mal T cells that pack the lymph nodes of *lpr/lpr* mice (*17*). It is also consistent with observations that the amount of *myb* RNA is high in immature, actively dividing hemopoietic cells of the chicken (*18*) and in thymus and immature, incompletely differentiated lymphocytic cells and tumors in mice (*19*).

Our results confirm the previous observation that the amount of *myb* RNA is substantial in the thymus (*18*), an organ with large numbers of proliferating T cells. However, high *myb* expression does not appear to be a feature of all cells that are undergoing proliferation because, of the lymph node cells from *lpr/lpr* mice that contained large amounts of *myb* RNA, only 6 percent were proliferating (*20*); also, mitogen-stimulated spleen cells did not express high *myb* even though they did express *myc* and *raf*. It is possible that *myb* is only expressed for a limited period during mitogen-induced proliferation and that, during the times chosen for study, transient *myb* expression was not detected. However, if that were the case, the high *myb* expression in *lpr/lpr* lymph nodes and in AILD would still indicate that a large number of cells is arrested in a particular stage of the cell cycle. Taken together, the data suggest that *myb* expression is high in cells for limited periods during differentiation rather than only during proliferation.

The results of other studies have implicated abnormal lymphoid differentiation as a common feature in autoimmune diseases (*1, 2, 21*). Individual accelerating genes, such as *lpr*, probably induce autoimmune phenomena by causing differential expression of certain genes (*2, 17*). As a step toward understanding the details of pathogenic gene expression, our results are apparently the first definitive association of increased oncogene expression with murine and human autoimmune disease. We suggest that the high levels of *myb* RNA in lymph nodes of *lpr/lpr* mice, known to be packed with the abnormal T cells associated with this autoimmune disease, may help identify the cells that act in the disease process and may provide one way to explore the genetic basis of autoimmune disease states.

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Prolonged Ca^{2+}-Dependent Afterhyperpolarizations in Hippocampal Neurons of Aged Rats

Abstract. *The possibility that calcium is elevated in brain neurons during aging was examined by quantifying afterhyperpolarizations induced by spike bursts in CA1 neurons of hippocampal slices from young and aged rats. The afterhyperpolarizations result from Ca^{2+}-dependent K^+ conductance increases and are blocked in medium low in Ca^{2+} and prolonged in medium high in Ca^{2+}. The afterhyperpolarization and associated conductance increases were considerably prolonged in cells from aged rats, although inhibitory postsynaptic potentials did not differ with age. Since elevated intracellular Ca^{2+} can exert deleterious effects on neurons, the data suggest that altered Ca^{2+} homeostasis may play a significant role in normal brain aging.*

Although it is well established that a variety of structural, chemical, and physiological changes occur in the mammalian brain during normal and pathological aging (*1*), little is known about the mechanisms that induce these age-dependent alterations. Among the few consistent microelectrophysiological changes observed in rats during aging are an impaired capacity of hippocampal synapses to exhibit frequency or long-term potentiation in response to repetitive stimulation (*2*) and altered patterns of transmitter release at the neuromuscular junction during stimulation (*3*). However, some nonsynaptic neurophysiological changes are also seen with aging (*4*).

Elevation of the extracellular Mg^{2+}:Ca^{2+} ratio selectively ameliorates the age-related deficit in hippocampal frequency potentiation, both in vitro (*5*) and in vivo (*6*). High Ca^{2+} impairs frequency potentiation in various preparations by saturation of binding sites for release or by rapid transmitter depletion (*7*); since Mg^{2+} competitively inhibits these Ca^{2+} actions (*7*), the beneficial effects of Mg^{2+} on hippocampal synaptic potentiation in aged rats have suggested that Ca^{2+} may be functionally elevated in aged hippocampal cells (*5, 6*). Other results, including changes in Ca^{2+} exchange or uptake in aged rat synaptosomal preparations (*8*), are at least consistent with the possibility that brain Ca^{2+} homeostasis is altered with age. If elevated brain Ca^{2+} does occur, the implications for both physiological and structural mechanisms of brain decline during aging might be substantial. That is, neurophysiological functions are likely to be affected by high Ca^{2+}, and a rise in intracellular Ca^{2+} seems to accelerate the structural disintegration of nonneural or neural cells that follows cellular trauma (*9*). These and other data have led to a growing interest in the hypothesis that an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) could be one important factor in the complex set of processes that pre-

Table 1. Mean (± S.E.M.) AHP amplitudes in CA1 neurons from young and aged rats. Measures were obtained of the maximum hyperpolarization during the first 200 msec (usually the peak of the IPSP) and at 600 and 1000 msec after the onset of a 40-msec depolarizing intracellular current pulse. Values from all cells per animal were averaged to yield one value per animal for two spikes and one for three spikes (*16*). After two spikes, there is a main effect of age on AHP amplitudes [$F(1, 24) = 5.98$, $P < 0.02$], but a greater interaction effect of age with AHP duration [$F(1, 24) = 6.89$, $P < 0.003$]. After three spikes, the main effect of age is not significant [$F(1, 12) = 1.13$, $P > 0.05$], but interacts significantly with AHP duration [$F(1, 12) = 9.16$, $P < 0.002$].

Spikes	Number of animals	Maximum IPSP (mV)	AHP (mV)	
			600 msec	1000 msec
Two				
Young	16	3.58 ± 0.16	0.55 ± 0.23	0.07 ± 0.05
Aged	10	3.43 ± 0.44	1.96 ± 0.45	1.00 ± 0.37
Three				
Young	9	4.42 ± 0.57	2.45 ± 0.65	0.77 ± 0.43
Aged	5	3.75 ± 0.40	3.85 ± 1.13	2.70 ± 0.58

sumably underlies normal age-related neural decline (*10*).

The results of the synaptic studies discussed above are suggestive, but many presynaptic and postsynaptic factors apart from $[Ca^{2+}]_i$ can affect synaptic efficacy; therefore no clear tests have yet been conducted of the hypothesis that elevated $[Ca^{2+}]_i$ is a consistent correlate of normal brain aging and that such elevation affects physiological processes. A prediction of this latter hypothesis is that Ca^{2+}-dependent physiological processes should be increased in aged brain cells or should mimic the pattern seen in young brain cells maintained in high Ca^{2+}. To specifically test this prediction, measurements are needed of a neurophysiological process that is more solely dependent upon $[Ca^{2+}]_i$ than is synaptic transmission.

One such process is the Ca^{2+}-dependent K^+ conductance [$G_{K(Ca)}$] increase and resulting afterhyperpolarization (AHP) that follows a burst of action potentials in vertebrate spinal (*11*) and hippocampal (*12*) neurons. In hippocampal neurons several kinds of Cl^- and K^+ conductances are activated by a burst of action potentials evoked by intracellular current injection; however, the long K^+ conductance increase that lasts beyond 500 msec, and for as long as several seconds after intracellular stimulation, seems to depend solely on Ca^{2+} (*12*). Further, the duration and amplitude of the Ca^{2+}-dependent AHP depend proportionately on $[Ca^{2+}]_i$ in a variety of preparations (*13*). One other long, non-Ca^+–dependent K^+ conductance has been described for hippocampal neurons, but that conductance is apparently activated only by feed-forward synaptic stimulation and is absent after intracellular stimulation (*14*).

Consequently, we tested the possibility that aged brain neurons are characterized by greater or more prolonged elevations of $[Ca^{2+}]_i$ by quantifying the amplitude and duration of the AHP and the associated $G_{K(Ca)}$ increases that are activated by intracellular stimulation of hippocampal neurons from aged and young rats. We report here that the AHP's and conductance increases are prolonged in hippocampal neurons from aged rats, as would be predicted by a hypothesis of increased $[Ca^{2+}]_i$ during normal aging.

Hippocampal slices were prepared and maintained conventionally (*15*), and cells from field CA1 were examined intracellularly with pipettes of 75- to 110-megohm impedance. Only cells meeting criteria for good penetrations were analyzed (*15*). In most studies, slices were maintained at 33° to 34°C in an artificial cerebrospinal fluid medium composed (in millimoles per liter) of NaCl, 122; KCl, 2.75; $CaCl_2$, 2.0; $MgSO_4$, 2.0; KH_2PO_4, 1.25; $NaHCO_3$, 30; and glucose, 10.0. In some experiments, slices were maintained in a low Ca^{2+}:high Mg^{2+} medium (1.4:2.6) (in millimoles per liter) or a high Ca^{2+}:low Mg^{2+} medium (2.6:1.4). We studied 60 cells from 24 young mature (5 to 8 months old) and 10 aged (27 to 30 months old) male Fischer 344 rats from the barrier-reared colony of the National Institute on Aging. Animals were housed behind an air barrier system in our facilities until used and were free from respiratory infections.

Input-output stimulation series of intracellular depolarizing current pulses (40-msec duration) were conducted for each cell. Although aged cells tended to exhibit lower thresholds, the difference between age groups in the amount of current needed to induce either two or three action potentials did not reach significance. The size and duration of the AHP seemed to depend directly on the number of spikes induced by the injected currents, and almost no AHP was seen unless the stimulating current reached threshold for spike generation. Therefore, in order to equalize the amount of

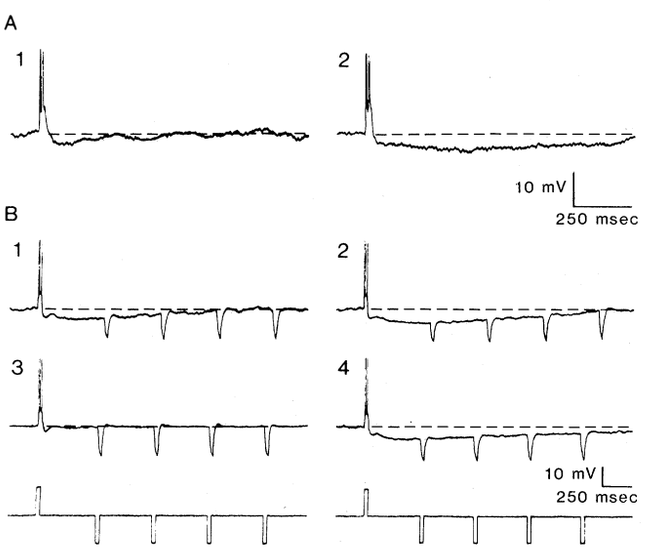

Fig. 1. Intracellular current-induced bursts of action potentials and subsequent AHP's in CA1 neurons of hippocampal slices from young and aged rats. (A) AHP's following a 0.4-nA current-induced burst of two spikes. (A_1) Cell from a young rat. (A_2) Cell from an aged rat. (B) AHP's and concomitant conductance increases following a 0.4-nA current-induced burst of three spikes. (B_1) Young rat cell in normal Ca^{2+}. (B_2) Aged rat cell in normal Ca^{2+}. (B_3) Young rat cell in low Ca^{2+}:high Mg^{2+}. (B_4) Young rat cell in high Ca^{2+}:low Mg^{2+}. Dashed lines show resting potentials before the burst. At the bottom are shown the initial intracellular depolarizing current pulse used to induce a spike burst and the subsequent 2-Hz train of 0.4-nA hyperpolarizing pulses used to assess input conductance during the AHP, for cells shown in (B).

depolarization used to trigger Ca^{2+} influx and the AHP for each cell (*12*), AHP's were analyzed separately after two and after three spikes. Input resistance was assessed while the current injection circuit was well balanced, with hyperpolarizing current pulses of the same magnitude (0.25 to 0.45 nA) and duration (40 msec) (sufficient to charge membrane capacitance) used as the depolarizing pulses. Responses were stored on frequency modulation tape and subsequently analyzed on a digital oscilloscope-microprocessor system (Norland) or on hard copies made with a light beam oscillograph. The AHP's were measured at the maximum amplitude within the initial 200 msec [generally the peak of the inhibitory postsynaptic potential (IPSP)], and at 600 and 1000 msec after the onset of the depolarizing current pulse (*16*).

No differences were seen in resting values for membrane potential [mean ± standard error of the mean (S.E.M.) for cells from young animals, 59.3 ± 0.65 mV, and from aged animals, 59.1 ± 1.73 mV] or in input resistance (young animals, 34.4 ± 2.51 megohms, and from aged animals, 32.1 ± 3.23 megohms).

However, significant differences were found in the AHP's of long duration that followed bursts of spikes (*16*). Quantitative analyses showed that, although maximum hyperpolarization during the IPSP phase did not differ with age, the duration of the AHP was significantly prolonged in cells from aged rats after bursts of either two or three spikes (Table 1). In some instances the increase in the duration of the AHP was striking (Fig. 1A). The interaction terms between age and duration were greater than the main effects terms for age alone (Table 1), indicating a larger effect of age on AHP duration than on AHP amplitude; this pattern held even for analyses of variance performed only on the 600- and 1000-msec points, when the AHP's are mediated solely by increases in $G_{K(Ca)}$. As others have reported (*11–14*), the AHP following an intracellular current-induced burst of spikes was accompanied by an input conductance increase that lasted approximately as long as the AHP (Fig. 1B). In 20 cells in which conductance was assessed with a 2-Hz train of hyperpolarizing pulses during the AHP, the amount and duration of the conductance increase closely paralleled the AHP.

To confirm that our AHP's were not contaminated by non-Ca^{2+}–dependent AHP's, and that the AHP can be prolonged by high Ca^{2+}, 24 cells from only young rats were investigated in the low Ca^{2+}:high Mg^{2+} or high Ca^{2+}:low Mg^{2+} mediums. In each cell studied in the low Ca^{2+}, AHP's and conductance changes were essentially completely blocked (Fig. $1B_3$). Conversely, in essentially each young rat cell examined in the high-Ca^{2+} medium, AHP's were longer than in normal Ca^{2+}; some AHP's were remarkably prolonged (≥3 seconds) in high Ca^{2+} (Fig. $1B_4$). Thus, the AHP's are highly sensitive to Mg^{2+}:Ca^{2+} balance, and the young cells in high Ca^{2+} medium resemble aged rat cells in normal Ca^{2+} medium.

A preliminary report has noted prolonged AHP's in spinal motoneurons of aged cats (*17*), but the observed AHP's were less than 100 msec long, and the aged neurons also exhibited slower conduction velocities and higher input resistance. Differences were therefore attributed to an age-related increase in the relative population of small motoneurons. However, since intracellularly evoked hippocampal AHP's lasting beyond 500 msec are not significantly contaminated by other conductances, and since CA1 cells exhibit no major age differences in resting input resistance or membrane potential, our results appear to directly address the question of altered Ca^{2+} homeostasis in aged brain neurons.

Several mechanisms could account for a prolonged $G_{K(Ca)}$, including (i) greater influx of Ca^{2+}; (ii) reduced Ca^{2+} extrusion or buffering; (iii) higher resting $[Ca^{2+}]_i$; or (iv) altered affinity of Ca^{2+} binding sites. Intrinsic alterations of K^+ conductance channels are unlikely to be involved in the prolonged conductance increase, since the repolarization of spikes did not differ, and since resting conductance or potential did not differ as a function of age; moreover, as noted, $G_{K(Ca)}$ has been found to vary primarily as a direct function of $[Ca^{2+}]_i$ under a wide variety of experimental conditions (*11–14*). It also seems unlikely that these results are limited to in vitro preparations since both the age-related impairment of hippocampal frequency potentiation and the effects of elevated Mg^{2+} on hippocampal frequency potentiation in aged rats are similar in vitro and in vivo (*2, 5, 6*).

In sum, the quantitative findings of this study seem to provide the first clear evidence that the impact of Ca^{2+} on a normal physiological process is increased in aged mammalian brain cells (due either to higher resting $[Ca^{2+}]_i$ or to larger transient increases in $[Ca^{2+}]_i$). Additional studies are needed to analyze underlying mechanisms and further rule out the possibility of subtle alterations in K^+ channels. Finally, elevation of $[Ca^{2+}]_i$ could be relevant not only to normal brain aging, but to age-correlated pathological conditions as well, since elevated Ca^{2+} has been detected in neurofibrillary tangle-bearing cells from the brains of demented subjects (*18*).

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16. Since it was readily apparent that cells from the same animal often exhibited similar values of resting potential and AHP length (that is, they were not independent), values for all cells from the same animal were averaged to provide a single value per condition. Animals were then used as the statistical population; two way analyses of variance with split-plot designs were used to assess the effect of age (between groups variable) and postactivation duration (within subjects variable) on AHP amplitudes.
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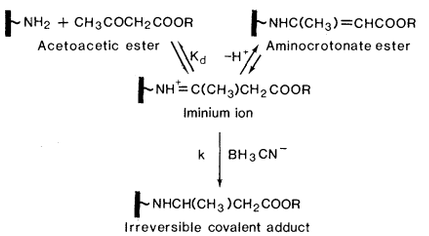
Selective Deficits in the Sense of Smell Caused by Chemical Modification of the Olfactory Epithelium

Abstract. *A chemically selective procedure for covalent modification of Schiff base–forming binding sites in proteins is demonstrated in vitro. In vivo studies show that the same procedure produces a selective anosmia ("odor blindness") when applied to the olfactory epithelia of experimental animals. Surgical experiments confirm that the sense of smell is specifically affected.*

The hypothesis that olfactory receptors are protein molecules is widely embraced (*1*). Here we report experimental findings to support that hypothesis. Our working model (*2*) is based upon the supposition that Schiff base–forming proteins in the olfactory epithelium bind carbonyl-containing odorant molecules. To assess this notion, we have developed a procedure to specifically and irreversibly modify proteins that bind simple ketones as iminium ions. We have probed its chemical selectivity and have explored the effects on the sense of smell of tiger salamanders (*Ambystoma tigrinum*). We have previously shown how to evaluate anosmias in this species with a behavioral assay (*3*).

The modification procedure is derived from well-known techniques for affinity labeling of Schiff base–forming enzymes (*4*). We have tested our sequence of reagents, acetoacetic ester followed by sodium cyanoborohydride ($NaBH_3CN$), by examining pure polypeptide proteins. As an example of a Schiff base–forming protein, the bacterial enzyme acetoacetate decarboxylase (AAD) (*5*) was chosen for model studies. AAD binds simple, uncharged ketones (*5–7*) at an active site lysine residue. Borohydride reducing agents convert reversible covalent complexes to irreversible covalent adducts (*2*).

Quantitative studies with pure AAD demonstrate the selectivity of irreversible modification. Nondialyzable radioactivity is incorporated into AAD after treatment with ^{14}C-labeled ethyl acetoacetate (EAA) (*8*) followed by $NaBH_3CN$. This treatment does not radioactively label lysine-rich proteins that lack specialized Schiff base–forming



binding sites (such as bovine serum albumin or ribonuclease A), nor does it label Schiff base–forming proteins that require electrically charged substrates (such as rabbit muscle aldolase or glucosephosphate isomerase) (*9*). Reaction 1 depicts a reaction mechanism for irreversible covalent modification. Acetoacetic ester binds reversibly to ϵ-amino groups of lysine residues (*10*) with dissociation constant K_d. An iminium ion is formed, which can be reduced by $NaBH_3CN$, but, unless it is in a specialized environment, the iminium ion rapidly loses a proton to form an aminocrotonate ester, which is not attacked by $NaBH_3CN$ at $pH \geq 6$. The active site of AAD represents a specialized environment (*11*), where the presence of a nearby positive charge facilitates attack by $NaBH_3CN$ (with pseudo-first-order rate constant **k**). In our in vitro studies, EAA was removed by dialysis in competition with reaction 1. Under these conditions, the extent of modification, ϕ, ought theoretically to obey Eq. 1, where S_0 represents the initial concentration of EAA and τ its residence time under dialysis conditions (*2*). Experimentally, Eq. 1 is obeyed, as the linear log-log plot in Fig. 1 demonstrates. The value of K_d was taken to equal the inhibition constant K_i determined from inhibition studies, 0.1 m*M* (*2*), and the graph becomes significantly curved if other values of K_d are used.

$$\phi = 1 - \left(\frac{K_d}{K_d + S_o}\right)^{k\tau}$$

We infer, therefore, that irreversible covalent modification occurs at the same site where EAA binds as a reversible inhibitor.

When the same sequence of reagents (0.5 m*M* aqueous acetoacetic ester followed by 50 m*M* aqueous $NaBH_3CN$) is applied to the olfactory epithelia of tiger salamanders, the animals develop selective anosmia that lasts for approximately 1 week. Application of either reagent alone has no behavioral effect.

A group of 12 salamanders was conditioned to avoid negatively reinforced presentations of cyclohexanone (CH) or dimethyl disulfide (DMDS) (*3*). Responses to presentations of a third odorant, *n*-butanol (BuOH), were not reinforced, but were monitored concurrently. Training and testing were performed with odorant concentrations at 2.0 percent of vapor saturation, with one session per day comprising ten presentations of each odorant in a randomized order. Criterion learning performance was defined as ≥80 percent avoidance of CH and of DMDS and ≤20 percent avoidance of BuOH. The score for each session is expressed as the frequency of avoidance for each odorant, always given in order, CH, DMDS, and BuOH.

All animals surpassed criterion by day 9 (mean scores were 9.0, 8.8, and 1.4)

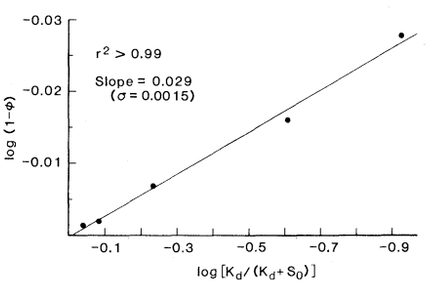


Fig. 1. Extent of modification, ϕ, of AAD as a function of initial concentration S_0 (from 0.010 m*M* to 0.74 m*M*) of ethyl [3-^{14}C]acetoacetate. Enzyme (2.4 activity units per nanomole) and radiolabel were treated with 30 m*M* $NaBH_3CN$ followed by dialysis. The slope represents the pseudo-first-order rate constant, **k**, for production of irreversible adduct times the dialysis residence time τ (Eq. 1). Each point represents a single determination, and the standard deviation (σ) represents the error of the slope from a least-squares fit.