

Deficits in memory and hippocampal long-term potentiation in mice with reduced calbindin D_{28K} expression

(calcium/synaptic plasticity/antisense transgenic mouse)

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ABSTRACT The influx of calcium into the postsynaptic neuron is likely to be an important event in memory formation. Among the mechanisms that nerve cells may use to alter the time course or size of a spike of intracellular calcium are cytosolic calcium binding or "buffering" proteins. To consider the role in memory formation of one of these proteins, calbindin D_{28K}, which is abundant in many neurons, including the CA1 pyramidal cells of the hippocampus, transgenic mice deficient in calbindin D_{28K} have been created. These mice show selective impairments in spatial learning paradigms and fail to maintain long-term potentiation. These results suggest a role for calbindin D_{28K} protein in temporally extending a neuronal calcium signal, allowing the activation of calcium-dependent intracellular signaling pathways underlying memory function.

Intracellular calcium (Ca²⁺) ions play a key role in neuronal events that are likely to involve the establishment of memory; these include controlling transmitter release, influencing intracellular signaling, altering membrane permeability, and activating genes and enzymes (1–3). Among the enzymes responding to intracellular free Ca²⁺ is calmodulin kinase (CaM kinase II), which transduces changes in Ca²⁺ into changes in phosphorylation state and activity of target proteins. Gene targeting studies have demonstrated that CaM kinase II is important for spatial memory (4). CaM is however only one among many calcium binding proteins in the central nervous system and among the proteins that can act to modify the shape and time course of an intracellular free Ca²⁺ spike is the calcium binding protein calbindin D_{28K} (5–7). Calbindin D_{28K} is localized in a number of forebrain areas implicated in learning and memory (8–10), and calbindin D_{28K} levels are reduced in the hippocampus and cerebral cortex of patients with Alzheimer disease (11). In this study, an antisense transgenic approach was used to reduce the expression of calbindin D_{28K} mRNA and protein, and the role of this protein in learning and memory was evaluated. Antisense transgenic mice were examined for deficits in spatial learning using water and radial eight-arm mazes (12, 13) and for their ability to maintain long-term potentiation (LTP) (14–16). This study shows that the antisense calbindin D_{28K} transgene substantially reduced the expression of calbindin D_{28K} mRNA and protein in the forebrain of the transgenic mice and that these mice have deficits in spatial learning paradigms and failed to maintain LTP.

MATERIALS AND METHODS

Creation of the Antisense Transgenic Mice. The immunological screening of a mouse cDNA library (Clontech) in λgt11

expression vector led to the identification of 24 positive clones. The insert of clone mCB14, representing calbindin D_{28K} from nt 419 to nt 1459, as assessed by sequence analysis (17), was subcloned in antisense orientation in the *AccI* site of pNF-LPr. This plasmid contains the human neurofilament L promoter and 5' flanking sequences controlling neuronal-specific transcription (18–20). Simian virus 40 splicing and polyadenylation signals were added 3' of the calbindin insert to generate the final construct pNF-L/CBas2 (see Fig. 1A). The *NotI*–*KpnI* fragment containing the NF-L promoter, antisense calbindin cDNA, and the splicing and polyadenylation signals was injected into the pronuclei of mouse fertilized oocytes. Southern blot hybridization to tail DNA with a ³²P-labeled *Bam*HI–*Bam*HI fragment of pNF-L/CBas2, revealed the presence of a 2.4-kb fragment that is unique to animals carrying the transgene. Copy number estimates were done by comparing the signals obtained from 10 μg of tail DNA to the signals obtained from the same amount of chicken DNA to which pNF-L/CBas2 was added to give 1, 5, 10, 50, or 100 copies per genome (see Fig. 1B).

In situ Hybridization and Immunocytochemistry. Synthetic antisense oligonucleotide probes, complementary to bp 207–237, 672–703, and 836–864 of the mouse calbindin D_{28K} sequence (17), were 3'-tailed with ³⁵S-labeled dATP using terminal deoxynucleotidyltransferase (Pharmacia), to a specific activity of >1 × 10⁸ dpm/μg and purified on Sephadex G-50 (fine) columns. Fresh frozen brain sections of control, heterozygous, or homozygous mice were fixed with 4% neutral-buffered paraformaldehyde, rinsed with phosphate-buffered saline (PBS), dehydrated, and then hybridized with labeled oligonucleotides. Sections were washed to a stringency of 1× standard saline citrate (SSC) at 55°C, quickly dehydrated, air-dried, and exposed to Hyperfilm β-max for 3 weeks. Levels of native sense mRNA were determined by computer-assisted image analysis (Magiscan; Joyce–Loebl). Integrated optical densities were measured and means were obtained for each class of animal.

Immunocytochemistry to visualize calbindin D_{28K}-like immunoreactivity was carried out on control and homozygous transgenic mice using an antibody raised against recombinant rat calbindin D_{28K} (7) using standard methods (11) and the Vectastain ABC kit (Vector Laboratories).

Morris Water Maze. Spatial learning was assessed in a Morris water maze (12) modified for use in mice. Escape from the tank was provided by a circular 6-cm diameter Perspex

Abbreviations: LTP, long-term potentiation; CaM, calmodulin; fEPSP, field excitatory postsynaptic potential.

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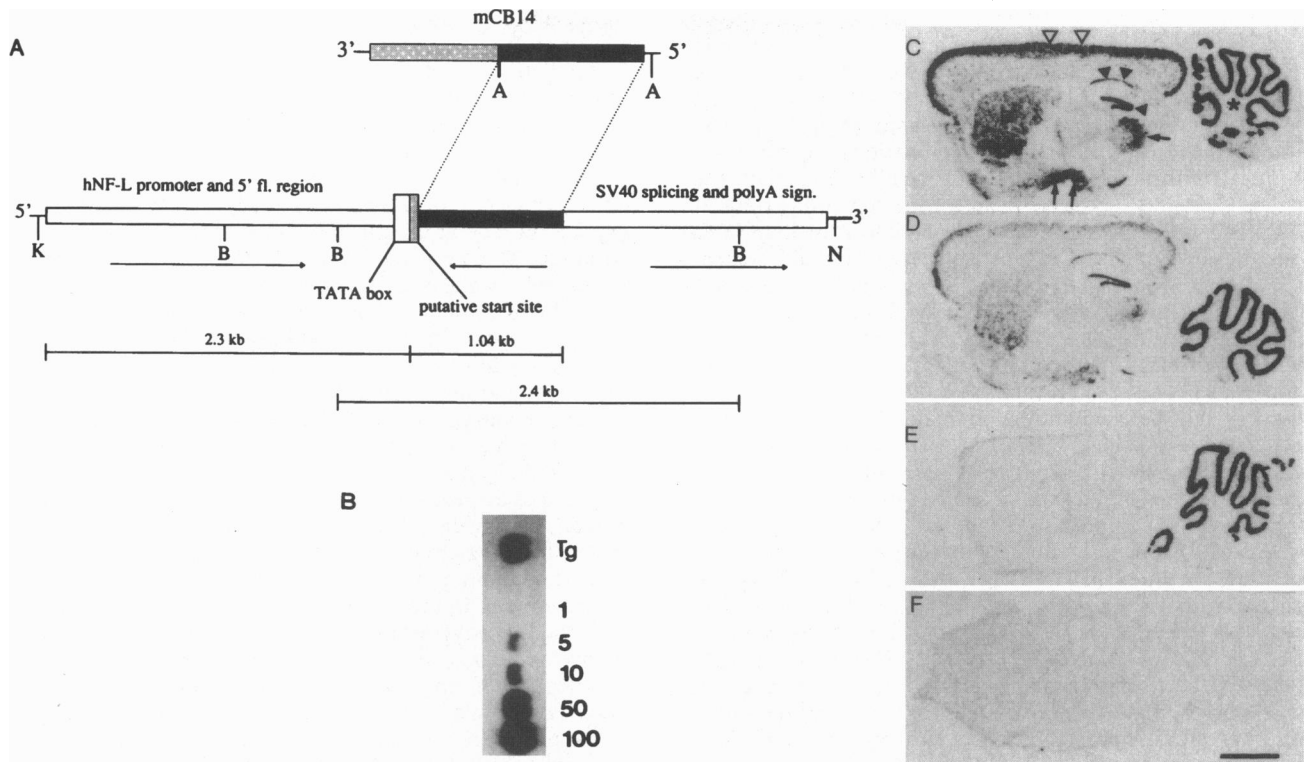


FIG. 1. The construct used to generate calbindin D_{28K} antisense transgenic mice (A). The construct contains a 1.7-kb mouse calbindin mouse calbindin D_{28K} cDNA in reverse orientation with simian virus 40 splicing and polyadenylation signals. Southern blot analysis of tail DNA showed between 35 and 50 copies of the construct per genome (B). *In situ* hybridization (C–E) showed a regional reduction of calbindin D_{28K} mRNA expression in heterozygotes (D) and homozygotes (E) relative to a control mouse (C). The specificity of calbindin D_{28K} mRNA signal was confirmed using an excess unlabeled probe (F).

platform, submerged by approximately 0.5 cm. For each animal, this platform was located centrally in one of the four quadrants of the pool (northeast, northwest, southwest, or southeast) and remained in that location throughout that animal's training. Animals were therefore only able to use distal cues in the room surrounding the tank to determine the location of the escape platform. On each trial the animal was placed into the maze near one of four possible points—north, south, east, or west—the location on each trial being determined randomly. Animals were allowed up to 60 s to locate the escape platform, and their escape latency was recorded. Animals failing to locate the platform within this limit were ascribed an escape latency of 60 s and removed from the tank to the platform. All animals were allowed to remain on the platform for 20 s. Animals received two trials a day, and training continued for 8 days.

Radial Eight-Arm Maze. Spatial working memory was also assessed in a radial eight-arm maze adapted for mice (13). Access from the arms to the central platform was provided by clear Perspex guillotine doors that could be raised and lowered remotely. The central platform itself was surrounded by a 20-cm high clear Perspex enclosure of the same diameter. Reinforcement was with 0.1 ml of 20% sucrose solution placed in a food well at the end of each arm. Animals were maintained on a 22.5-hr schedule of water deprivation throughout the period of testing in the radial-arm maze; food was available ad libitum. Animal weights did not differ significantly between groups: homozygotes, 29.2 g; controls, 32.0 g. At the start of each trial all eight arms were baited, and all guillotine doors were closed. The animal was then placed in the central enclosure and left for 20 s before all eight doors were opened simultaneously. After the animal's entry into an arm (defined strictly as placing all four feet onto the arm), all doors were reclosed except for that of the chosen arm. This door remained

open until the animal returned to the central enclosure, at which point it was closed. The animal was then held in the central enclosure for 20 s (to prevent adoption of response strategies such as always turning left) until all eight arms were reopened and the animal made its second choice. For a single trial, this sequence was repeated until the animal had visited all 8 baited arms or until the animal had made 24 choices. A correct response was recorded after entry into a previously unvisited arm; an error was recorded after entry into an arm that had already been visited. Chance performance in this task leads on average to 5.3 correct responses in the first 8 choices. Prior to training, all animals were given 6 min access to the unbaited maze with all doors open to habituate them to the environment; the number of arms entered during this phase did not differ between homozygous mice and controls, again providing evidence against any form of nonspecific motor impairment. Animals were then given one training trial a day (thus minimizing interference in memory from previous trials), and training continued for 10 days.

Black/White Discrimination. Acquisition of the black/white discrimination was also performed in the radial-arm maze, but with some modifications (13). A Y-maze was formed by using just two adjacent arms of the radial maze and the central enclosure. One of these arms was entirely covered with black cardboard, and the other was covered with thick translucent tracing paper. While these coverings totally obscured surrounding spatial cues in the room, they provided a completely dark arm and a contrasting light arm. Each animal was placed in the central enclosure and remained there for 20 s until the doors to the light and dark arms were raised simultaneously. After the animal's entry into one of the arms, the door to the other arm was closed, whereas the door of the chosen arm remained open until the animal returned to the central enclosure or until 60 s had elapsed. The animal was

then removed from the apparatus. One-half of each group of animals, homozygotes and controls, received reinforcement in the light arm; the other half received reinforcement in the dark arm. Reinforcement again was 0.1 ml of a 20% sucrose solution placed in a food well at the end of the appropriate arm. The position of the arms in the apparatus was varied randomly from trial to trial with the constraint that for half of the trials, the reinforced arm was on the left, and for the remaining trials, it was on the right. This removed any possible contribution to solving the discrimination from spatial or positional information. Animals were again on a 22.5-hr schedule of water deprivation throughout training with food available ad libitum. Animals received five trials a day, continued for 4 days.

Electrophysiology. Three-month-old wild-type ($n = 10$) and homozygous ($n = 12$) mice were used alternately. Each day, a mouse was anesthetized with halothane and decapitated. The hippocampus was quickly removed and placed in ice-cold medium. Slices (300–400 μm thick) were cut and placed in a holding chamber for at least 1 hr. A single slice was then transferred to the recording chamber. The slice was held between two nylon nets, submerged beneath a continuously superfusing medium that had been warmed to 29–31°C and pre-gassed with 95% O_2 /5% CO_2 . The composition of the medium was 119 mM NaCl, 3.5 mM KCl, 1.3 mM MgSO_4 , 2.5 mM CaCl_2 , 26.2 mM NaHCO_3 , 1.0 mM NaH_2PO_4 , and 11 mM glucose. Extracellular recordings were obtained from apical dendritic layers of CA1 area using glass micropipettes filled with 2 M NaCl and having resistances of 2–6 M Ω . Field excitatory postsynaptic potentials (fEPSPs) were evoked by electrical stimulation of CA1 afferent fibers (Schaffer collaterals and commissural fibers) located in the stratum radiatum. Test stimuli were adjusted to get a fEPSP with an amplitude minimum of 0.5 mV and were applied every 15 s. Averaged fEPSPs (eight averaged traces) were stored on a digital oscilloscope every 3 min, and the initial slope of the field was calculated. Control traces were recorded for at least 15 min before conditioning tetanic stimulation. The tetanic stimulation consisted of two high-frequency trains (two stimulus trains of 100 pulses at 100 Hz separated by 20-s interval, at twice test intensity). Testing with single pulses was then resumed for at least 70 min to determine the level of stable LTP. In further experiments in both populations of mice, a second stimulating electrode was positioned in the stratum radiatum on the side opposite to the recording electrode to activate independent inputs. No high-frequency stimulation was applied at this site. The size of the fEPSPs in response to single pulses delivered to this electrode was unchanged throughout the whole experiment, confirming the input specificity of the LTP in our experimental conditions.

RESULTS

Examinations of the founder mouse Tg28 used to generate the calbindin D_{28K} -deficient mouse line used herein showed that the founder contained between 35 and 50 copies of the antisense DNA in its genome (Fig. 1B). Both heterozygous and homozygous mice derived from the founder showed a marked reduction of calbindin D_{28K} mRNA in the forebrain (Fig. 1C–E). Forebrain and midbrain regions of homozygous mice ($n = 4$) showed no expression of calbindin D_{28K} mRNA above tissue background and expression as determined by image analysis was greatly reduced in the forebrain and midbrain of heterozygous mice ($n = 3$) in comparison to control animals ($n = 4$). [Control versus heterozygote versus homozygote: $F(2, 8) = 126.8$; $P < 0.0001$; Fig. 1D.] Interestingly, the level of calbindin D_{28K} mRNA was much less affected in the Purkinje cells of the cerebellum where the sense mRNA signal is strongest (Fig. 1C–E), although it was reduced in both homozygous and heterozygous mice relative to controls (10.9% and 9.9%, respectively; $F(2, 8) = 5.8$; $P = 0.027$). All

the calbindin *in situ* signal in the mice brains was specific as shown in the displacement control (Fig. 1F).

Paralleling the depletion of calbindin D_{28K} mRNA, there was a considerable reduction in calbindin D_{28K} protein, as revealed by immunohistochemistry (data not shown). The loss of calbindin D_{28K} protein matched well with the loss of calbindin D_{28K} mRNA signal, indicating that the antisense construct was, as expected, reducing both mRNA and protein expression. The areas affected included the hippocampus and striatum. The only region where the protein content was preserved was the cerebellum where the Purkinje cells stained normally. Routine histology showed that there were no obvious gross histological abnormalities or cell loss in any of the forebrain/midbrain structures including the cerebral cortex, thalamus, and hippocampus.

Mice homozygous for the antisense construct were examined for behavioral performance. The mice had no obvious motor deficits and were examined in a water maze test (Fig. 2A), a radial eight-arm maze test (Fig. 2B), and a simple Y-maze black/white discrimination test (Fig. 2C) for deficits in spatial learning/processing (water maze and radial eight-arm maze test) or for a general impairment in learning ability (Y-maze test). In the water maze test of spatial learning (Fig. 2A), the homozygous mice were impaired in their acquisition of this task relative to controls. A mixed ANOVA revealed that the degree of impairment in the escape latencies of the homozygous mice varied across trials [$F(7, 140) = 3.35$; $P < 0.005$] producing a significant retardation on trial blocks 4 and 5 [analysis of simple effects F values of (1, 138) = 19.33 and 7.13, respectively; $P < 0.01$]. The initial and final levels of escape latency in the two groups were equivalent indicating that the homozygous mice were not impaired in their ability to perform the task due to nonspecific motor decrements (Fig. 2A) but showed a selective deficit in the acquisition of spatial learning. This was confirmed in a transfer or probe test (12) in which the mice were allowed to swim freely in the maze for 60 s with the platform removed. This measures the tendency of the mice to persist in searching in the area of the pool where the platform had been located during training and revealed that good spatial learning appeared to have been achieved by both transgenic and control mice that spent, respectively, 62% and 68% of the 60-s trial swimming in the quadrant of the pool where the platform had been located. This pattern of results was also demonstrated in the radial eight-arm maze (Fig. 2B) in which homozygous mice again demonstrated a retardation in the acquisition of a spatial working memory task relative to controls (open squares, $n = 8$). A mixed ANOVA revealed a significant retardation in homozygous mice [$F(1, 12) = 4.88$; $P < 0.05$] relative to controls, a difference that proved to be significant only for trial blocks 3 and 4 [$F(1, 44) = 7.04$ and 4.91, respectively; $P < 0.05$]. Again initial and final levels of performance did not differ between homozygous mutant mice and controls, indicating that the deficit was unlikely to be due to nonspecific deficits but represents a selective impairment in the acquisition of the spatial working memory task. However, in a simple Y-maze black/white discrimination (Fig. 2C), there was no evidence of any deficit in homozygous mice, with both groups acquiring the discrimination by the fourth block of five trials. A mixed ANOVA revealed a significant effect of training block [$F(3, 57) = 8.91$; $P < 0.001$] with performance on block 4 significantly greater than on blocks 1 to 3 (post hoc Newman–Keuls pair-wise comparisons, $P < 0.05$). Hence the retardations observed in the acquisition of spatial learning and spatial working memory tests by homozygous mice are not the result of a general learning deficit but seem to be the result of a selective impairment in spatial processing. Furthermore, it cannot be argued that this pattern of results occurs due to differential task difficulty, as homozygotes demonstrated normal levels of performance on the black/white discrimination for which controls required the highest number of trials in

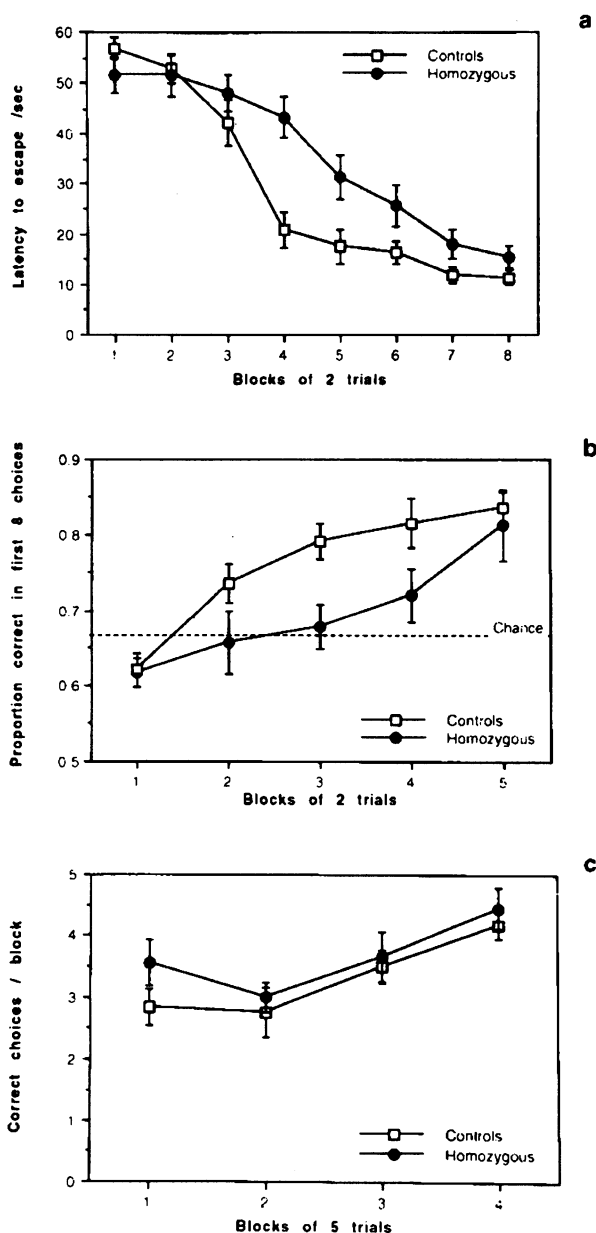


FIG. 2. (A) The rate of acquisition of a water maze test of spatial learning is impaired in homozygous mice (●), $n = 10$) relative to control mice (○, $n = 12$). Each point represents the mean \pm SEM of escape latency (see text for details). Note that the mice do not differ in initial or final escape latency indicating that homozygous mice do not show nonspecific sensory impairment. (B) Retardation of performance in a radial eight-arm maze test. Homozygous mice (●, $n = 6$) were impaired relative to controls (□, $n = 8$) in ability to learn this task. In contrast both groups were similar in performance in a simple Y-maze black/white discrimination test (C). In this test homozygous mice (●, $n = 10$) acquired the task as well as controls (□, $n = 12$).

acquisition but showed deficits in the water maze and radial-arm maze tasks, which controls acquired more rapidly.

Since LTP has received much attention as a leading candidate mechanism for memory storage (although it has been questioned whether there is any significant role for LTP in real learning and memory), we also examined LTP in the CA1 region of hippocampus (15, 16) (Fig. 3). No difference in the basal properties of the fEPSPs was observed between control and homozygote mice. The amplitude and the slope of basal fEPSPs were similar in the two groups, 0.82 ± 0.04 mV ($n = 36$) and 0.64 ± 0.05 V·s⁻¹ ($n = 33$) for the control wild type versus 0.81 ± 0.04 mV ($n = 36$) and 0.64 ± 0.05 V·s⁻¹ ($n = 33$)

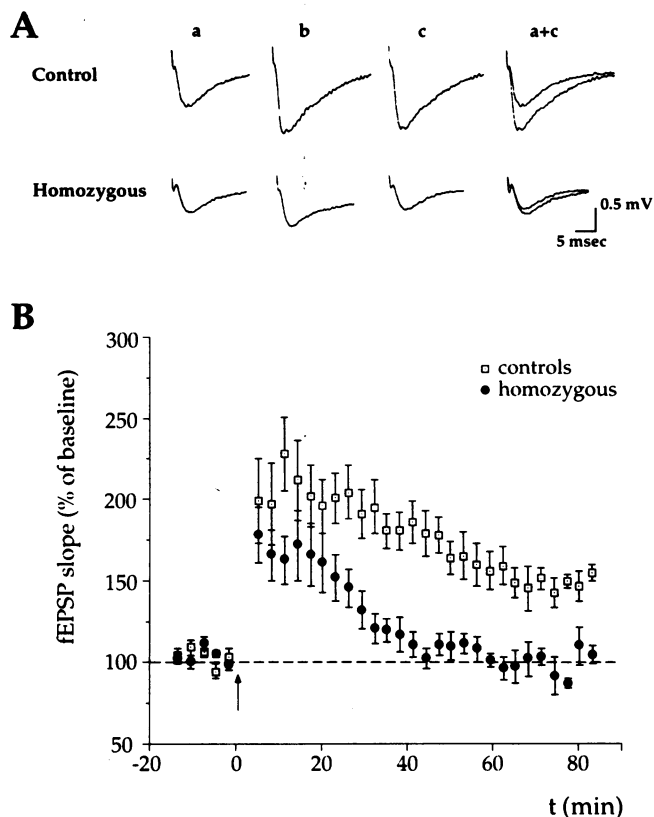


FIG. 3. CA1 hippocampal synaptic plasticity in wild-type control and homozygous transgenic mice. (A) Examples of averaged fEPSPs recorded in response to constant test stimuli ($1.3\text{-}\mu\text{A}$ intensity; 0.02-ms duration; eight traces averaged) in stratum radiatum of CA1 in a control and a homozygous mouse. Traces illustrate the fEPSPs recorded before (trace a) or after (trace b, 20 min; trace c, 60 min) high-frequency stimulation of afferent fibers. Notice an increase in the size of the fEPSPs shortly after tetanic stimulation in both groups of mice (trace b) but the lack of persistent potentiation in homozygous mice (trace c) as illustrated on the superimposed traces (traces a and c). (B) Synaptic strength expressed as the percent change of the fEPSP slope as a function of time before and after tetanic stimulation (arrow). Slopes of each fEPSPs are calculated as an average of the percentage change (mean \pm SEM) from average baseline responses obtained during at least 15 min before high-frequency stimulation, as a function of time. In each experiment, the slope of eight averaged fEPSPs is measured every 3 min. Finally, each point of the graph represents the average slope \pm SEM of 14 fEPSPs recorded in control and 12 fEPSPs recorded in homozygous mice. High-frequency stimulation is applied at the time indicated by the arrow. Note the occurrence of a LTP of the synaptic transmission in hippocampus from control mice and the presence of a short-term (but not a long-term) potentiation in the homozygous mice.

for the homozygous. The fEPSPs were obtained with comparable mean stimulation intensities in both groups (2.1 ± 0.12 μA and 2.0 ± 0.15 μA , respectively). Fiber volley amplitude 0.39 ± 0.05 mV ($n = 7$) for the wild type versus 0.41 ± 0.05 mV ($n = 15$) for the homozygous and the ratio of fEPSP slope to fiber volley amplitude (0.49 ± 0.06 for wild type versus 0.45 ± 0.05 for the homozygous) were comparable in both populations.

A robust LTP after high-frequency stimulation was observed in all wild-type mice slices studied. In contrast, a persistent potentiation of the fEPSP was observed in only 4 slices among 12 in the homozygous mice. In all other homozygous mice, a strong potentiation of the fEPSP developed after high-frequency potentiation but lasted for only 20–30 min (Fig. 3). For instance, the mean fEPSP slope, 20 min after tetanic stimulation, was $156.7 \pm 18.2\%$ of the average slope

before tetanic stimulation in homozygous versus $191.8 \pm 16.8\%$ in control mice. The difference between the two values was not statistically significant ($P = 0.17$, *t* test). In contrast, mean values after 60 min were $92.3 \pm 7.3\%$ and $153.3 \pm 11.3\%$ of basal for homozygous and wild-type mice. The difference between these values was highly significant ($P < 0.001$, *t* test).

DISCUSSION

In this study we have examined the effects of reducing calbindin D_{28K} expression in a novel antisense transgenic mouse. The antisense transgenic approach used here has been used successfully in the past. For example, Pepin *et al.* (21) reduced type II glucocorticoid receptor expression in the brains of transgenic mice. This general approach may have utility as it is much simpler than the normal homologous recombination approach in embryonic stem cells. In addition, as this method does not completely abolish expression, it may be less vulnerable to the possibilities of developmental compensation that sometimes complicates interpretation of null mutant knock-outs. Interestingly, this antisense transgene also shows regional selectivity, the depletion of mRNA and protein is marked in the majority of the central nervous system but is only slight (-10%) in the cerebellum. This observation may also provide a clue to the mechanisms by which this antisense transgene is working. It is usually assumed that the antisense mRNA will interact with the sense mRNA to form a duplex RNA that is degraded [and indeed we failed with *in situ* hybridization with sense oligonucleotides to detect significant expression of antisense mRNA, although reverse transcription-coupled PCR (RT-PCR) does show the presence of antisense RNA] and we suggest that this mechanism is probably operating herein, as the depletion of sense mRNA is minimal in the cerebellum, which has a very high level of sense mRNA. In contrast, the level of sense mRNA is at least 5–6 times lower in the rest of the central nervous system (compare cerebral cortex signal with that in cerebellum, Fig. 1C) and in the cerebellum the level of expression of the antisense RNA from the NFL promoter is presumably insufficient to saturate the sense mRNA in the Purkinje cells. The observation that heterozygotes are intermediate in level of depletion (Fig. 1D) of calbindin D_{28K} mRNA would suggest that it is the level of expression of the antisense mRNA that determines the extent of depletion of calbindin D_{28K} mRNA and, hence, calbindin D_{28K} protein. The heterozygotes as well as being intermediate between controls and homozygotes in calbindin D_{28K} mRNA expression are also midway between homozygotes and controls in impairment on spatial learning tests. This dose–response relatedness indicates that the spatial learning impairments are likely to be due to the transgene. This is in contrast to the CaM kinase knock-outs (4) that do not show a simple relationship between gene dose and behavioral deficits and where interpretation of the behavioral deficits in these knockouts is complicated by their altered fear and aggression and hyperexcitability (22, 23). Additional control experiments have also shown that the expression of the related mRNA for calretinin (which is approximately 60% homologous at the sequence level with calbindin D_{28K}) is not affected in these mice, indicating the specificity of this approach for calbindin D_{28K} (unpublished data).

The behavioral tests used here show that the homozygous mice are deficient in spatial processing but not impaired generally in learning ability (simple Y-maze test) or motor performance. These data suggest that calbindin D_{28K} is involved in the spatial learning tasks that are believed to involve the hippocampus (12). This suggestion is supported by the observation that homozygotes failed to maintain LTP. How then may calbindin D_{28K} be acting to influence synaptic plasticity and learning? We report a dramatic decrease in LTP in the transgenic mice while the short-lasting enhancement of the synaptic response (often called short-term potentiation)

persists. How can we explain the lack of LTP in the transgenic mice? This could not be due to a loss of synaptic connections or excitability since comparable stimulus intensities elicit comparable responses in terms of fEPSP slope and amplitude in both groups of mice. A first hypothesis is based on the fact that stabilization of LTP seems to require some critical threshold level of calcium (24). For instance, it has been shown that a short period of calcium entry does not result in LTP but only in short-term potentiation expression (25). On the other hand, a markedly enhanced Ca^{2+} -dependent inactivation of Ca^{2+} currents has been reported in neurons depleted of their calbindin D_{28K} content (26). Such a stronger inactivation of Ca^{2+} currents might occur in the transgenic mice resulting in only a weak rise in intracellular calcium that would not reach the threshold level required for the maintenance of LTP.

Another hypothesis would be that calbindin D_{28K} , besides its proposed role as a calcium buffer, may play an important role in spatially transferring and/or releasing Ca^{2+} within the neuronal cytoplasm, thus prolonging an intracellular calcium signal (our data on calcium handling by calbindin D_{28K} transfected GH₃ cells support this suggestion) (7). Lack of this “slow release” calcium buffer would prevent, for example, activation of Ca^{2+} -activated kinases such as protein kinase C or Ca^{2+} -CaM kinase II, proteins required for the maintenance of LTP (27, 28). Interestingly, our results suggest that calbindin D_{28K} may act through activation of protein kinase C rather than CaM kinase II since in transgenic mice lacking CaM kinase II, the LTP as well as the short-term potentiation is blocked (4). It is also interesting to note that reduction of calbindin D_{28K} expression is observed in normal aging and in Alzheimer disease, so that a reduction in expression of this protein may contribute to the cognitive decline in these groups (10, 11).

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