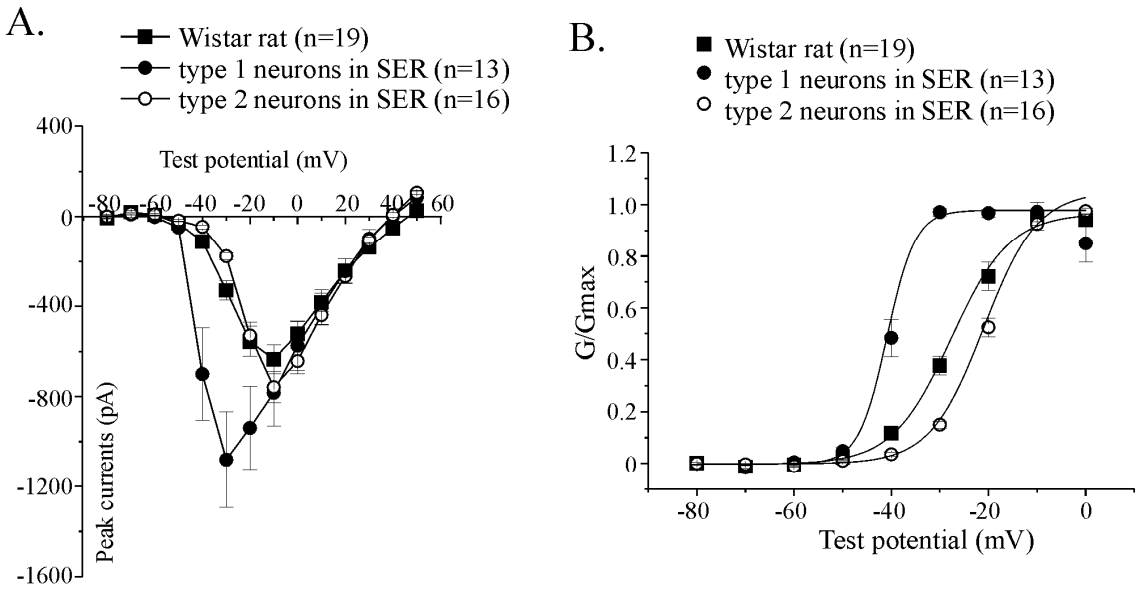

Fig.2 Yan et al.

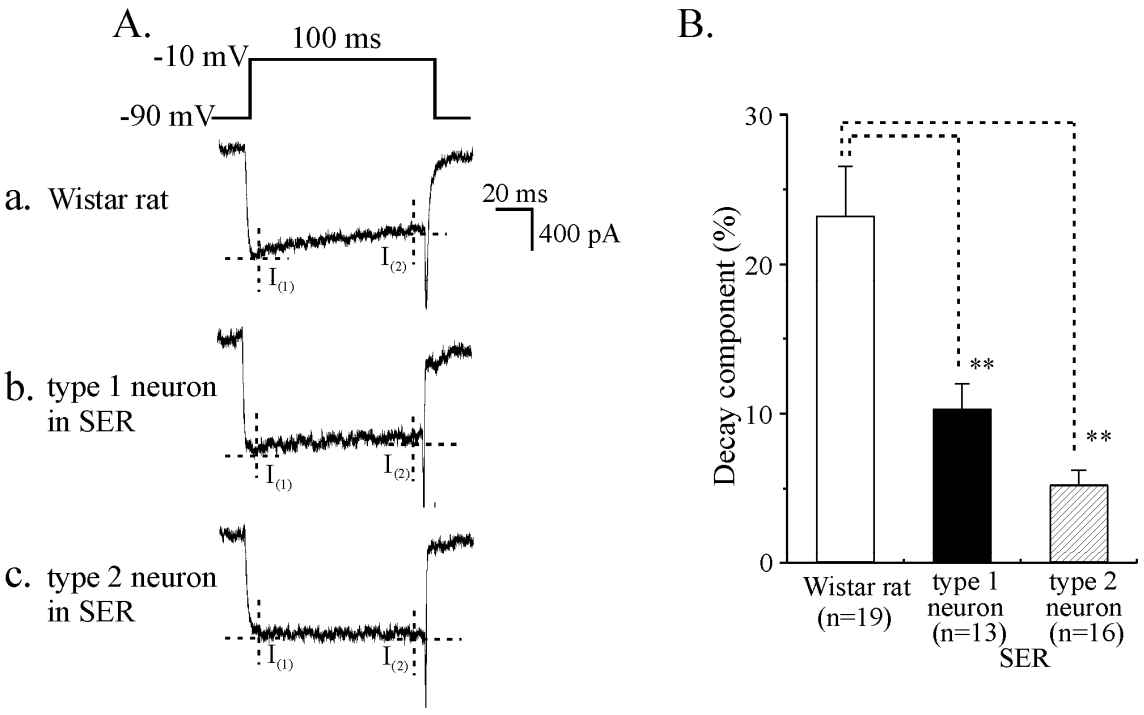

Fig.3 Yan et al.

Table 1.

The number of neurons showing peak currents under the test potentials in the I-V relation.

	Test potential (mV)					Total
	-40	-30	-20	-10	0	
Wistar rat			4*	14	1	19
SER Type 1	3	10				13
Type 2				14	2	16

* Number of neurons.

Table 2.

Activation kinetics in hippocampal CA3 neurons of Wistar rats, and SER type 1 and 2 neurons.

	$V_{1/2}$(mV)	K	n
Wistar rat	-27.41±1.90	6.34±0.65	19
SER Type 1	-41.08±1.21**	2.81±0.41**	13
Type 2	-23.07±0.75	5.56±0.42	16

** P<0.01 vs. Wistar rat.

Activation kinetics: $G/Gmax=1/\{1+\exp[(V-V_{1/2})/K]\}$, see method.