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Reversible antisense inhibition of Shaker-like Kv1.1 potassium channel expression impairs associative memory in mouse and rat

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ABSTRACT **Long-term memory is thought to be subserved by functional remodeling of neuronal circuits. Changes in the weights of existing synapses in networks might depend on voltage-gated potassium currents. We therefore studied the physiological role of potassium channels in memory, concentrating on the Shaker-like Kv1.1, a late rectifying potassium channel that is highly localized within dendrites of hippocampal CA3 pyramidal and dentate gyrus granular cells. Repeated intracerebroventricular injection of antisense oligodeoxyribonucleotide to Kv1.1 reduces expression of its particular intracellular mRNA target, decreases late rectifying K^+ current(s) in dentate granule cells, and impairs memory but not other motor or sensory behaviors, in two different learning paradigms, mouse passive avoidance and rat spatial memory. The latter, hippocampal-dependent memory loss occurred in the absence of long-term potentiation changes recorded both from the dentate gyrus or CA1. The specificity of the reversible antisense targeting of mRNA in adult animal brains may avoid irreversible developmental and genetic background effects that accompany transgenic "knockouts".**

Drosophila memory mutants (primarily the Shaker mutants) (1), *Hermissenda* associative conditioning (2) and rabbit nictitating membrane conditioning (correlated with enhanced post-synaptic responses due to persistent reduction of voltage-dependent K^+ current in hippocampal cells) (3) have all strongly suggested a role for specific potassium channels in learning and memory. In recent years, studies of Shaker-like genes in the mammalian brain revealed a family of potassium channel related proteins that differ in both their electrophysiological properties and in their neuroanatomical distribution (4, 5). In this study we concentrated on the Kv1.1 that contributes to late rectifying potassium current(s) and is highly localized to dendrites both in the hippocampal CA3 pyramidal cells and dentate gyrus granular cells (6, 7).

In contrast to *Drosophila* memory mutant (8) and mouse transgenic manipulations (9) that can impact on the development of brain networks as well as network function, antisense ODNs have the potential of reversibly disrupting the expression of proteins in a fully differentiated brain without deranging its genetically specified neuronal or synaptic architecture (10). *In vivo* antisense ODN administration has in the past been compromised by (*i*) the instability of the short single stranded oligonucleotides involved and (*ii*) poor uptake of the oligonucleotides into cells (11). Biochemical modification of the phosphodiester backbone to better resist nucleases and coincubation with cationic lipids to facilitate intracellular uptake have improved *in vivo* oligo efficacy (12–14). These methods were applied here to block specific K^+ channels with anatomically specific localization to induce memory loss without affecting sensory motor and state-dependent behavior.

MATERIALS AND METHODS

Mice Microinjections. Male Swiss-Webster mice (22–28 g) from Morini (San Polo d'Enza, Italy) were kept at constant temp (23 ± 1°C), a 12 hr light/dark cycle, with *ad libitum* access to food and water. All experiments were carried out according to the guidelines of the European Community Council. Intracerebroventricular administration was performed according to Haley and McCormick (15). Under ether anesthesia, a 0.5-mm external diameter, hypodermic needle attached to a 10-μl syringe was inserted perpendicularly 2 mm on the right, from a line drawn through the anterior base of the ears penetrating no more than 2 mm into the brain, injecting 5 μl/30sec. To verify the injection location, mice were injected with 1:10 India ink and their brains examined microscopically after sectioning.

Rats Injections and Surgery. Male Wistar rats (250–300 g) from Charles River Breeding Laboratories were housed individually with *ad libitum* access to food and water, constant temperature (23 ± 1°C), and a 12 hr light/dark cycle. All experiments were carried out according to National Institutes of Health guidelines. Rats were surgically implanted with two stainless still guide cannulas 0.5 mm above each lateral ventricle using a stereotactic apparatus (Kopf Instruments, Tujunga, CA), Coordinates according to Paxinos and Watson (16): Anterior–posterior = 0.5 mm, lateral = 1.5 mm, horizontal = 3.2 mm. The cannulas were fixed in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Rats were allowed a recovery period of 7 days before the first ODN injection. ODNs were injected every 48 hr for 7 days (2.5 μl per cannula, 1 μl/min) before the first training day. Injections were continued during training every 2 days, training were carried out in the morning and the rats were injected in the afternoon.

Antisense Oligodeoxyribonucleotides (ODNs). 24 mers, were designed to hybridize to the AUG translation initiation codon of the mRNA encoding Kv1.1. The ODNs were checked for absence of significant homology with other mammalian sequences presented in the GenBank database, minimization of potential hairpin and duplex formation. They were phosphorothioate protected by a 3′- and 5′-end double substitution, synthesized on a 10 μmol scale and purified on an HPLC (Genosys, The Woodlands, TX). The ODNs were incubated at 37°C for 30 min, in the presence of the cationic lipid DOTAP (13 μM) (Boehringer Mannheim). The sequence of the ODNs: rat: 5′-TGACATCACCGTCATGATGGATGC-3′ mice: 5′-CGACATCACCGTCATGATGAAAGC-3′ The control was

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Abbreviations: ODN, oligodeoxyribonucleotide; PGK1, phosphoglycerate kinase 1; LTP, long-term potentiation; RT, reverse transcription.

a fully degenerated ODN: 5′-NNNNNNNNNNNNNNNNNN-NNNNNNN-3′ where n = G, C, A, or T.

Reverse Transcription (RT)–PCR. RNA, from rats hippocampi or mouse brain that underwent behavior testing (see below) and were sacrificed immediately after training, was extracted by RNAzol, treated with RNase-free DNase to remove any residual genomic DNA and reverse transcribed to single-stranded cDNAs using random hexamers. PCR was performed on a Perkin–Elmer/Cetus thermal cycler: 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification products arising from RT-PCR were electrophoresed on a 2% agarose gel, stained by ethidium bromide and analyzed by densitometry. For sequence analysis, amplification products were isolated, subcloned into the pCR-Script SK^+ cloning vector (Stratagene), and sequenced with T3 and T7 primers. In a preliminary series of experiments, the RT-PCR protocols used for the analysis of the rat mRNA were optimized so that the efficiency of amplification of Kv1.1, PGK1, and β-actin mRNAs remained in the linear range. Primers used to identify the rat and mouse (data not shown) Kv1.1 mRNAs by RT-PCR were based on the reported sequence (17, 18) and generated a 326- and a 189-bp cDNA fragment, respectively. Oligonucleotide sequences and location of the primers were as follows: 5′-GTAGACCTCTG-AACCTTCTGG-3′ (rat Kv1.1, residues 86–106), 5′-AGAG-TCTTGAGCTGCGTCTC-3′ (rat Kv1.1, residues 392–411), 5′-GCTCTCTCCTGGCCTCCT-3′ (mouse Kv1.1, residues 544–561), and 5′-GTTTCGAAGCGCAGCCCG-3′ (mouse Kv1.1, residues 715–732). Forward and reverse primers were upstream from and downstream to the segment of Kv1.1 mRNA targeted by the antisense ODNs. To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat phosphoglycerate kinase 1 (PGK1) (19) or mouse β-actin (20) were included in the PCR reactions and generated a 183- and 232-bp PCR product, respectively. Oligonucleotide sequences and location of the primers were as follows: 5′-AGGTGCT-CAACAACATGGAG-3′ (PGK1, residues 777–796), 5′-TACCAGAGGCCACAGTAGCT-3′ (PGK1, residues 940–959), 5′-GCGGGAAATCGTGCGTGACATT-3′ (β-actin, residues 2106–2127) and 5-GATGGAGTTGAAGGTAGTT-TCGTG-3′ (β-actin, residues 2409–2432).

Water Maze Learning. The procedures were modified from Morris (21, 22). Rats were trained in a pool 1.5 m in diameter and 0.6 m high, containing water at 26 ± 1°C. The pool was in the center of a room containing various salient cues. A 10-cm square transparent platform was hidden in a constant place in the pool with its top surface submerged 1 cm below the water level. The rats were given three consecutive training trails each day for three consecutive days starting each time from random locations around the pool. Each trial lasted up to 120 sec. If the rat did not find the island within this time, it was guided to the island. In any case, the rats were given 30 sec on the island to learn its location. On the fourth day the island was removed and the search strategy of the rat monitored to check if the rat used spatial memory and searched for the island in the quadrant where the island was previously located.

Passive-Avoidance Test. This was performed according to the step-through method described by Jarvick and Kopp (23) with slight modifications. The apparatus consists of two compartments, one dark and one lighted, separated by a guillotine door. The dark compartment has a pitfall floor to a cold water bath (10°C). Latency times for entering the dark compartment were measured in the training test and after 24 hr in the retention test. A memory index was expressed as the difference between retention and training latencies.

Spontaneous Locomotor Activity. This was measured using the hole board test (Ugo Basile, Varese, Italy) The hole board consists of a 40-cm square plane with 16 flush mounted cylindrical holes (diameter, 3 cm) distributed 4-by-4 in an equidistant, grid-like manner. Mice were placed in the center of the board and left to move freely for 5 min. Two electric eyes dividing the plane into four quadrants, automatically monitored the movement of the mice on the surface of the plane. Miniature photoelectric cells, in each of the 16 holes, recorded the exploration of the holes (head plugging activity).

Motor Coordination Learning. This was measured using a rota-rod (Ugo Basile) rotating at a speed of 16 rpm (3 cm in diameter with a nonslippery surface). Motor coordination was assessed by the number of falls in 30 sec.

Intracellular Recordings from Dentate Gyrus Granule Cells. Recordings were obtained with 75–80 MΩ glass micropipette electrodes filled with 2 M potassium acetate. The electrodes were inserted into the slice in 5 μm increments using a Kopf Instruments hydraulic microdrive. The amplifier was put into oscillation

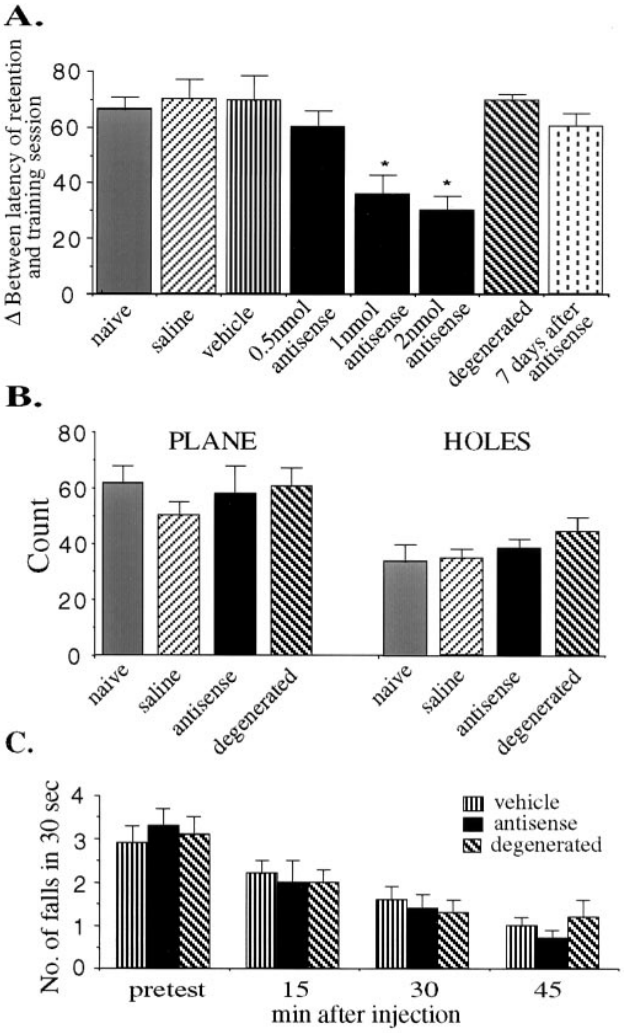

FIG. 1. The effect of antisense inhibition on mouse passive avoidance conditioning. (*A*) Passive-avoidance tests were performed according to the step-through method. Latency times for entering the dark compartment were measured in the training test and after 24 hr in the retention test. A memory index was expressed as the difference between retention and training latencies ± SEM (naive, n = 12; saline, n = 18; vehicle, n = 27; 0.5 nmol antisense, n = 20; 1 nmol antisense, n = 25; 2 nmol antisense, n = 36; degenerated ODN, n = 38; 7 days after antisense injection, n = 39). (*B*) Spontaneous locomotor activity was measured using the hole board test. Mice were placed in the center of the board and left to move freely for 5 min. Two electric eyes automatically monitored the movement of the mice on the surface of the plane. Miniature photoelectric cells, in each of the 16 holes, recorded the exploration of the holes (head plugging activity) (n = 10 in each group). (*C*) Motor coordination was measured using a rota-rod. Motor coordination was assessed by the number of falls in 30 sec (n = 10 in each group).

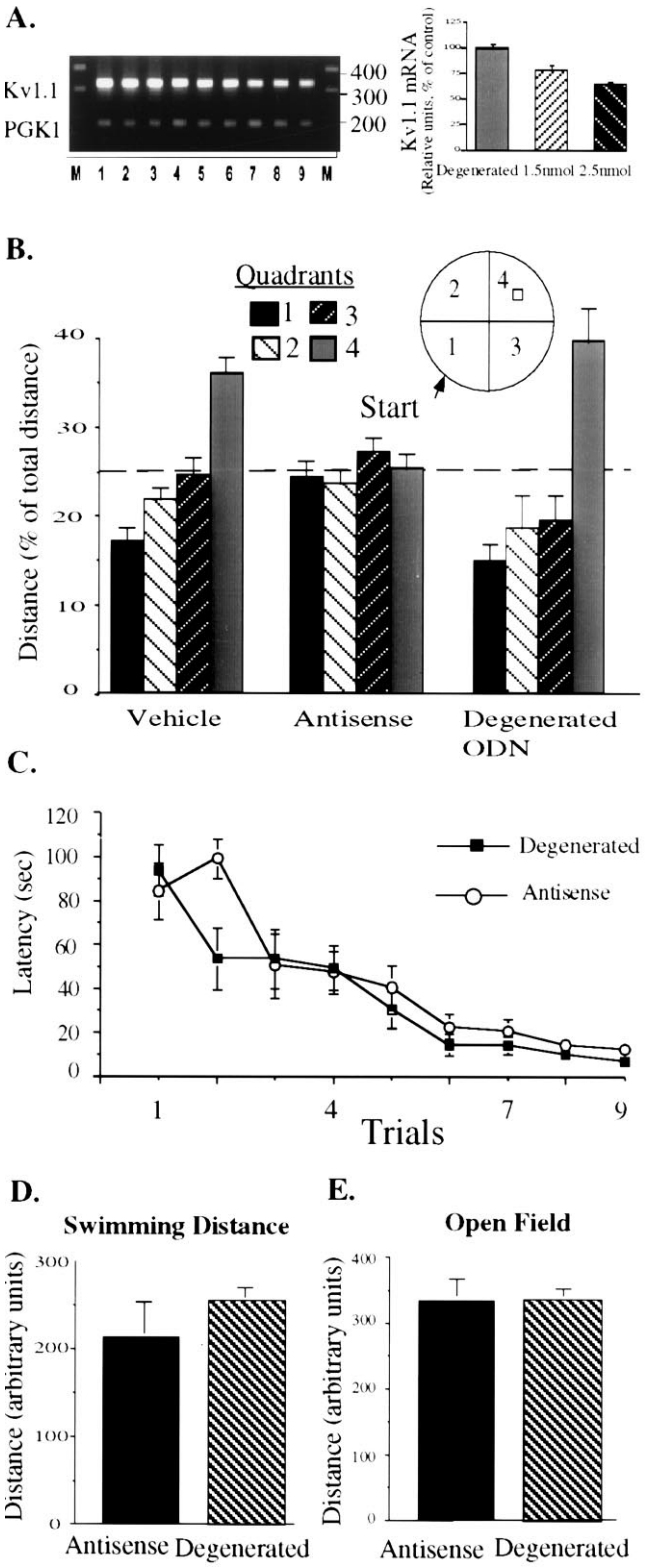

FIG. 2. Analysis of Kv1.1 mRNA by RT-PCR and the effect of antisense on rat spatial memory. (*A*) Agarose gel of amplification products arising from RT-PCR with PGK1 and Kv1.1 primers from rats injected with either degenerated ODN (lanes 1–3), 1.5 nmol antisense ODN (lanes 4–6), or 2.5 nmol antisense ODN (lanes 7–9) that underwent water maze testing (see below). (*B*) Quadrant analyses were performed on the fourth day, after three training days in the water maze, in the absence of the island. The square in quadrant 4 indicates the previous position of the submerged platform (antisense, $n = 18$; degenerated ODNs, $n = 19$; vehicle, $n = 10$). (*C*) Water maze rats were trained three consecutive training trails each day for three consecutive days to locate a submerged island ($n = 20$ in each group). (*D*) Distance of swimming in the first encounter with the pool ($n = 9$ in each group). (*E*) Open field exploratory behavior was monitored in the same pool as the water maze but without the water, a day after the quadrant analysis was monitored ($n = 9$ in each group).

until a granule cell was penetrated (as indicated by a membrane resting potential of −60 to −70 mV). Stimulating electrodes in the medial perforant pathway were Teflon-insulated PtIr wire 100 μm in diameter. Slices were prepared and pathway isolation was obtained according to Dahl and Sarvey (24).

Field Recording to Induce Long-Term Potentiation (LTP) in the Dentate Gyrus and CA1. Population spikes and EPSPs were recorded in the dentate gyrus granule cell layer to single pulse stimulation of the medial perforant pathway, and in the CA1 to a single train stimulation of the Schaeffer Collateral pathway. The medial perforant pathway location in the molecular layer was confirmed using the dentate gyrus profile (24). To prevent confounding current spread in the molecular layer, stimulation intensity was kept to less than that necessary to evoke 50% of the maximum medial perforant pathway response. CA1 LTP and paired-pulse facilitation were measured also at 50% of maximum response. Tetanization in the dentate gyrus consisted of five trains, 10 pulses, each 200 Hz. Tetanization for LTP in CA1 consisted of one 100 Hz train, during one sec. Both tetanizations were induced at the baseline intensity. Paired-pulse facilitation was performed with a double pulse 20 ms apart.

RESULTS AND DISCUSSION

Two ODNs were designed to target the translation start site region of Kv1.1 mRNAs, each one specific to either the rat or the mouse sequence. The ODNs were designed to have minimal sequence homology to other genes. Behavioral analyses of the rodents injected with the antisense revealed clear memory impairment in two different learning paradigms, mouse passive avoidance conditioning and rat spatial memory in the Morris water maze (Figs. 1 and 2). The design of ODNs and mouse experiments were conducted at the University of Florence, while the rat experiments were conducted independently at the National Institutes of Health. Repeated i.c.v. injection of these ODNs every 48 hr caused a marked reduction of cerebral Kv1.1 in both species as measured by RT-PCR. The degree of inhibition depended on the dose of the ODN injected. Rat hippocampal Kv1.1 mRNA was reduced by 22 ± 4 and 36 ± 2% 48 hr after the last of five injections, 1.5 nmol or 2.5 nmol, respectively (Fig. 2*A*). Mouse brain Kv1.1 mRNA was reduced by 37.5 ± 2.4, 48 hr after the last of three injections ($n = 3$ in each group) (data not shown).

Mice were conditioned using the light/dark box apparatus to passively avoid the dark compartment, which is associated during training with the punishment of falling into cold water. If mice were i.c.v. injected with the antisense ODN once every 72 hr, on three successive occasions, and trained 48 hr after the last injection, they showed a significantly shorter latency time, 48 hr later, to enter the dark compartment, i.e., they showed less memory of the conditioned response, compared with vehicle or degenerated ODNs injected controls (Fig. 1*A*). This effect was dose dependent in that 1 nmol per i.c.v. injection inhibited passive avoidance memory by 34% (ANOVA, $P < 0.01$) whereas 2 nmol inhibited the memory by 57% (ANOVA, $P < 0.01$). When the last antisense injection was performed 7 days before testing, the antisense had no effect on mouse performance in the passive avoidance test, indicating that the inhibitory effect is reversible (Fig. 1*A*). The mice injected with antisense ODN had no other apparent behavioral deficit as measured by spontaneous motility and inspection activity on the hole board test (Fig. 1*B*) as well as performance on the rota-rod test (Fig. 1*C*). Moreover, short-term procedural memory

of the mice was not affected by the i.c.v. injection of antisense ODN as indicated by repeated rota-rod testing (Fig. 1*C*). All three mouse groups (i.e., antisense, degenerated ODNs, and vehicle-treated) showed similar ability to progressively learn the rota-rod exercise (ANOVA with repeated measurements shows a significant linear effect of session $F_{3.46}$, $P < 0.0001$, and no effect of treatment and no interaction between groups).

We then examined the effect of antisense inhibition of the Kv1.1 gene expression on a second rodent species in a memory test that requires different neuronal circuitry than passive avoidance, i.e., rat spatial memory assesed in the Morris water maze, that is a hippocampal-dependent form of learning (21, 22). Antisense ODN (either 1.5 or 2.5 nmol per injection) repeatedly injected via a cannula into the rat lateral ventricle caused a clear loss of spatial memory as probed by quadrant analysis testing one day after all training was completed. Antisense injected rats spent no greater proportion of their time in the platform quadrant than chance levels, i.e., 25% of the time. Injection of 0.5 nmol of antisense ODN did not affect the rats' performance in quadrant analysis, $n = 10$ for each group (data not shown). Trained rats injected with complete sequence-degenerated ODNs, however, showed the same preference as did the vehicle injected controls (Fig. 2*B*) spending more than $43 \pm 5\%$ of the time in the quadrant in which the platform was located during training (ANOVA to quadrant preference in control rats $P < 0.01$). Acquisition was not affected by the antisense ODN as demonstrated by the learning latency to swim to the platform (ANOVA with repeated measures show a significant linear effect of session $F_{8.128}$, $P < 0.002$, and no effect on treatment or interaction between groups) (Fig. 2*C*). These rat spatial maze learning results are similar to those obtained when mice were tested with a rota-rod (Fig. 1*C*). Both sets of results indicate that memory (i.e., retention), but not acquisition is affected by the antisense treatment. Other behaviors as monitored by exploratory behavior in an open field (Fig. 2*E*), or swimming patterns [distance (Fig. 2*D*) and pool coverage when the rats are first introduced to the water pool, were not affected by the antisense treatment (data not shown)].

To assess the physiological impact of antisense treatment, rat hippocampal slices were analyzed with field and intracellular recordings. Intracellular recordings from dentate granular cells, one of the primary loci for those K^+ channels targeted by the antisense (6, 7), showed a consistent broadening of the spike waveform in antisense-injected compared with rats injected with degenerated ODNs or noncannulated rats (Fig. 3*A*). This broadening was due to a prolonged repolarization phase of the spike (Student's *t* test, $P < 0.001$) (Fig. 3*B*) and the absence of any measurable after-hyperpolarization (Student's *t* test, $P < 0.001$) (Fig. 3*C*). The clear and consistent difference in spike waveform for antisense treated cells was most likely due to blockade of late rectifying K^+ channels distributed in the dendritic branches and is consistent with the spike waveform described in the *Drosophila shaker* mutant (25). Inhibition of soma voltage-dependent K^+ channels might have been expected to increase input resistance, which was not observed (resting input resistance in noninjected rats, $43.6 \pm 2.69 M\Omega$, $n = 7$; antisense, $39.4 \pm 1.61 M\Omega$, $n = 9$; degenerated ODNs $40.7 \pm 3.1 M\Omega$, $n = 7$). Other electrophysiological measurements that would reflect cell integrity such as resting potential (noninjected, -62.2 ± 0.46 mV, $n = 7$; antisense, -63.6 ± 0.92 mV, $n = 9$; degenerated ODNs, -63.5 ± 0.86 mV, $n = 7$) or peak spike amplitude (noninjected, 14 ± 0.63 mV, $n = 7$; antisense, 16.3 ± 1.43 mV, $n = 9$; degenerated ODNs, 18.4 ± 0.97 mV, $n = 7$) were not significantly different for antisense and untreated control animals.

Since long-term changes of synaptic function (e.g., LTP, long-term depression, and long-term transformation, refs. 26–28) have been proposed as models for memory we further assessed the electrophysiological impact of Kv1.1 inhibition on LTP. Extracellular field recordings were made from the CA1 and dentate gyrus regions of the hippocampus. Antisense inhibition of Kv1.1 did not affect CA1 or dentate gyrus LTP.

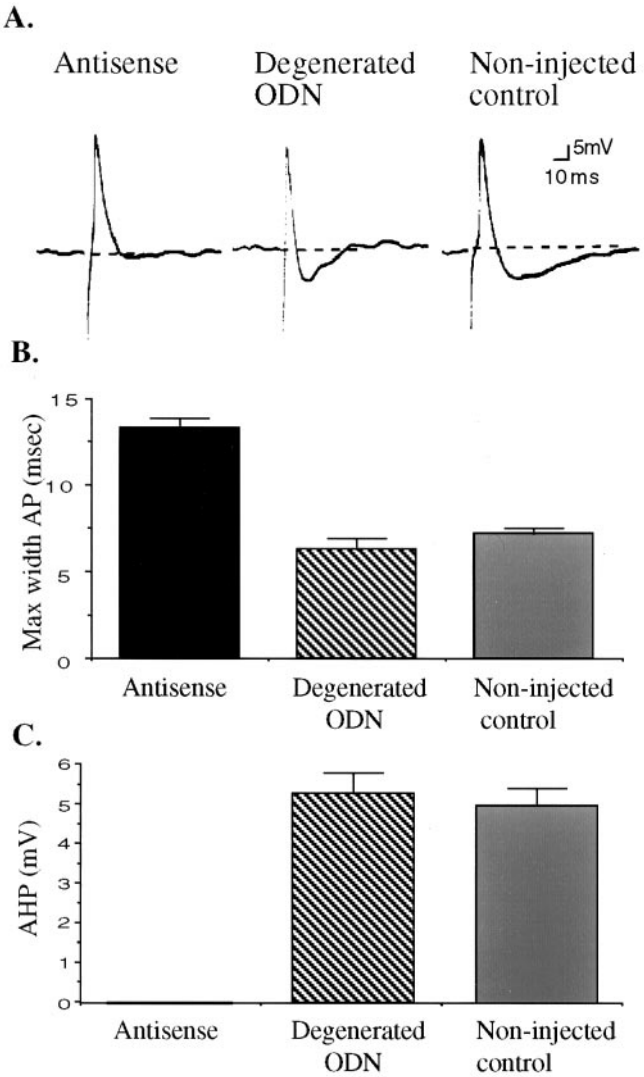

FIG. 3. Intracellular recordings from dentate gyrus granule cells. (*A*) Intracellular recordings from dentate gyrus granule cells. (*B*) Maximum width of the action potential. (*C*) Amplitude of the after hyperpolarization (noninjected, $n = 7$; antisense, $n = 9$; degenerated ODN, $n = 7$).

LTP was comparable in both aforementioned hippocampal regions to LTP obtained in slices from rats injected with the degenerated ODNs (Fig. 4). In contrast to spike waveform broadening, field population spike profiles were characteristic of normal dentate gyrus granular cell layer responses to stimulation of the medial perforant pathway (Fig. 4*A*). The current required to elicit baseline dentate excitatory postsynaptic potentials for both degenerated and antisense ODN's was comparable. The CA1 LTP, maximum excitatory postsynaptic potential amplitude, and paired pulse facilitation (Fig. 4*B*) all showed no antisense effects.

All of these results taken together suggest that the specific antisense ODN designed to bind mRNA for the potassium Kv1.1 subunit does reach and obstruct its expected intracellular mRNA target, thereby altering late voltage-dependent K^+ channels and causing memory impairment in two species. In the hippocampus, Kv1.1 channels are restricted not only to particular cells (i.e., CA3 pyramidal cells, dentate gyrus granule cells), but to local neuronal sites, namely post-synaptic dendrites. Reversible, specific antisense "knockdown" was achieved in nondeveloping mature brain networks, thus avoiding irreversible alterations of developing brain structures as effected in varying degrees by "knockout" strategies. Thus this

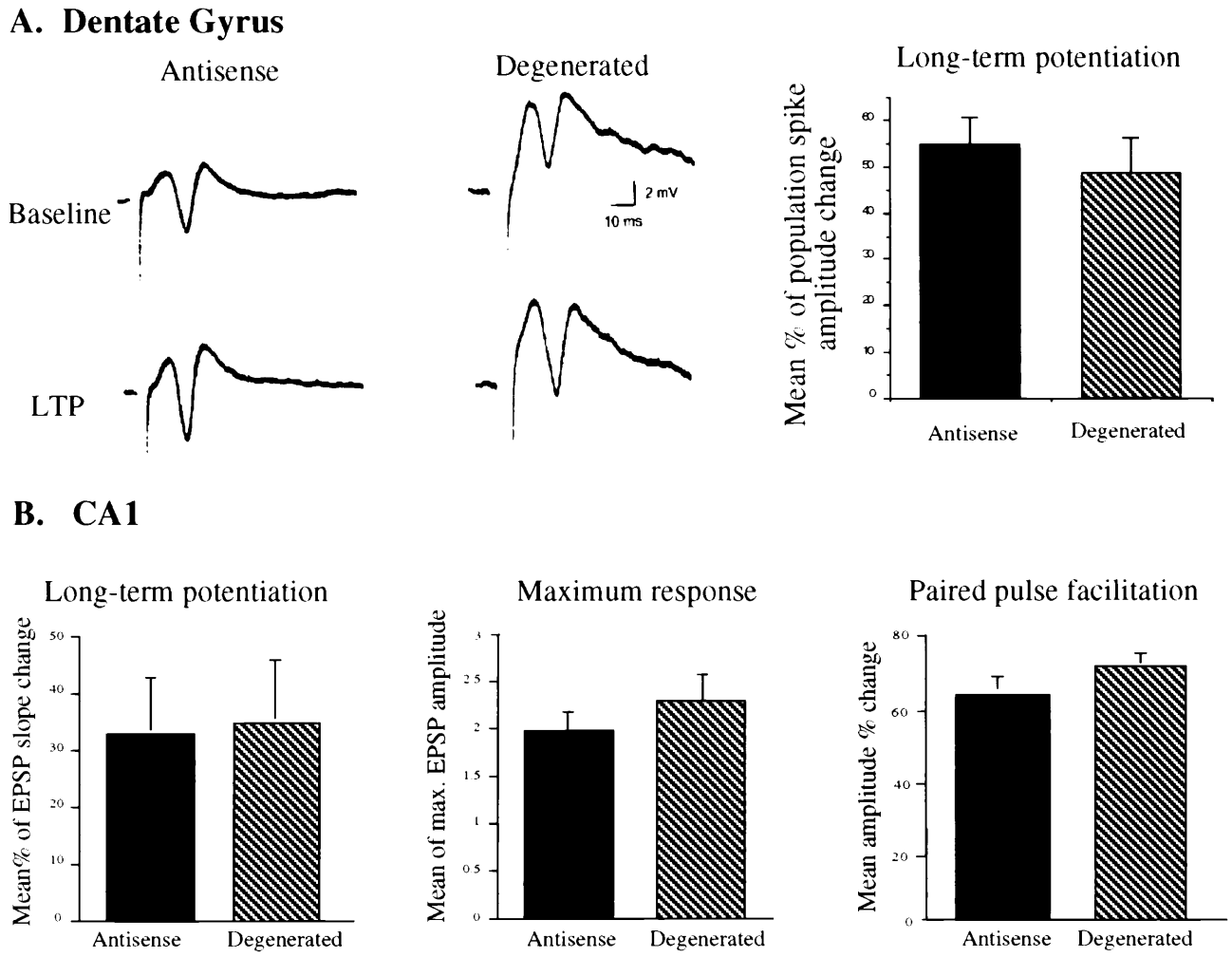

FIG. 4. Field recording to induce LTP in the dentate gyrus and CA1. Population spikes were recorded in the dentate gyrus granule cell layer in response to single pulse stimulation of the medial perforant pathway (*A*), and EPSPs in the CA1 in response to a single train stimulation of the Schaeffer Collateral pathway (*B*). LTP is shown as a percent of the baseline response 20 min (CA1) or 60 min (dentate gyrus) after tetanization. Paired-pulse facilitation was performed with a double pulse 20 ms apart (CA1: denatured ODN, $n = 11$ and antisense, $n = 15$; dentate gyrus: $n = 8$ in each group).

reversible "knockdown" of Kv1.1 may account for the memory loss in the two species. Within the limits of the present studies, these channels are apparently essential for passive avoidance and spatial memory, but not for LTP, demonstrating a clear dissociation between the two phenomena.

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