Increase in Single L-Type Calcium Channels in Hippocampal Neurons During Aging

Olivier Thibault and Philip W. Landfield*

Voltage-activated calcium (Ca^{2+}) influx is increased in mammalian CA1 hippocampal neurons during aging. However, the molecular basis for this elevation is not known. The partially dissociated hippocampal ("zipper") slice preparation was used to analyze single Ca^{2+} channel activity in CA1 neurons of adult and aged rats. Total L-type Ca^{2+} channel activity in patches was found to increase with aging, primarily because of an increase in the density of functional channels. Learning in aged animals was inversely correlated with channel density. This increase in functional Ca^{2+} channels with aging could underlie the vulnerability of neurons to age-associated neurodegenerative conditions.

There is increasing evidence that dysregulation of several aspects of Ca^{2+}-dependent processes, including voltage-activated Ca^{2+} influx, occurs in brain neurons during aging (*1*). Voltage-activated Ca^{2+}-dependent after-hyperpolarizations (AHPs), Ca^{2+} action potentials, and macroscopic Ca^{2+} currents have consistently been observed to be larger and more prolonged in hippocampal CA1 neurons from aging rats (*2*) and rabbits (*3*). The aging-related increase in AHPs seems to involve, in part, L-type channels (*2*, *3*), which are one of several types of high-threshold Ca^{2+} channels found in neurons (*4*). It has been well established that elevated Ca^{2+} is neurotoxic (*5*), and aging appears to be the major risk factor for Alzheimer's disease (AD) and several other neurodegenerative and traumatic conditions (*6*), which suggests that increased Ca^{2+} influx could contribute to heightened vulnerability of aging hippocampal neurons.

However, the molecular basis of the aging-dependent increase in the Ca^{2+}-dependent AHPs and other Ca^{2+} potentials and currents is not understood. To date, it has not been feasible to use single-channel patch-clamp recording techniques to determine whether changes in density, conductance, or other channel properties underlie the change in Ca^{2+} influx with aging. Dissociated cell preparations used for patch-clamp studies of adult brain neurons (*7*) may be too traumatic to use in studies of aging brain cells (*8*). It is also difficult to obtain the high signal-to-noise ratios required for recordings of single Ca^{2+} channels from nondissociated slice preparations, which are being used increasingly for whole-cell recording (*9*) but have been used in only a few studies of single Ca^{2+} channels (*10*).

We used partially dissociated hippocampal slice preparations to obtain the large number of high-quality, single-channel recordings needed for a reliable statistical comparison of neurons in aged, mid-aged, and young adult brain neurons. This slice preparation, originally developed by Gray, Johnston, and colleagues in guinea pigs (*11*), is often termed the "zipper slice" for its tendency to "unzip" along the major cell layers, exposing pyramidal cell somata. We adapted this preparation for single-channel studies in hippocampal CA1 neurons of adult and aging rats (*12*).

Recording methods were based on established cell-attached patch procedures (*13*). The dihydropyridine (DHP) agonist Bay K 8644 was used to induce the L-type current to overwhelmingly dominate total patch current (*4*, *14*, *15*). Because L-type channels in brain neurons can continue to open after repolarization to −70 mV [termed repolarization openings (ROs) (*15–17*)], we also examined the repolarization period. The methods used were similar to those described previously for cultured hippocampal neurons

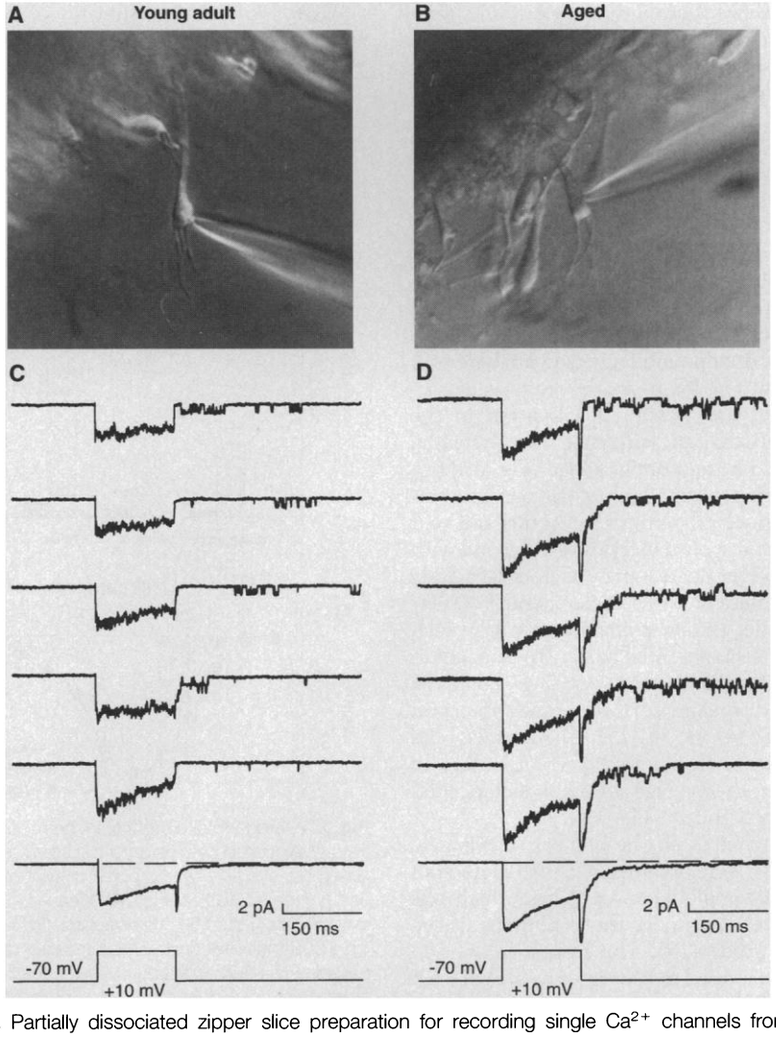

Fig. 1. Partially dissociated zipper slice preparation for recording single Ca^{2+} channels from CA1 neurons. (**A**) Exposed CA1 pyramidal neuron from a young adult animal during recording with a cell-attached pipette. (**B**) Similar neuron during recording from an aged rat. (**C** and **D**) Five representative leak-subtracted recordings from a multichannel patch on a neuron. (C) shows recordings from a young adult animal and (D) shows recordings from an aged animal during repetitive depolarizations (−70 mV to +10 mV). The first 15 depolarizations (30-s intervals) were used to create an average current ensemble for each patch (shown below the five single traces). Voltage protocol is shown at the bottom.

Department of Pharmacology, College of Medicine, University of Kentucky, Lexington, KY 40536–0084, USA.

*To whom correspondence should be addressed.

(*17*) and included use of 20 mM Ba^{2+} as the charge carrier. This Ba^{2+} concentration is lower than that often used for studies of single Ca^{2+} channels and enhances the RO activity of L-type channels (*17*).

Thirty depolarization pulses to +10 mV [from a holding potential (V_h) of −70 mV] were applied to each patch (at 30-s intervals) and an ensemble average of pseudomacroscopic patch current was constructed (Fig. 1). This average was integrated and divided by the pulse or window duration to calculate average total patch current (*I*) for each neuron during the 150-ms depolarization pulse and during the 300-ms postpulse repolarization window (*18*).

Analysis of variance of the ensemble average current showed that *I* during the depolarization pulse and repolarization window was increased with aging (Fig. 2A; depolarization pulse: $F = 7.43$, $P < 0.001$; repolarization: $F = 6.48$, $P < 0.005$). Post hoc analyses showed that these results were largely due to an increase that occurred in the aged group. (ROs were present in 37% of young adult, 52% of mid-aged, and 72% of aged neuron patches.)

To determine whether the increased *I* was due to a shift in voltage dependence rather than to an overall increase in current flux, we evaluated the voltage dependence of channel activation by plotting the current-voltage (*I-V*) relation in patches maintained through a full *I-V* analysis. We used a smaller but comparable group of patches than in the ensemble average analysis of *I*. No shift was found in the voltage dependence of Ca^{2+} channel activation in the *I-V* analyses, indicating that *I* was increased throughout the voltage range (Fig. 2B).

To dissect out which of the multiple single-channel properties that contribute to *I* was most responsible for the change in *I* with aging, we evaluated these factors separately (*18*). Values of *i*, the single-channel current amplitude, were measured directly from each trace at each potential of the *I-V* protocol in which L-type channel openings could be clearly distinguished (open time >5 ms in Bay K 8644) (*4, 14, 15*). The average *i* did not differ with age at any voltage step. In addition, the average slope conductance did not vary with age (Fig. 3A).

Estimates of *N*, the number of available channels, were obtained with the method of maximal simultaneous openings, which was highly reliable under the conditions of the present study (*19*). The resistance of each pipette was used to calculate the area of each patch (*20*) for estimates of the density of available channels ($N/\mu m^2$). Average pipette resistance was similar among all age groups: young adult, 4.96 ± 0.07 megohm; mid-aged, 4.85 ± 0.09 megohm; and aged, 4.95 ± 0.10 megohm, yielding mean patch areas of 2.77, 2.82, and 2.77 μm^2, respectively. We also monitored patches carefully to ensure that more patch area was not pulled into the pipette in aged neurons (*20*) and ruled out the possibility that any observed effects reflected age differences in sensitivity to Bay K 8644 (*21*). The density of available channels was increased about threefold in the aged neurons (Fig. 3B). This increase in $N/\mu m^2$ accounted for most of the aging-related increase in *I* (Fig. 2A), and therefore appears to underlie much of the change in voltage-gated Ca^{2+} influx with aging.

An indirect estimate of p_O (the probability that a single channel is open) was obtained for the average p_O over the entire 150-ms pulse (as opposed to the maximal p_O at I_{max}) by using the values for *N*, *I*, and *i* obtained at steps to +10 mV (*18*). This indirect estimate did not significantly increase with aging, although a nearly significant trend was observed (mean ± SEM): young adult p_O, 0.33 ± 0.03; mid-aged p_O, 0.36 ± 0.04; and aged p_O, 0.44 ± 0.04. More direct estimates of p_O as a function of aging will require analyses in patches with fewer channels.

Before recording, 10 aged animals were studied for performance in a spatial-learning water-maze task sensitive to aging differences (*22*). Successful recordings were obtained from eight of these animals, in which the rank order of $N/\mu m^2$ was inversely correlated with the rank order of performance in the water maze (Fig. 4). Young adult and mid-aged animals generally perform uniformly well on this task (*22*). The inverse correlation of a higher $N/\mu m^2$ with maze performance in a task known to involve the hippocampus (*22*) raises the possibility that the increase in L-type Ca^{2+} channel density may be related to impaired neuronal function. In fact, L-type Ca^{2+} channel antagonists can improve learning behavior in aged animals (*23*). Our results, therefore, could point to a single-channel basis for those psychopharmacological observations.

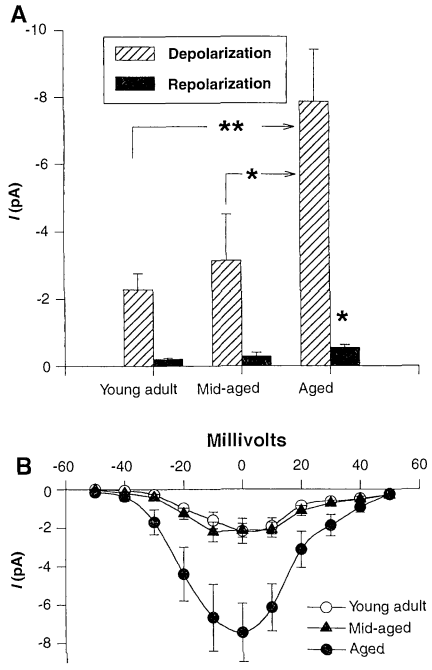

Fig. 2. Average total current (*I*) in the multichannel patch (calculated by division of the integral of each ensemble average by the pulse or window duration) increases with aging. (**A**) Mean ± SEM for current during the 150-ms depolarization and during a 300-ms window after repolarization to resting potential ($n = 35$ young adult, 19 mid-aged, and 25 aged patches). Values for *I* during both the depolarization pulse and the repolarization window differed significantly as a function of age (asterisk, $P < 0.05$; double asterisk, $P < 0.01$ by Bonferroni post-hoc comparison). (**B**) *I-V* relation for 10 patches from each age group. Plots of *I* (mean ± SEM) during the depolarization pulse show that no shift occurred in voltage dependence.

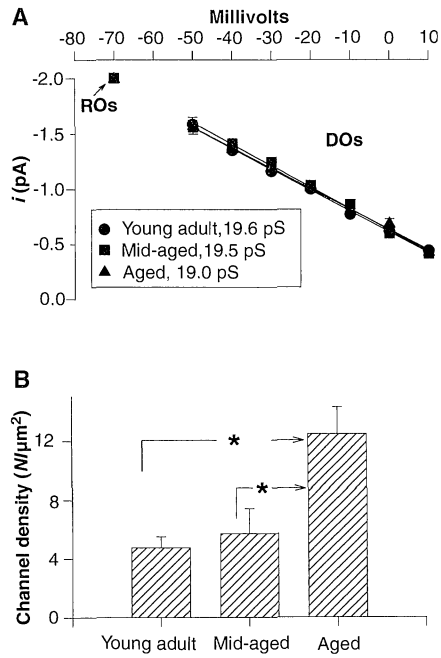

Fig. 3. Aging-related increase in channel density. (**A**) Mean ± SEM values for single-channel current amplitudes (*i*) during depolarization pulses to multiple test voltages in patches from animals of the three age groups. Individual values of *i* represent the mean amplitude of all clearly resolvable L-type openings during the pulse [depolarization openings (DOs)] for each patch at each voltage and for ROs after the pulse at −70 mV ($n = 5$ to 20 patches per age group at each voltage). Mean slope conductance for each group was calculated from the average of individual patch slope conductances. No significant age differences were seen in average single-channel current amplitude at any voltage or in slope conductance. (**B**) We obtained L-type channel density by estimating *N* from maximum simultaneous openings (*19*) and calculating patch area from pipette resistance (*20*). Channel density increased substantially as a function of aging (shown by asterisks, $F = 9.5$, $P < 0.001$) and appears to account for most of the increase in *I* (Fig. 2A) ($n = 35$ young, 19 mid-aged, and 25 aged patches).

Thus, a substantial aging-related increase occurs in N, the magnitude of which appears sufficient to account for most of the elevation in I. An increase in the density of functional channels does not necessarily imply more channel protein molecules per unit membrane. Studies of Ca^{2+} channel radioligand binding in brain cells have not observed an aging-related increase (*24*), but binding studies may overestimate functional channel density (*25*). Consequently, the aging-dependent increase in N could involve recruitment of previously "silent" channels. Alterations in phosphorylation state can modulate Ca^{2+} channel function and availability (*26*), and such alterations can occur with aging (*27*). On the other hand, evidence of an aging-related increase in mRNA of the α_{1D} subunit of L-type channels in CA1 of F344 rats has also been found (*28*). Additional studies will be required to understand the basis of the increased $N/\mu m^2$ in aged neurons. Moreover, changes in L-type Ca^{2+} channels alone probably do not account for all aspects of aging-dependent change in voltage-gated Ca^{2+} influx (*4*), much less all aspects of dysregulation of brain neuronal Ca^{2+} homeostasis (*1*).

Nevertheless, brain neurons with elevated Ca^{2+} channel density are likely to be subject to enhanced Ca^{2+} influx during and after each depolarization, which in turn could impair neuronal and behavioral function (*2, 3, 23*) and might also accelerate gradual deterioration of neuronal structure (*5*). In addition, persistent challenges by high Ca^{2+} influx could result in the eventual decline of buffering and extrusion mechanisms needed to respond to periodic toxic insults (*1*). Thus, an elevation in the density of available L-type Ca^{2+} channels appears to be a candidate mechanism for aspects of aging-dependent vulnerability to neurotoxic influences.

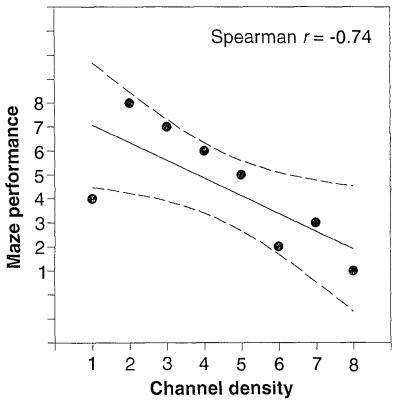

Fig. 4. Rank scores of channel density (8 is highest) and Morris water maze performance (8 is best) for aged animals for which scores on both variables were available. Animals were ranked on the basis of performance over the last three acquisition trials and on $N/\mu m^2$ (Fig. 3B). The task depends significantly on hippocampal function and is impaired with aging in F344 rats (*22*). A significant negative correlation ($r_s = -0.74$, $P < 0.05$, Spearman's nonparametric test) was found between maze performance and increasing channel density, indicating that channel density was highest in neurons from the most impaired animals. Dotted lines represent 95% confidence intervals.

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EGTA, 10 mM Hepes, and 1 μM tetrodotoxin (TTX). Pipettes were filled with 20 mM $BaCl_2$, 90 mM choline chloride, 10 mM tetraethylammonium (TEA), 10 mM Hepes, and 500 nM Bay K 8644. The pH was 7.3 for all solutions. If necessary, osmolarity was adjusted with sucrose to 300 mosM for the bath and 290 mosM for the pipette solution. We accomplished leak subtraction off-line by averaging 10 to 15 recorded leak currents of opposite polarity (same magnitude) and adding them to the records with channel openings.

18. Average current in a patch is given by $I = Np_O i$.
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20. Patch area (a) is inversely correlated with pipette resistance (R) according to the relation $a = 12.6\,(1/R + 0.018)$ [B. Sakmann and E. Neher, in *Single Channel Recordings*, B. Sakmann and E. Neher, Eds. (Plenum, New York, 1983), pp. 37–51]. We carefully monitored patches for visible membrane in the pipette tip and for unusual suction durations. All patches for which extensive membrane (other than the small omega-shaped dome) was visible in the pipette tip or that required inordinately long suction for a seal were excluded from the study. There were relatively few of these, with a similar incidence in each group, and nearly all were excluded, in any case, by the criterion of a minimum seal resistance of 15 gigohm. In general, the suction time to seal was at least as rapid in the aged group as in the others, as indicated by the relatively high yield in this group (*12*).
21. To determine whether altered sensitivity to Bay K 8644 could account for aspects of these changes, we also measured the mean channel open time (*14*) during the step to −30 mV in patches used for the *I-V* analysis (Fig. 2B) and at −70 mV during the repolarization period (ROs) after steps to +10 mV in all patches in which ROs were present. In these multichannel patches, open time was most accurately measured at −30 mV and at −70 mV, when there were few simultaneous openings. The mean ± SEM values for open time at −30 mV were: young adult, 6.5 ± 1.4 ms; mid-aged, 6.5 ± 1.5 ms; and aged, 5.7 ± 0.8 ms; and at −70 mV (ROs) were: young adult, 5.5 ± 0.6 ms; mid-aged, 5.0 ± 0.5 ms; and aged, 5.7 ± 0.7 ms. These values do not differ statistically, indicating that Bay K 8644 affected the different age groups similarly. This conclusion is further supported by the lack of age difference in voltage dependence (Fig. 2B), because a shift in voltage dependence is also a hallmark of DHP agonists (*14*).
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Binding of APC to the Human Homolog of the *Drosophila* Discs Large Tumor Suppressor Protein

Akihiko Matsumine, Akiko Ogai, Takao Senda, Nobuaki Okumura, Kiyotoshi Satoh, Gyeong-Hun Baeg, Takeo Kawahara, Shigeru Kobayashi, Masato Okada, Kumao Toyoshima, Tetsu Akiyama*

The adenomatous polyposis coli gene (*APC*) is mutated in familial adenomatous polyposis and in sporadic colorectal tumors, and its product binds to the adherens junction protein β-catenin. Overexpression of APC blocks cell cycle progression. The APC–β-catenin complex was shown to bind to DLG, the human homolog of the *Drosophila* discs large tumor suppressor protein. This interaction required the carboxyl-terminal region of APC and the DLG homology repeat region of DLG. APC colocalized with DLG at the lateral cytoplasm in rat colon epithelial cells and at the synapse in cultured hippocampal neurons. These results suggest that the APC-DLG complex may participate in regulation of both cell cycle progression and neuronal function.

The tumor suppressor gene *APC* is mutated in most cases of familial adenomatous polyposis (FAP), a dominantly inherited disease characterized by multiple adenomatous polyps in the colon (*1*, *2*). The *APC* gene is also somatically mutated in the majority of sporadic colorectal tumors (*2*). Mutation of *APC* is thought to be an early event in tumorigenesis (*3*).

The product of *APC* is a 300-kD homodimeric protein localized in the cytoplasm (*4*, *5*). The APC protein interacts with the adherens junction protein β-catenin, which suggests that APC may be involved in cell adhesion (*6*). APC also associates with microtubules and with a protein of unknown function, EB1, in cells overexpressing transfected *APC* (*7*). Overexpression of APC blocks progression from the G_0-G_1 to the S phase of the cell cycle (*8*).

To identify other proteins that associate with APC, we performed a two-hybrid screen of a human brain cDNA library using various regions of APC as "bait" (*9*). One clone that scored positive for interaction with the COOH-terminal region of APC contained a portion of the cDNA encoding DLG (amino acids 199 to 507), the human homolog of the *Drosophila* discs large tumor suppressor protein (*10*, *11*). To confirm that APC and DLG associate directly, we expressed each as a glutathione-S-transferase (GST) fusion protein and examined its ability to interact with the other protein, produced by in vitro translation (Fig. 1). In vitro–translated full-length DLG associated specifically with the COOH-terminal domain of APC (amino acids 2475 to 2843, APC-C369) fused to GST but not with GST alone. Likewise, in vitro–translated APC-C369 interacted with GST-DLG but not with GST alone.

DLG contains three DLG homology re-

A. Matsumine, A. Ogai, K. Satoh, G.-H. Baeg, T. Akiyama, Department of Oncogene Research, Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan.
T. Senda, T. Kawahara, S. Kobayashi, Department of Anatomy I, Nagoya University School of Medicine, Nagoya, Aichi 466, Japan.
N. Okumura and M. Okada, Division of Protein Metabolism, Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan.
K. Toyoshima, The Center for Adult Diseases, Osaka 537, Japan.

*To whom correspondence should be addressed.