Effects of thyroid hormone replacement on associative learning and hippocampal synaptic plasticity in adult hypothyroid rats

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Abstract

Activity-dependent changes taking place at the hippocampal perforant pathway-dentate gyrus synapse during classical eyeblink conditioning were recorded in adult thyroidectomized (hypothyroid) and control (euthyroid) rats, and in animals treated with thyroid hormones 20 days after thyroidectomy (recovery rats). The aim was to determine the contribution of thyroid hormones and the consequences of adult-onset hypothyroidism to both associative learning and the physiological potentiation of hippocampal synapses during the actual learning process in alert behaving animals. Control and recovery rats presented similar learning curves, while hypothyroid animals presented lower values. A single pulse presented to the perforant pathway during the conditioned-unconditioned inter-stimulus interval evoked a monosynaptic field EPSP in dentate granule cells (whose slope was linearly related to the rate of acquisition in the control group), but not in hypothyroid and recovery animals. Input-output relationships and long-term potentiation evoked by train stimulation of the perforant pathway were significantly depressed in hypothyroid animals. Thyroid hormone treatment failed to normalize these two neurophysiological abnormalities observed in hypothyroid animals. In contrast, paired-pulse facilitation was not affected by thyroidectomy. Results indicate that thyroid hormone treatment after a short period of adult hypothyroidism helps recover some hippocampally dependent functions, such as classical conditioning, but not other hippocampal properties, such as the synaptic plasticity evoked during associative learning and during experimentally induced long-term potentiation. Present results have important clinical implications for the handling of patients with adult-onset thyroid diseases.

Introduction

Both clinical and experimental reports have described impairment of cognition, learning and memory in adult hypothyroid humans and animals. Adult hypothyroidism also induces a depression-like disorder, as well as slowing of thoughts and movements (Gerges & Alkadhi, 2004; Montero-Pedrazuela *et al.*, 2006; Roberts & Ladenson, 2004). Besides, it is known that adult-onset hypothyroidism prevents the generation of experimentally evoked early and long-term potentiation (LTP) in the hippocampal CA1 region of the rat, in association with changes in the expression of NR1 glutamatergic receptor subunits (Gerges & Alkadhi, 2004; Gerges *et al*., 2001; Lee *et al.,* 2003). In addition, adult-onset hypothyroidism induces a reduction of signaling molecules essential for learning and memory, and LTP (Alzoubi *et al*., 2008; Gerges *et al*., 2005). However, there are few studies on the impact of adult-onset hypothyroidism on associative learning, and none on the activity-dependent synaptic changes underlying memory processes.

In the hippocampal circuit, deficiency of thyroid hormones [thyroxine (T4) and 3,5,3′-triiodothyronine (T3)] at adult stages impairs normal neurogenesis in the dentate gyrus, reducing proliferation and maturation of newly generated neuroblasts (Desouza *et al*., 2005; Montero-Pedrazuela *et al*., 2006). In this regard, it has been reported that the number of adult-generated neurons increases in the rat dentate gyrus during the acquisition of hippocampally dependent associative learning (Gould *et al*., 1999; Shors *et al*., 2001). Although thyroid hormone replacement seems to restore neurogenesis and some hippocampal functions, there are still some conflicting reports regarding the ability of thyroid hormone administration to restore all the molecular, electrophysiological, behavioral, and learning and memory impairments produced by clinical and experimental hypothyroidism (Alzoubi *et al*., 2005; Capet *et al*., 2000; Leentjens & Kappers, 1995; Montero-Pedrazuela *et al*., 2006; Roberts & Ladenson, 2004; Tagay *et al*., 2005).

Confirming an early study (Weisz *et al*., 1984), it has been demonstrated in both mice and rats that the acquisition of different types of learning modifies the strength of hippocampal synapses (Gruart *et al*., 2006; Whitlock *et al*., 2006). Here, we were interested in investigating the consequences of adult hypothyroidism (with and without thyroid hormone replacement) on associative learning, the physiological potentiation of hippocampal synapses during the actual learning process, LTP, and other tests of adaptive synaptic function in the dentate gyrus of the hippocampus of awake rats. Animals were classically conditioned using a hippocampally dependent (Moyer *et al*.,

1990) trace paradigm, presenting a tone as a conditioned stimulus (CS) and an electric shock to the supraorbital nerve as an unconditioned stimulus (US). Conditioned responses were determined from the electromyographic (EMG) activity of the ipsilateral orbicularis oculi muscle. We also recorded the field excitatory postsynaptic potential (fEPSP) evoked in the dentate granule cell layer by a single pulse presented to the perforant pathway within the CS-US interval. Thus, we could follow activity-dependent changes in synaptic strength across the successive conditioning sessions (Gruart *et al*., 2006). We determined whether changes in synaptic plasticity during associative learning in control (Ctl), hypothyroid (Hypo), and recovery (Rec) animals were accompanied by similar changes in LTP evoked in the dentate gyrus by high-frequency stimulation (HFS) of the perforant pathway.

Material and Methods

Animals

Experiments were carried out on adult male Wistar rats obtained from an official supplier (IIB Animal House, CSIC, Madrid, Spain). Before electrode implantation, animals were housed in separate cages (n = 3 per cage), but after surgery they were kept in individual cages. Rats were maintained on a 12 h light/dark cycle with constant ambient temperature (22 ± 1 ºC) and humidity (50 ± 7%). To follow the increase in body weight during the experiment and to characterize the thyroidal status of the animals, they were weighed twice a week. Electrophysiological and behavioral studies were carried out in accordance to the guidelines of the European Union Council (86/609/EU) and recent Spanish regulations (BOE 252/34367-91, 2005) for the use of laboratory animals in chronic experiments. Experiments were also approved by the Institution Committee for animal care and handling.

Thyroidectomy and hormonal treatment

Rats were divided in three experimental groups: euthyroid or controls (Ctl; n = 10), hypothyroid (Hypo; n = 12), and thyroid hormone-treated hypothyroid or recovered animals (Rec; n = 11). Only rats that completed all phases of the study were used in the analyses.

Thyroidectomy (Hypo and Rec groups) was carried out on postnatal days 61-63 (P61-P63) following procedures used in the laboratory by some of us for years (Berbel *et al*., 1993; Íñiguez *et al*., 1992; Montero-Pedrazuela *et al*., 2006). In short, animals

were anesthetized with a mixture of ketamine (100 mg/kg; Imalgene 1000, Merial, Lyon, France), medetomidine (0.1 mg/kg; Domtor, Orion Pharma, Espoo, Finland) and atropine sulfate (0.05 mg/kg; B. Braun Medical SA, Rubi, Spain) injected i.p. The neck hair was shaved off and the skin treated with an antiseptic. An incision was made in the skin over the trachea, and muscles were separated to allow a view of the thyroid gland, which was dissected out by pulling on the lobes from the bottom upwards. Special care was taken to leave the parathyroids intact and not to damage the recurrent nerve (Montero-Pedrazuela *et al.*, 2006). No drug was given to accelerate the disappearance of thyroid hormones remaining in tissues. For the Ctl group, a similar procedure was carried out without removing the thyroid gland.

Both Hypo and Rec animals were fed a low iodine diet starting the day of surgery to prevent any synthesis of thyroid hormones from possible remnant tissue (Martínez-Galán *et al*., 1997). This low iodine diet was maintained until the animals were sacrificed (P140-P143). Rec rats were treated with a combination of thyroid hormones (T4 and T3; T2376 and T6397 respectively, Sigma, Madrid, Spain) in the drinking water (0.18 μg T4/mL and 0.03 μg T3/mL). The solution was prepared daily in sterile 0.01% bovine serum albumin (A4503, Sigma) and kept in the dark to prevent hormone degradation. This treatment started 20 days after thyroidectomy (i.e. at P80-P83) and was maintained across the subsequent 60 days. With this treatment, the rats received approximately 2.4 μg of T4 and 0.4 μg of T3/100 g of body weight per day (see Escobar-Morreale *et al.*, 1996; and Montero-Pedrazuela *et al*., 2006 for details). This replacement treatment has previously been used successfully to recover thyroid hormone levels and some hippocampal structural and functional abnormalities observed in thyroidectomized rats using the same experimental procedures as in the present work (Montero-Pedrazuela *et al*., 2006). T4 levels of all the animals were measured after sacrifice (see below).

Electrode implantation

At P96-P100, animals were anesthetized with 4% chloral hydrate (0.5-1 mL/100 g, i.p.; Sigma) following a protective injection of atropine sulfate (0.1 mg/100 g, i.m.). Animals were implanted with bipolar EMG recording electrodes in the left orbicularis oculi muscle and with bipolar stimulating electrodes on the ipsilateral supraorbital nerve (Fig. 1A). Electrodes were made of 50 μm, Teflon-coated, annealed stainless steel wire (A-M Systems, Carlsborg, WA, USA) with their tips bared of the isolating cover for ≈ 0.5 mm. The electrode tips were bent as a hook to facilitate a stable insertion in the upper

eyelid. Animals were also implanted with bipolar stimulating electrodes in the dorsomedial aspect of the right angular bundle (6.8 mm posterior and 3 mm lateral to Bregma; depth from brain surface, 2 mm; Paxinos & Watson, 1986), and with four recording electrodes aimed at the granular cell layer of the dorsal dentate gyrus (3.6 mm posterior and 1.2 mm lateral to Bregma; depth, 3.4 mm). These electrodes were made of 25 μm, Teflon-coated tungsten wire (Advent Research Materials, Eynsham, England). Finally, animals were implanted with a 0.1 mm bare silver wire as ground. All wires were soldered to three four-pin sockets (RS Amidata, Madrid, Spain), and the sockets were fixed to the skull with the help of three small screws and dental cement (Gruart *et al*., 2006; Valenzuela-Harrington *et al*., 2007).

Recording and stimulation procedures

The EMG recordings were carried out using Grass P511 differential amplifiers with a bandwidth of 0.1 Hz-10 kHz (Grass-Telefactor, West Warwick, RI, USA). As a control procedure, reflex blinks were evoked by stimulation of the supraorbital nerve with single 500 μs, 2 × threshold, square, cathodal pulses presented at a rate of 1/30 s delivered from a CS-20 stimulator (Cibertec, Madrid, Spain).

Hippocampal recordings were carried out with high-impedance (2×10^{12} Ω, 10 pF) probes connected to four Grass P511 differential amplifiers. As indicated above, electrodes were surgically implanted in the dentate gyrus using as a guide the field potential depth profile evoked by paired (40 ms of inter-stimulus interval) pulses presented at the ipsilateral perforant pathway. For this, we used a dual-pulse CS-20 stimulator provided with ISO-200 isolation units (Cibertec, Madrid, Spain). The bunch of recording electrodes was fixed at the site where a reliable monosynaptic field EPSP was recorded. The actual localization of the electrodes was checked in tissue sections after the sacrifice of the animals (see below and Fig. 1).

In order to check the auditory conditions of thyroidectomized animals, the auditory brainstem response was checked in all the animals using a modular multifunctional TDT system 3 (Tucker-Davis Technologies, Alachua, FL, USA) at the end of the studies. This study was carried out following procedures described elsewhere (Cediel *et al*., 2006).

Input-output curves and paired-pulse stimulation

For input/output curves (Fig. 2A), animals (n = 10 per group) were stimulated at the perforant pathway with single pulses, at increasing intensities (0.02-0.3 mA). For each

intensity, the stimulus was repeated ≥ 5 times with time intervals ≥ 30 s, to avoid as much as possible interferences with slower short-term potentiation (augmentation) or depression processes (Zucker & Regehr, 2002). We also checked the effects of paired pulses at different (10, 20, 40, 100, 200, and 500 ms) inter-stimulus intervals, but using intensities corresponding to 40% of the amount necessary to evoke a saturating response (Fig. 2B). Also in this case, the pair of pulses was repeated ≥ 5 times with time intervals ≥ 30 s.

Classical conditioning

For the classical conditioning of eyelid responses, the animal was placed in a Faraday box (30 × 30 × 30 cm), and the stimulating and recording wires were connected to the implanted sockets. Experimental sessions started 14 days after surgery (from P113 on). Classical conditioning was achieved with a trace paradigm (Fig. 3A). For this, a tone (50 ms, 2.4 kHz, 85 dB) was presented as a CS. The US started 500 ms from CS onset, and consisted of a 500 μs, 3 × threshold, square, cathodal pulse (< 0.8 mA). A total of 2 habituation, 10 conditioning, and 5 extinction sessions were performed (Fig. 3C, D). A conditioning session consisted of 60 CS-US presentations separated at random by 30 ± 5 s. Conditioning sessions lasted for ≈ 30 min. For habituation and extinction sessions, the CS was presented alone, also for 60 times per session at intervals of 30 ± 5 s. As a criterion, we considered a "conditioned response" the presence, during the CS-US period, of EMG activity lasting > 20 ms and appearing > 50 ms after CS onset. In addition, the integrated EMG activity recorded during the CS-US interval had to be ≥ 2.5 times greater than the averaged activity recorded 500 ms before CS presentation (Gruart *et al*., 2006; Valenzuela-Harrington *et al*., 2007).

Synaptic field potentials in the dentate gyrus were evoked during habituation, conditioning, and extinction sessions by a single 100 μs, square, biphasic (negative-positive) pulse applied to the perforant pathway 300 ms after CS presentation (Figs. 3, 4). For each animal, the stimulus intensity was set well below the threshold for evoking a population spike, usually 30-40% of the intensity necessary for evoking a maximum fEPSP response (range: 40-350 μA; Gureviciene *et al*., 2004). An additional criterion for selecting stimulus intensity was that a second stimulus, presented 40 ms after a conditioning pulse, evoked a larger (> 40%) synaptic field potential (Bliss & Gardner-Medwin, 1973).

Long-term potentiation

Field EPSP baseline values (Fig. 5A) were collected 15 min prior to LTP induction using single 100 μs, square, biphasic pulses. Pulse intensity was set at 40% of the amount necessary to evoke a maximum fEPSP response (0.05-0.25 mA) — that is, well below the threshold for evoking a population spike (Gruart *et al*., 2006; Gureviciene *et al.,* 2004; Madroñal *et al*., 2007). An additional criterion for selecting stimulus intensity was that a second stimulus (presented 40 ms later) evoked a larger (> 40%) synaptic field potential than the first, conditioning one (Bliss & Gardner-Medwin, 1973). For LTP induction, each animal was presented with an HFS session consisting of five 200 Hz, 100 ms trains of pulses at a rate of 1/s. This protocol was presented six times, at intervals of 1 min. Thus, a total of 600 pulses were presented during a complete HFS session. In order to avoid evoking large population spikes and/or the appearance of electroencephalographic seizures, the stimulus intensity during HFS was set at the same as that used for generating baseline recordings. After each HFS session, the same single 100 μs, square, biphasic stimulus was presented every 20 s for 30 min during the first LTP session and for 15 min the two following days (Fig. 5B). To avoid any interference with previous conditioning tests, LTP was carried out at P136, i.e. 5 days after the last extinction session.

Histology and immunohistochemistry

At the end of the experiments (P140), animals were deeply re-anesthetized with a mixture of ketamine (100 mg/kg) and medetomidine (0.1 mg/kg) and perfused transcardially with saline following with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7. The brains were removed, postfixed overnight in the same solution, and serially sectioned on a vibratome at 50 μm in the coronal plane. Selected sections including the dorsal hippocampus were mounted on gelatinized glass slides and stained using the Nissl technique with 0.1% toluidine blue, to determine the location of stimulating and recording electrodes (Fig. 1B, C).

In order to evaluate alterations in inhibitory cortical circuits due to thyroidal status or in T3-modulated proteins involved in LTP induction we decided to analyze the expression of several proteins by immunohistochemistry with specific and well reported antibodies: parvalbumin (PV; P-3088, Sigma; Celio, 1986), GABA transporter type 1 (GAT-1; ab426; Abcam plc, Cambridge, UK, Manzano *et al*., 2007) and NMDA receptor subunit 1 (NR1; AB9864, Chemicon, Temecula, CA, USA; Nácher *et al*.,

2007). PV is a calcium binding protein expressed by both the soma and processes of the subpopulation of GABAergic interneurons (chandelier and basket cells) representing the most powerful inhibitory neurons of the cerebral cortex (Celio, 1986). GAT-1 is the predominant type of GABA transporter in the cerebral cortex which has been shown to be a good marker of GABAergic terminals (Guastella *et al*., 1990). NR1 is a constitutive subunit of the ionotropic NMDA glutamate receptor-channel complex (Cull-Candy *et al*., 2001; Monyer *et al*., 1992) Alterations in the expression (protein or mRNA) of all these proteins have been previously detected in animal models of adult hypothyroidism and thyroid hormone receptor deficient and mutant animals (Guadaño-Ferraz *et al*., 2003; Lee *et al*., 2003; Venero *et al*., 2005).

For immunohistochemistry a total of four animals per condition were used. One series (four to five sections) from the dorsal hippocampus from each animal were batch-processed and analyzed for each antibody as previously described (Venero *et al*., 2005) using the specific primary antibodies described above: mouse anti-PV (1/5000), rabbit anti-GAT-1 (1/750), and rabbit anti-NR1 (1/500). Briefly, sections were pretreated with a solution of 10% methanol and 3% hydrogen peroxide in PB to remove endogenous peroxidase activity, then preincubated in a blocking solution (5% normal horse serum (S-2000, Vector Laboratories, Burlingame, CA, USA) for the study with mouse anti-PV and 5% normal goat serum (S-1000, Vector Laboratories) for the studies with rabbit anti-GAT-1 and rabbit anti-NR1, 0.1% Triton X-100, and 4% BSA in PB) for 2 h at room temperature. Sections were then incubated overnight at 4°C in the above solution containing the specific primary antibodies. The sections were subsequently washed in PB, incubated in a species-specific biotinylated secondary antibodies (horse anti-mouse, BA-2001, or goat anti-rabbit, BA-1000, Vector Laboratories), diluted 1:200 in PB for 1 h at room temperature, and processed by the avidin-biotin-peroxidase method (Vectastain Elite, PK-6100, Vector Laboratories). The sections were mounted on glass slides, dehydrated, cleared in xylene, and coverslipped with Depex. No staining due to the omitted primary antibody was observed in control experiments with any of the antibodies used. In all cases, the analysis was conducted blindly as to the animal's thyroidal status.

Quantitative measurements of the density of PV, GAT-1, and NR1 immunopositive (+) terminals in the hippocampal CA1 region and dentate gyrus were performed by using the Neurolucida software (MicroBrightField, Inc., Colchester, VT, USA). From each individual section, B/W images were captured from 6 randomly selected microscope fields from the stratum piramidale and the granular cell layer at

high magnification using a Nikon Eclipse 80i microscope (60×, numerical aperture 0.85). Similar measurements were made in the white matter for background subtraction. The cell bodies from PV stained cells were excluded from the measurements. Counting of PV-labeled somata was performed in the dorsal hippocampus in three 275 μm^2 randomly selected areas of each section in the CA1 field and in the granular cell layer of the dentate gyrus directly from light microscope slides (20x, numerical aperture 0.5).

Microphotographs were acquired using a digital camera Nikon DS-Fi1 and the figures were prepared with Adobe Photoshop 7.0.1.

Hormonal determinations

On the day of killing, blood and liver tissue were obtained from each animal prior to perfusion to measure T4 levels following previously established methods (Morreale de Escobar *et al*., 1985). In brief, liver tissue and plasma underwent an extensive extraction and purification protocol. Afterwards, T4 content was determined in duplicate at two dilutions by a highly sensitive and specific radioimmunoassay using specific antibodies (Ruiz de Oña *et al*., 1991). The limit of detection was 2.5 pg/tube.

Data analysis

The extracellular EMG and dentate gyrus recordings were stored digitally on a computer through an analog/digital converter (CED 1401 Plus, Cambridge, England), at a sampling frequency of 11-22 kHz with an amplitude resolution of 12 bits. Commercial computer programs (Spike 2 and SIGAVG from CED) were modified to represent EMG and fEPSP recordings. Data were analyzed off-line for quantification of conditioned responses and the fEPSP slope with the help of homemade representation programs (Domínguez-del-Toro *et al*., 2004; Gruart *et al*., 2006). The slope of evoked fEPSPs was collected as the first derivative (mV/s) of fEPSP records (mV). For this, 5 successive evoked field synaptic potentials were averaged, and the mean value of the slope was determined for the rise time period (i.e., the period of the slope between the initial 10% and the final 10% of the evoked field potential). Computed results were processed for statistical analysis using the SPSS for Windows package (SPSS Inc., Chicago, IL, USA). Unless otherwise indicated, data are represented by the mean ± SEM. Collected data were analyzed using a two-way ANOVA test, with time or session as repeated measure, coupled with contrast analysis (*post hoc* Bonferroni test) when appropriate. Repeated-measures ANOVA allowed checking the statistical differences of the same group across sessions. Regression analysis was used to study the evolution of

fEPSP slopes across conditioning sessions (Fig. 4) for the three experimental conditions.

Student's *t*-test was used to evaluate intra-group weight gain and one-way ANOVA was used for inter-group weight analyses and hormonal determinations. Immunohistochemistry data were analyzed using one-way ANOVA or ANOVA mixed model with the number of sections as repeated measures. A *post hoc* Bonferroni test was used when appropriate.

Results

Recording of EMG and synaptic field potentials in alert behaving rats

In a series of preliminary recording sessions, we checked that electrode implantation in the upper eyelid did not prevent eyelid responses. In fact, the electrical stimulation (2 × threshold) of the supraorbital nerve evoked an early (6-8 ms) activation of the orbicularis oculi muscle, followed by a second, more variable (15-25 ms) EMG activation (not illustrated). These two successive muscle activations correspond to the R1 and R2 components of the blink reflex described in different species of mammals (Gruart *et al*., 1995, 2000, 2006; Valenzuela-Harrington *et al*., 2007), including humans (Kugelberg, 1952). Thus, the thin wires used for EMG recordings and trigeminal nerve stimulation did not interfere with eyelid kinematics.

The electrical stimulation of the perforant pathway evoked monosynaptic fEPSPs in the dentate gyrus consisting of a positive wave, with a latency of ≈ 2 ms (see Figs. 2, 3). This early positive wave was interrupted by a sharp negative wave representing the population spike only when high stimulus intensities were used (Krug *et al*., 2001). As already indicated in Methods, we used here stimulus intensities of about 40% of the amount necessary to evoke a maximum fEPSP, thereby avoiding the presence of population spikes. According to previous descriptions (de Jonge & Racine, 1985; Gruart *et al*., 2006), in the absence of classical conditioning or of the presentation of the HFS protocol, the slope of fEPSPs evoked by the stimulation of the perforant pathway remained stable across successive recording days. For example, during habituation sessions the percentage of variation in fEPSPs slopes was ≤ 12.5% as compared with the mean value (100%), with no statistically significant trend towards a decrease or increase ($F_{2,38} = 1.442$; $P \geq 0.53$).

Because we used a tone as CS for classical eyeblink conditioning, we checked that thyroidectomy at adult stages did not affect the auditory system functionality. A loss in functionality has repeatedly been described in hypothyroidism during development in both experimental animals and humans (Deol, 1973; Rovet *et al*., 1996). The auditory brainstem response test was carried out in the three experimental groups plus an additional control group of non-operated rats (n ≥ 10 animals/group). Auditory thresholds determined for euthyroid control (Ctl, 38 ± 1.22 dB), hypothyroid (Hypo, 38 ± 1.29 dB), and recovery (Rec, 39 ± 1.11 dB) groups, and for the additional control (37 ± 2.54 dB) group, showed no significant differences. Accordingly, a tone could be used as an appropriate CS in the three experimental groups.

Input/output relationships at the perforant pathway-dentate gyrus synapse in the three groups of animals

In a first series of experiments, we studied the changes in the slope of fEPSPs evoked at the dentate gyrus by single-pulse stimulation of the perforant pathway at increasing intensities. As illustrated in Fig. 2A for control animals (Ctl, black circles), the slope of fEPSPs (in mV/s) evoked in the dentate gyrus increased steadily with current strength until reaching asymptotic values, being significantly larger than baseline values from 0.08 to 0.3 mA ($F_{13,117} = 14.61$; $P < 0.001$). In contrast, fEPSPs evoked in hypothyroid animals (Hypo, black triangles) did not increase in slope (or in amplitude) until high stimulus intensities were used (0.18-0.3 mA; $F_{13,117} = 31.15$; $P < 0.001$). Surprisingly, fEPSPs evoked in recovered animals (Rec, white triangles) needed even larger stimulus intensities (0.22-0.3 mA; $F_{13,117} = 21.15$; $P < 0.001$) to be significantly larger than baseline values. On the other hand, fEPSPs evoked in Ctl animals were significantly larger than the corresponding fEPSPs evoked in both Hypo and Rec groups for a wide range of intensities (0.1-0.3; $F_{28,252} = 76.889$; $P \leq 0.001$; asterisks in Fig. 2A), whilst fEPSPs evoked in Hypo animals showed larger slopes than those evoked in the Rec group for a narrower range of intensities (0.24-0.3; $F_{28,252} = 76.889$; $P \leq 0.001$; plus signs in Fig. 2*A*). These data suggest that input/output relationships at the perforant pathway-dentate gyrus synapse are affected by thyroid hormones deficiency in Hypo animals. Interestingly enough, the deficit in input/output relationships was not compensated by hormone administration. In fact, this deficit was increased in Rec animals.

Paired-pulse facilitation

Paired-pulse stimulation is a well-known form of short-term modulation of hippocampal synapses, and it is used as an indirect measurement of changes in the probability of release of neurotransmitter at the presynaptic terminal (Lauri *et al*., 2007; Thomson, 2000; Zucker & Regehr, 2002). In a second series of experiments, we checked whether this short-term form of synaptic plasticity was affected by thyroid hormones deficiency. For this, animals from the three groups were stimulated with pairs of pulses of increasing intervals (10-500 ms), but at a fixed intensity, corresponding to some 40% of that necessary to evoke maximum fEPSP responses. As illustrated in Fig. 2B, the three groups of animals presented significant (one-way ANOVA, $P < 0.001$) increases in response to the 2nd pulse as compared with that evoked by the 1st one at short time intervals (10-50 ms). No significant differences were observed between the slopes of the fEPSPs evoked in the three groups ($F_{10,90} = 0.258$; $P \geq 0.988$). These results indicate that paired-pulse facilitation is preserved in both Hypo and Rec animals at the same levels as those observed in the Ctl group.

Classical eyeblink conditioning

In a third series of experiments, we investigated the learning capabilities of the three experimental groups in a typical associative learning task: the classical conditioning of eyelid responses, using a trace (CS, tone; US, shock) paradigm (Figs. 1A and 3A). The time interval between the end of the CS and the beginning of the US was 450 ms. The percentage of conditioned responses for euthyroid control animals (Fig. 3D, black circles) increased steadily across conditioning sessions, being significantly different from habituation values from the 3rd to the 10th conditioning sessions and from all of the extinction sessions ($F_{16,144} = 32.559$; $P < 0.001$), with a profile similar to previous descriptions in this species, using similar trace conditioning procedures (Valenzuela-Harrington *et al*., 2007). No differences due to the thyroidal status were observed during the habituation time (Fig. 3D). The two-way ANOVA of conditioned responses reached during conditioning and extinction sessions by the three groups yielded significant differences ($F_{32,288} = 3.876$; $P < 0.001$). A *post hoc* Bonferroni test indicated that the Hypo group (Fig. 3D, black triangles) presented an initial increase in the percentage of conditioned responses that reached asymptotic values at a rather low level (30-35% of conditioned responses), significantly below asymptotic values reached by the other two groups (Ctl and Rec groups; for the 6th to the 10th conditioning sessions and for the 1st extinction session; $P \leq 0.001$; Fig. 3D). In contrast, the Rec group (Fig. 3D, white

triangles) reached percentages of conditioned responses across conditioning similar to the values obtained by the Ctl group ($P \geq 0.354$). In summary, hypothyroid animals were unable to reach the same asymptotic level of conditioned responses presented by euthyroid (Ctl) animals. Interestingly, the group treated with thyroid hormones (Rec group) showed similar performances to that of the Ctl group.

Evolution of fEPSPs evoked at the perforant pathway-dentate gyrus synapse across classical eyeblink conditioning

As illustrated in Fig. 3A, B the electrical stimulation of the perforant pathway 300 ms after CS presentation evoked a noticeable fEPSP in the dentate gyrus of the three experimental groups. Although the stimulus presented to the perforant pathway disrupted the regular theta rhythm recorded in the dentate gyrus area, the rhythm reappeared in phase ≈ 300 ms afterwards.

In accord with an early study (Weisz *et al*., 1984), fEPSPs evoked by the electrical stimulation of the perforant pathway increased progressively in slope in the Ctl group (taking the slope of fEPSPs collected during the two habituation sessions as 100%) across conditioning sessions (Fig. 3C). Importantly, this increase in fEPSP slopes was significantly different ($F_{16,144} = 8.87$; $P < 0.001$) from baseline values from the 6th to the 10th conditioning sessions. In contrast, fEPSPs evoked in the Hypo group resulted in a small, non-significant (< 115%; $F_{16,144} = 4.15$; $P \geq 0.457$) increase in slope across conditioning. Similar results (< 107.5% of increase in fEPSP slopes with respect to baseline values; $F_{16,144} = 2.18$; $P \geq 0.658$) were obtained in the Rec group.

Although fEPSP slopes of fEPSPs evoked in the Ctl group across conditioning reached larger values than those collected from the other two groups, no significant differences were observed between the groups ($F_{32,608} = 1.336$; $P \geq 0.105$; Fig. *3C*). A re-analysis of the collected data indicated the presence of noticeable differences in fEPSP slope evolution at the different recording sites of the dentate gyrus even for the same animal and group. In coincidence with the authors of a recent report (Whitlock *et al*., 2006), we noticed that fEPSP slopes increased across conditioning in some recording electrodes, but decreased in another, with no significant changes in the rest. For this reason, we decided to carry out a separate linear regression analysis of fEPSPs evoked at individual electrodes across the 10 conditioning sessions. As illustrated in Fig. 4, the number of electrodes crossing the dashed lines (indicating ± 1 SD for data collected from each group) increased across conditioning for the three experimental

groups. Nevertheless, we noticed marked differences between the Ctl and the other (Hypo, Rec) two groups.

Using these analytical procedures, we found that in Ctl animals, 70% (13 out of 20) of recording electrodes showed a significant increase (values for the r_9 coefficient ranging from 0.78 to 0.89, $P \leq 0.05$) in slopes (range: 1.9% to 12.4%) across conditioning sessions, whilst 20% (4/20) presented decreasing fEPSP slopes (range: −1.6% to −5.1%; r_9: 0.79 to 0.96; $P \leq 0.05$). As a whole, of the 20 electrodes analyzed in the Ctl group, 11 (55%) increased (n = 7) or decreased (n = 4) in fEPSP slopes, exceeding the ± 1 SD determined for data collected from the group (Fig. 4A). In contrast, in the Hypo group, only 5 out of 20 electrodes (25%) analyzed increased (n = 3) or decreased (n = 2) in fEPSP slopes across conditioning. The fEPSPs recorded in the rest of the electrodes (n = 15; 75%) did not present any significant change across conditioning. Moreover, 70% of the r_9 coefficients (14/20) showed values ≤ 0.6, suggesting a large variability in the collected data, a fact not observed in the Ctl group ($r_9 \geq 0.78$). Similar results were obtained from the Rec group — only 25% of the electrodes analyzed (5/20) increased (n = 2) or decreased (n = 3) in fEPSP slopes during the successive conditioning sessions, and 65% of the r_9 coefficients (13/20) presented values ≤ 0.6, suggesting a variability in fEPSP slopes not noticed in the Ctl group.

In short, in euthyroid (Ctl) animals, 55% fEPSP slopes changed significantly, mostly in the increasing direction, across classical eyeblink conditioning at the perforant pathway-dentate gyrus synapse (Fig. 4A). In contrast, the percentage of electrodes presenting a significant change across conditioning in the Hypo (Fig. 4B) and Rec (Fig. 4C) groups was very low (25%). Furthermore, fEPSPs recorded from the Hypo and Rec groups along the 10 conditioning sessions showed a larger variability in slopes than those from Ctl animals.

Characteristics of long-term potentiation evoked in control, hypothyroid, and recovery animals

To further investigate the deficit in synaptic plasticity observed in Hypo and Rec rats, we decided to carry out a comparative study of the effects of an HFS session applied to the perforant pathway in the Ctl, Hypo, and Rec groups (n = 10 animals/group; Fig. 5). For baseline records, the perforant pathway was stimulated every 20 s for 15 min. The stimulus consisted of a single, 100 μs, square, biphasic pulse. Stimulus intensity was set at a 40% of the amount necessary to evoke a maximum response (Fig. 5A). After the HFS session, the same single stimulus was presented at the initial rate (3/min) for

another 30 min (Fig. 5A). Additional 15-minute sessions were carried out 24 h and 48 h later.

With this protocol, we found that LTPs evoked in Ctl animals were significantly different from those in Hypo and Rec animals (two-way ANOVA, $F_{48,432} = 3.549$; $P < 0.001$; see asterisks in Fig. 5B, top and middle graphs). A *post hoc* Bonferroni analysis showed specifically that the Ctl group yielded an LTP response > 125% of baseline values as quantified 15 min following the HFS session ($P < 0.01$; Fig. 5B). The LTP response of the Ctl group was still significantly larger than baseline values 2 days after the HFS train ($P < 0.01$; Fig. 5B; Ctl: black circles). In contrast, both the Hypo and Rec groups were unable to evoke LTP at the perforant pathway-dentate gyrus synapse. For example, the increase in fEPSP slopes 15-30 min after the HFS session in Hypo rats was negligible (≈ 103%; $P = 0.42$), and appeared well below baseline values two days later (Fig. 5B; Hypo: black triangles). The Rec group also showed no evidence of an LTP following the HFS session (Fig. 5B; Rec: white triangles). As illustrated in Fig. 5C, fEPSP slopes collected from the Ctl group were significantly larger than the corresponding values recorded from the Hypo and the Rec groups up to 48 h after the HFS session (see asterisks in Fig. 5C, $P < 0.001$), but no significant ($P \geq 0.145$) differences were observed between the latter two groups.

Changes in body weight and in T4 levels evoked by thyroidectomy and hormonal treatment

One-way ANOVA analysis followed by a *post hoc* Bonferroni test showed differences in body weight and T4 levels between groups ($F_{2,34} = 53.850$, $P < 0.001$ and $F_{2,37} = 46.380$, $P < 0.001$, respectively). At the time of killing (P140-P143), the Ctl group presented a 177.0% increase in body weight from the day of sham surgery (P61-P63, $t_{24} = 6.285$, $P < 0.001$), whilst the Hypo group increased only 105.6% ($t_{20} = 0.857$, $P = 0.409$ as compared with the day of thyroidectomy and $P < 0.001$ as compared with the Ctl group at the time of killing). The Rec group showed intermediate values (153.6%). This increase in body weight was statistically different from that of Ctl animals ($P = 0.012$), as well as from that of Hypo animals ($P < 0.001$). A *post hoc* Bonferroni test also showed that T4 levels were considerably reduced in Hypo rats ($P < 0.001$) as compared with Ctl and Rec animals in both plasma and liver. In fact, all Hypo rats presented plasma and liver T4 levels of 1.1 ± 0.29 ng/mL and 1.12 ± 0.2 ng/g respectively, well below values reached by Ctl (16.39 ± 0.97 ng/mL and 19.08 ± 1.49 ng/g) and Rec (14.74 ± 2.33 ng/mL and 21.74 ± 1.35 ng/g) animals. T4 levels in Rec

animals showed no difference with those in Ctl animals (P = 0.513), which indicates that the replacement treatment with thyroid hormones has successfully recovered thyroid hormone levels, as found previously (Montero-Pedrazuela *et al*., 2006).

Immunohistochemical analyses

The density of immunoreactive terminals for GAT-1 and NR1 both in the stratum piramidale of the CA1 region and in the granular layer of the dentate gyrus did not show significant differences between the three groups of animals by using one-way ANOVA analysis (GAT-1: $F_{2,47}$ = 2,807, P = 0.071 for CA1 and $F_{2,\ 53}$ = 0.121, P = 0.887 for dentate gyrus; NR1: $F_{2,22}$ = 1.999, P = 0.159 for CA1 and $F_{2,\ 20}$ = 1.916, P = 0.174 for dentate gyrus; see Table 1). However, the density of PV+ perisomatic terminals was modified due to the thyroidal status both in hippocampal CA1 region and dentate gyrus ($F_{2,\ 43}$ = 12.287, P < 0.001, and $F_{2,\ 42}$ = 20.508, P < 0.001, respectively; see Table 1 and Fig. 6). Specifically, and following a *post hoc* Bonferroni test, there was an increase in the PV+ terminals density in the Hypo rats as compared to Ctl animals (P < 0.001 for both CA1 and dentate gyrus), that was not recovered after thyroid hormones treatment in hippocampal CA1 region and dentate gyrus (P = 0.036 and P = 0.028 respectively as compared to Ctl animals and P = 0.074 and P = 0.002 respectively as compared to Hypo animals; see Table 1 and Fig. 6).

The one-way ANOVA did not reveal any alterations in the number of PV+ cells due to thyroidal status in any of the regions analyzed ($F_{2,8}$ = 0.338*;* P = 0.723 for dentate gyrus and $F_{2,9}$ = 1.212*;* P = 0.342 for CA1). The average number (mean ± S.D.) of PV immunoreactive cells per mm^2 in the dentate gyrus was 116.6 ± 1.9 for Ctl animals, 118.7 ± 22.2 for Hypo animals, and 107.7 ± 23.8 for Rec animals. In the CA1 region (which included all strata from the stratum oriens to the stratum lacunosum-moleculare) the average number of PV+ cells per μm^2 was 1.01 ± 0.05 for Ctl animals, 1.14 ± 0.09 for Hypo animals, and 1.12 ± 0.20 for Rec animals.

In summary, the present results indicate that adult-onset hypothyroidism by experimental thyroidectomy impairs both associative learning capabilities and a number of specific synaptic functions, as activity-dependent synaptic changes in strength during learning and experimentally evoked LTP. Thyroid hormone replacement treatment helps recover associative learning capabilities, but did not offset other deficits observed in hippocampal synapses of thyroidectomized animals, such as modifications in input/output relationships, and changes in synaptic strength evoked during associative learning and during experimentally induced LTP, suggesting that specific subcellular

and molecular processes underlying the latter phenomena are not recovered by thyroid hormone administration after a short period of adult hypothyroidism. Indeed, these functional alterations correlate with an increase of perisomatic terminals expressing the calcium binding protein parvalbumin in the hippocampal CA1 region and dentate gyrus.

Discussion

It has been convincingly shown that trace conditioning is a hippocampally dependent form of associative learning (Clark & Squire, 1998; Moyer *et al*., 1990; Solomon *et al*., 1986) involving the presence of plastic changes in selected relays, including the CA3-CA1 (Gruart *et al*., 2006; Whitlock *et al*., 2006) and the perforant pathway-dentate granule cell (Weisz *et al*., 1984) synapses. As confirmed here in euthyroid alert behaving rats, fEPSPs evoked at the perforant pathway-dentate granule cell synapse increase their slopes across conditioning sessions, indicating the involvement of this synapse in the learning process. Furthermore, the present study demonstrates that the acquisition of this type of associative learning and the accompanying activity-dependent synaptic changes in the dentate gyrus are severely impaired by adult thyroidectomy. A replacement therapy with thyroid hormones allowed the acquisition of the trace conditioning task, but without the changes of synaptic strength observed at the perforant pathway-dentate granule cell synapse in Ctl rats. The latter result is very important, because it indicates that trace conditioning can be acquired in the absence of evoked LTP at this synapse, a fact suggesting that both processes can be differentiated experimentally. In this regard, it has been recently shown in alert behaving mice that experimentally evoked LTP and activity-dependent synaptic changes during actual learning present opposite presynaptic mechanisms that explain their different evolution (decreasing vs. increasing in synaptic strength) across time (Madroñal *et al*., 2009).

The cellular and molecular basis of LTP, associative learning, and other synaptic properties of hippocampal circuits have been studied in detail, and are still a matter of intense debate (Bliss & Collingridge, 1993; Citri & Malenka, 2008). As shown here, thyroidectomy evokes an increase of perisomatic terminals expressing PV in the hippocampal CA1 region and dentate gyrus, suggesting an increased role of GABAergic neurons that is not rescued by the administration of thyroid hormones. Moreover, thyroid hormone treatment did not allow a recovery in the input-output relationships and the LTP levels observed in Ctl animals, which were significantly impaired following thyroidectomy. In contrast, other functional properties of hippocampal

synaptic circuits, such as paired-pulse facilitation, were apparently not affected in thyroidectomized animals. The paired-pulse facilitation at brief interstimulus intervals observed here for the three experimental groups was similar to that reported previously in neocortex *in vivo* (Baranyi *et al*., 1999), and *in vitro* (Markram *et al*., 1997), and in CA1 *in vitro* (Magee & Johnston, 1997). Finally, and from a clinical point of view, it is important to mention here that the present results suggest that thyroid hormone replacement therapy does not completely compensate for normal euthyroid synaptic functioning.

Involvement of the perforant pathway-dentate gyrus granule cell synapse in associative learning

It has recently been shown in both mice (Gruart *et al*., 2006) and rats (Whitlock *et al*., 2006) that the strength of CA3-CA1 synapses is increased during different learning tasks. In accord with early electrophysiological recordings carried out during classically conditioned nictitating membrane responses (Weisz *et al*., 1984), the present results indicate that selected dentate gyrus recording sites steadily potentiated their fEPSPs in response to perforant pathway inputs across trace conditioning sessions. In contrast to studies carried out at the CA3-CA1 in behaving mice using a similar conditioning task (Gruart *et al*., 2006), but in agreement with data collected from behaving rats (Whitlock *et al*., 2006), not all dentate gyrus sites recorded here showed a significant increase in the slope of fEPSPs evoked by perforant pathway stimulation. In fact, some recording sites presented decreasing fEPSP slopes across conditioning, while others presented no significant changes. These findings suggest that, at least in the rat, specific hippocampal sites are reserved for specific learning tasks.

Our results indicate that the number of PV+ cells does not change, but there is a significant increase in the density of PV+ perisomatic terminals, suggesting an increasing role of GABAergic neurons in the modulation of hippocampal circuits, including the perforant pathway-dentate gyrus synapse (Jinno & Kosaka, 2006; Toledo-Rodriguez *et al*., 2005). These results can explain, in part, the results obtained in both Hypo and Rec groups for experimentally evoked LTP.

A putative explanation of the present results with respect to the Rec group is that, as already proposed, the full hippocampal pathway including the perforant pathway-dentate gyrus synapse is required for experimentally-evoked, stressful LTP and for rapid one-trial contextual learning (McHugh *et al*., 2007; Nakashiba *et al*., 2008). It is also possible that the monosynaptic entorhinal cortex → CA1 → entorhinal

cortex pathway would be sufficient to compensate the absence of plasticity noticed in the perforant pathway-dentate gyrus synapse in the Rec group.

An interesting issue regarding perforant pathway-dentate gyrus granule cell synaptic plasticity during associative learning is related to adult-generated granule neurons and hippocampally dependent learning. It has been repeatedly asserted that the number of adult-generated granule neurons increases in parallel to the acquisition of hippocampally dependent learning, and that the generation of new granule neurons is necessary for some kinds of hippocampal-dependent learning and memory processes, including classical eyeblink conditioning (Gould *et al*., 1999; Kempermann, 2002; Saxe *et al*., 2006; Shors *et al*., 2001). According to the present results, there is an accompanying increase in the synaptic strength at selected dentate gyrus sites during classical eyeblink conditioning. However, it should be kept in mind that similar activity-dependent synaptic changes have also been observed in hippocampal areas (i.e., the CA3-CA1 synapse; Gruart *et al*., 2006; Whitlock *et al*., 2006), but not accompanied by a corresponding increase in neurogenesis. Nevertheless, adult thyroidectomy seems to impair associative learning (present results), as well as to decrease by some 30% the number of newly generated neuroblasts in the dentate gyrus (Montero-Pedrazuela *et al*., 2006). It has also been reported that the experimental reduction (using the DNA methylating agent methylazoxymethanol acetate) of the rate of proliferation in the adult dentate gyrus impairs trace eyeblink conditioning in adult rats (Shors *et al*., 2001).

Although the impairment of neurogenesis caused by thyroidectomy can be reversed after a thyroid hormone replacement treatment (Montero-Pedrazuela *et al*., 2006), present results indicate that the ability to develop classically conditioned eyelid responses is recovered in thyroidectomized treated rats without any sign of recovery in LTP at the dentate gyrus. It is still possible that adult-born neurons could make distinct contributions to different hippocampal functions. For example, focal X-ray administration or genetic ablation of glial fibrillary acidic protein-positive neural progenitor cells impaired contextual fear conditioning, but not cued conditioning or spatial learning tasks (Saxe *et al*., 2006). Moreover, anxiety and memory deficits observed in dominant negative mutant thyroid hormone receptor $\alpha 1$ mice were mitigated by treatment with high levels of thyroid hormones, a fact that has been correlated with a normalization of GABAergic inhibitory interneurons in the hippocampal CA1 area (Venero *et al*., 2005). Thus, as shown here, thyroid hormone treatment could make selective and differential contributions to functional recovery at selected hippocampal circuits and/or functions.

Effects of hypothyroidism on experimentally evoked long-term potentiation

We observed here a clear impairment of LTP induction in the perforant pathway-dentate gyrus synapse in Hypo animals. In a series of seminal studies, it has been shown that adult-onset thyroidectomy-induced hypothyroidism interferes with early- (Gerges *et al*., 2001) and late-LTP (Alzoubi *et al*., 2008; Gerges & Alkadhi, 2004) at the CA3-CA1 synapse, but not at the perforant pathway-dentate granule cell synapse. Since those experiments were carried out in urethane-anesthetized rats for short periods of time (up to 6 h), an easy comparison can not be made with our study, carried out in behaving animals. The alert behaving condition could be a very demanding task for the experimental animals, placed in an open area with a certain level of stress (Gruart *et al*., 2006; Valenzuela-Harrington *et al*., 2007). Moreover, our surgical thyroidectomy procedure reduces thyroid hormones levels by some 90% as compared with euthyroid levels, while in those other studies the reduction of thyroid hormone levels was around 40%, so fewer detrimental effects of thyroid hormone deficiency would be expected. Adult-onset hypothyroidism also impairs LTP of the rat dorsal hippocampal-medial prefrontal cortex pathway *in vivo* (Sui *et al*., 2006), suggesting a general functional affecting of cortical circuits by low levels of circulating thyroid hormones.

According to the present results, LTP was not recovered in Rec animals even following a prolonged replacement treatment with thyroid hormones (8 weeks), using concentrations of thyroxine similar to those in previous replacement therapies (Alzoubi *et al*., 2005, 2008) that were sufficient for LTP recovery in the CA3-CA1 synapse in adult thyroidectomized animals. As indicated above, adult hypothyroidism in those studies was milder than in ours. On the other hand, the HFS protocol used here evoked fEPSPs that reached slopes and/or amplitudes below baseline values in Hypo animals, as previously noticed by others (Gerges & Alkadhi, 2004). Moreover, it has been reported that in hypothyroid rats there is a facilitation of expression of long-term depression at the CA3-CA1 synapse (Alzoubi *et al*., 2007).

Adult-onset hypothyroidism induced by an anti-thyroid drug did not modify basal synaptic transmission at the dorsal hippocampal-medial prefrontal cortex pathway in anesthetized rats, but significantly reduced paired-pulse facilitation (Sui *et al*., 2006). In the present experiments, input-output curves were significantly depressed in Hypo and Rec animals, but paired-pulse facilitation remained within control values. Although both experiments were carried out in rats, significant functional differences could be expected between the awake versus the anesthetized state. Moreover, there are

important functional differences between these two types of synapse. Finally, neither stimulus intensities selected here for the paired-pulse test, nor hypothyroidism induction were comparable.

It is possible, and experimentally easily, to induce adult-hypothyroidism with antithyroid drugs. Here, we chose thyroidectomy to avoid the possible additional effects of the administered drugs on thyroid hormones cerebral metabolism. It is important to stress that thyroidectomy in adult subjects is currently used as a therapeutic tool in patients with thyroid carcinoma. In the present work we also explore the deleterious effects of a short period of adult-hypothyroidism on neurological functions because short periods of adult hypothyroidism are also used therapeutically as established protocols in the following and treatment of thyroid carcinoma.

Differential compensatory effects of replacement treatment with thyroid hormones on associative learning and underlying synaptic plasticity

Thyroid hormones seem to act at many different molecular levels in hippocampal neurons. For example, it has been shown in rats that the thyroid hormone-responsive protein (THRP) is preferentially expressed during early LTP stages, and that T3 injection in the dentate gyrus increases THRP mRNA as well as producing a long-lasting enhancement of the synaptic efficacy of granule cells. Apparently, the reduced LTP expression in the dorsal hippocampus is related with decreased THRP levels (Tang *et al*., 2001). Adult thyroidectomy produces a selective decrease in the mRNA expression of certain NMDA subunits (such as the NR1), but not of others (NR2A, NR2B) in the dentate gyrus (Lee *et al*., 2003). Specific cellular messengers such as calcineurin and calmodulin kinase II (CaMKII) are also decreased in the hippocampus of hypothyroid rats (Gerges *et al*., 2005). It has already been proposed that calcineurin and CaMKII are balanced differently in the CA1 and dentate gyrus area in adult hypothyroid rats (Gerges *et al*., 2005). Other signaling molecules, essential for learning and late-LTP (such as CREB and MAPKp44/42), were reduced in the hippocampal CA1 area by adult hypothyroidism (Alzoubi *et al*., 2008). Thyroid hormone treatment seems to restore some proteins to their control levels, but not others as PKCγ (Alzoubi *et al*., 2005). Finally, thyroid hormones also seem to play more general roles in the excitability of cortical circuits. For example, they up-regulate Na^+-current densities and increase the rise rate, amplitude, and firing frequencies in cortical (including hippocampal) cultured cells (Hoffmann & Dietzel, 2004).

Although other authors have shown a reduction in NR1 mRNA levels in the dentate gyrus due to hypothyroidism (Lee *et al*., 2003), our results show no alterations in NR1 protein levels in the animal groups studied. However, our results show in Hypo animals an increase in PV+ terminal density in the granular cell layer without changes in the number of PV+ cells and GAT-1 + terminal densities, a fact that could be ascribed to an increased (inhibitory) contribution of GABAergic neurons (Jinno & Kosaka, 2006; Toledo-Rodriguez *et al*., 2005). Thyroid hormones treatment was not capable of recovering this effect. It has been proposed by other groups that alterations in PV immunostaining in some regions of the hippocampus is correlated with functional alterations of the circuits in which PV+ cells are involved. Nikonenko *et al*. (2006) using a mouse deficient for the cell adhesion molecule CHL1 showed that the increase of terminal density and total number of PV+ interneurons produce an LTP reduction in CA3-CA1 synapses, suggesting that enhanced inhibition is the cause of LTP impairment. It is important to bear in mind that the expression of the calcium binding proteins in cortical interneurons is closely related to their physiological properties (Andrioli *et al*., 2007; Caillard *et al*., 2000). The alterations in the expression of PV in the interneurons may affect the firing properties of these interneurons and alter the network response to excitatory neurotransmission.

This lack of recovery of activity-dependent synaptic plasticity reported here could be explained by an insufficient dose or duration of the hormonal treatment. However, in our study we used an effective replacement treatment with a combination of specific doses of T4 and T3 that restored the plasma and liver hormonal levels, and that in a previous study was capable of restoring hormonal levels in the cerebral cortex (Escobar-Morreale *et al*., 1996). In addition, this treatment successfully recovered the proliferative capacity of the adult dentate gyrus and the maturation of newly generated neuroblasts observed in thyroidectomized rats using similar experimental animal procedures (Montero-Pedrazuela *et al*., 2006). Another explanation for the persistent impairment in activity-dependent synaptic plasticity after thyroid hormone treatment could be the presence of permanent neural damage after a short period of adult hypothyroidism. Most hypothyroid patients show an excellent prognosis and almost a full recovery from all symptoms after an adequate thyroid hormone treatment. However, the patients very often complain about their sense of well-being, and there are even some case reports describing persistent learning and memory impairments after thyroid hormone treatments that recover plasmatic hormonal levels (Capet *et al*., 2000; Leentjens & Kappers, 1995; Roberts & Ladenson, 2004; Tagay *et al*., 2005). Thus,

available information and present results in adult experimental rats strongly suggest that thyroid hormone restorative therapies do not completely repair the different hippocampal dysfunctions evoked by thyroidectomy, a fact frequently reported in clinical studies (Alzoubi *et al*., 2005; Capet *et al*., 2000; Leentjens & Kappers, 1995).

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation [BFU2005-01024, BFU2005-02512, BFU2004-05944, and BFU2007-62979] and by the *Comunidad Autónoma de Madrid/CSIC*, Spain [CCG07-CSIC_SAL-1845]. We thank Ms. María Esteban and Ms. Silvia Murillo for their technical assistance, Mrs. Laura Barrios for her assistance with the statistical analyses, and Mr. Roger Churchill for his editorial help.

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Table 1. Density of PV, GAT-1 and NR1 positive terminals in the dentate gyrus and hippocampal CA1 region (± SD; arbitrary units)

	PV+ terminal density		GAT-1+ terminal density		NR1+ terminal density	
Group	Dentate gyrus	CA1	Dentate gyrus	CA1	Dentate gyrus	CA1
Ctl	3.46±0.99	3.06±0.70	6.70±1.01	6.14±0.21	1.69±0.72	2.84±1.47
Hypo	5.50±0.83***	5.17±0.89***	6.47±1.40	5.76±0.25	1.87±0.28	3.36±1.95
Rec	4.54±1.62*	3.94±1.23*	6.66±1.26	6.46±0.94	1.77±0.55	2.28±0.56

Significant differences as compared to Ctl group are shown as $*P < 0.05$ and $***P < 0.001$.

Figure legends

FIG. 1. Experimental design. (*A*) Animals were implanted with bipolar EMG recording electrodes in the orbicularis (O.O.) muscle of the upper left eyelid. For trace eyeblink conditioning, a tone was used as a CS. The loudspeaker was located 30 cm in front of the animal's head. The CS was followed 500 ms from its onset by a US consisting of an

electrical shock presented to the supraorbital nerve. For synaptic activation during trace conditioning and for evoking LTP (see top diagrams), animals were also implanted with stimulating (St.) and recording (Rec.) electrodes aimed to activate right medial perforant pathway projections to the dentate gyrus. (*B*, *C*) Two photomicrographs illustrating the location of recording (*B*) and stimulating (*C*) sites (arrows). Calibration bar is 500 μm. Abbreviations: D, L, M, V, dorsal, lateral, medial, ventral; DG, dentate gyrus; US, unconditioned stimulus; CS, conditioned stimulus; EMG, electromyography.

FIG. 2. Input/output curves and paired-pulse facilitation at the perforant pathway-dentate gyrus synapse for the three groups of animals. (*A*) Relationships between the intensity (in mA) of single stimuli presented to the perforant pathway and the slope of the fEPSPs evoked at the dentate gyrus in control (Ctl, black circles), hypothyroid (Hypo, black triangles), and recovery (Rec, white triangles) groups. Illustrated data correspond to mean ± SEM values collected from n = 10 animals/group. *, significant differences ($P \leq 0.001$) between the Ctl and the other two groups; $^{+}$, significant differences ($P \leq 0.001$) between the Hypo and the Rec groups. 1 and 2 indicate the two intensity values selected for evoking the representative averaged (3 times) records illustrated at the top for the 3 experimental groups. (*B*) Evolution of the paired-pulse ratio [(2nd/1st) × 100] with increasing inter-stimulus intervals in the three groups of animals (lettering and symbol codes as in *A*). Illustrated data correspond to mean ± SEM values collected from n = 10 animals/group. Representative averaged (n = 3) records collected from a control animal are illustrated at the top.

FIG. 3. Learning curves and evolution of fEPSPs evoked at the perforant pathway-dentate gyrus synapse across the classical conditioning of eyelid responses in the three experimental groups. (*A*) At the top is illustrated a schematic representation of the conditioning paradigm, indicating the presentation and characteristics of CS and US stimuli. Examples of EMG and dentate gyrus extracellular recordings collected from the 8th conditioning session of control (Ctl), hypothyroid (Hypo), and recovery (Rec) animals are also illustrated. The moment at which an electrical pulse was presented to the perforant pathway (St. PP) is indicated by arrows. Calibrations as indicated. (*B*) Field EPSPs evoked at the perforant pathway-dentate gyrus synapse in representative animals of the Ctl (black circle), Hypo (black triangle), and Rec (white triangle) groups and collected from the 1st (1) and 9th (2) conditioning sessions. Illustrated traces correspond to the average of ≥ 10 records. (*C*) Evolution of fEPSPs evoked in the

dentate gyrus area by a single pulse presented to the perforant pathway 300 ms after CS presentation in the three groups of animals. Illustrated data correspond to mean ± SEM values collected from n = 20 implanted electrodes/group. Sessions 1st and 9th of the conditioning protocol (which are represented in 3B) are indicated as 1 and 2 under square brackets. (*D*) Evolution of the percentage (%) of conditioned responses during the successive conditioning and extinction sessions for the three groups of animals. Abbreviations and codes as indicated in (*C*). Illustrated data correspond to mean ± SEM values collected from n = 10 animals/group. *, significant differences ($P \leq 0.001$) between the Hypo and the other two (Ctl and Rec) groups.

FIG. 4. A linear regression analysis of the evolution of fEPSP slopes evoked at the perforant pathway-dentate gyrus synapse across conditioning sessions in the three experimental groups. (*A*-*C*) Each regression line corresponds to data collected from one of the 20 recording electrodes included in the analysis illustrated in Fig. 3*C*. Arrows and dashed lines indicated ± 1 SD for data collected from each group.

FIG. 5. LTP induction in the dentate gyrus area following HFS of the perforant pathway in the three experimental groups. (*A*) Representative fEPSPs recorded from control (Ctl, black circle), hypothyroid (Hypo, black triangle), and recovery (Rec, white triangle) groups before (Baseline) and 15 min (Day 1), one day (Day 2), and 2 days (Day 3) after HFS. Each trace is the average of 20 records. For comparative purposes, baseline records are included as dashed traces in the other three sets of records. Calibration at the bottom is for all of the recordings. **(***B*) Graphs illustrating the time course of changes in fEPSPs (mean ± SEM) following HFS stimulation of the perforant pathway. The HFS train was presented after 15 min of baseline recordings, at the time marked by the dashed line. Field EPSPs are given as a percentage of the baseline (100%) slope. For the sake of clarity, the three experimental groups (n = 10 animals/group) are represented in pairs (top: Ctl against Hypo; middle: Ctl against Rec; and bottom: Rec against Hypo). *, significant differences ($P \leq 0.001$) between the Ctl and the other two (Hypo and Rec) groups. (*C*) Quantitative analysis of fEPSP evolution at the indicated times. Each bar corresponds to data collected for 15 min during the periods indicated in *B* (Baseline, 1, 2, and 3). *, significant differences ($P < 0.001$) between Hypo and Rec groups as compared with Ctl values.

FIG. 6. The density of PV+ terminals is increased in the dentate gyrus and CA1 region

of thyroidectomized rats and is not recovered by thyroid hormones treatment. This figure shows representative microphotographs of coronal sections of the dorsal hippocampus immunostained for PV and developed by the peroxidase method. The expression of PV in the dentate gyrus (a, c, e) and CA1 hippocampal (b, d, f) regions is illustrated for Ctl (a,b), Hypo (c,d) and Rec (e,f) rats. MoL: molecular layer; GL, granular layer; PoL: polymorphic layer; SO: stratum oriens; SP: stratum piramidale; SR: stratum radiatum. The arrows point to PV+ terminals around pyramidal and granular neurons. Scale bar, 20 μm.

List of abbreviations used in the manuscript

CS, conditioned stimulus
Ctl, control group
EMG, electromyography
HFS, high frequency stimulation
Hypo, hypothyroid group
LTP, long-term potentiation
Rec, recovery group
US, unconditioned stimulus

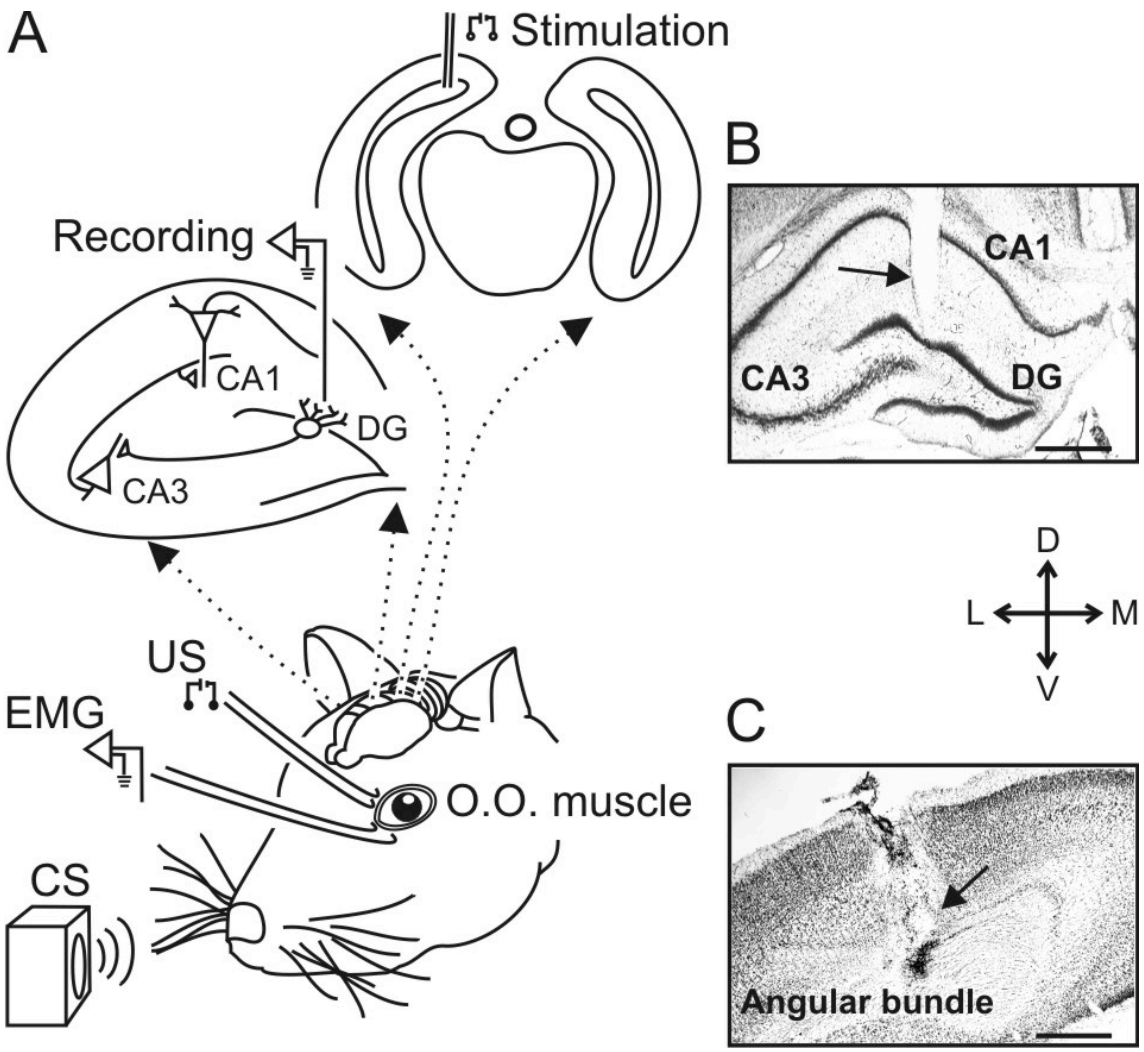
A
Stimulation
Recording
CA1
DG
CA3
US
EMG
O.O. muscle
CS
B
CA1
CA3
DG
D
L
M
V
C
Angular bundle

FIGURE 1

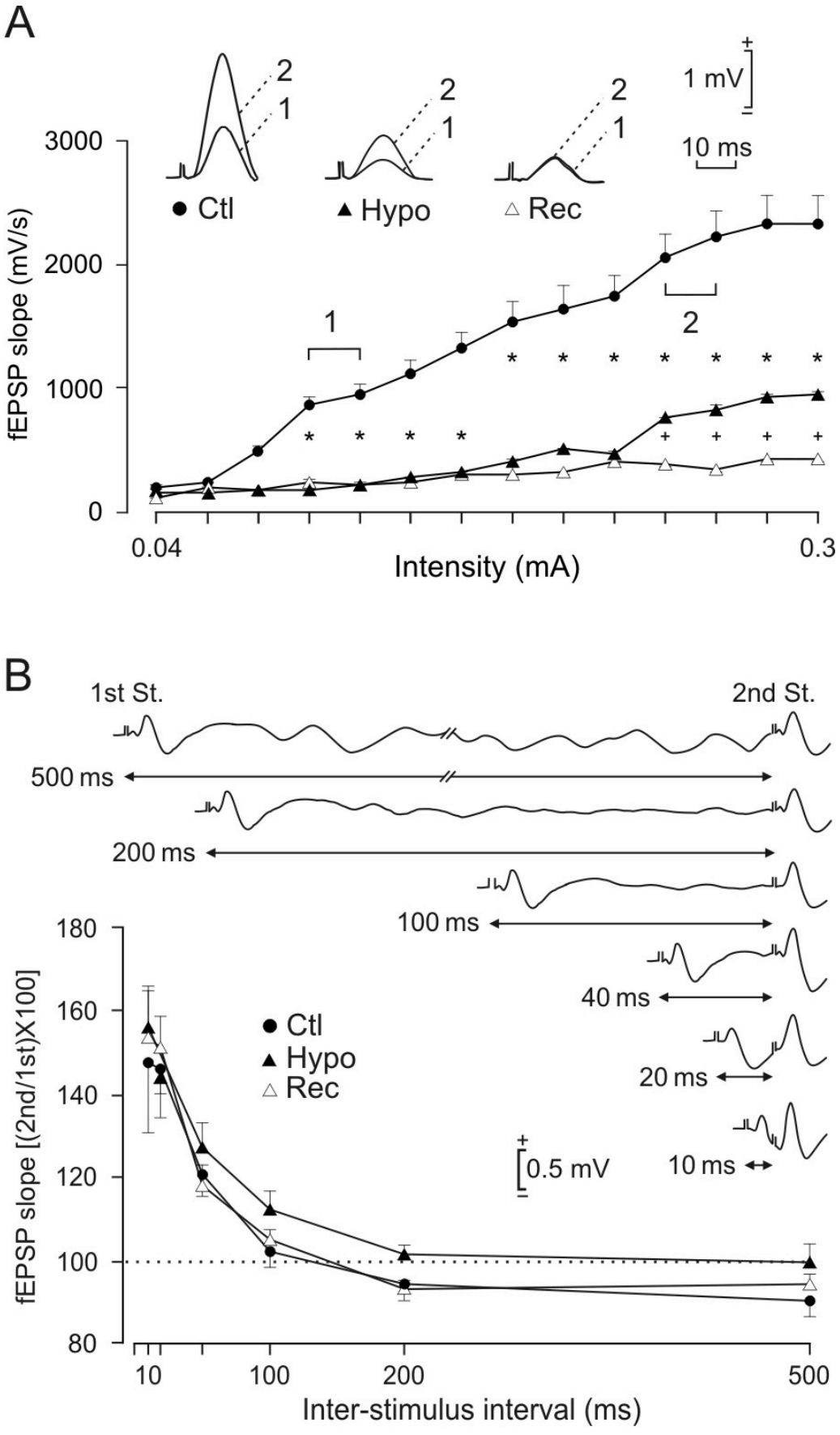
A
2
1
2
1
2
1
1 mV
10 ms
3000
2000
1000
0
fEPSP slope (mV/s)
Ctl
Hypo
Rec
1
2
0.04
0.3
Intensity (mA)
B
1st St.
2nd St.
500 ms
200 ms
100 ms
40 ms
20 ms
10 ms
0.5 mV
180
160
140
120
100
80
fEPSP slope [(2nd/1st)X100]
Ctl
Hypo
Rec
10
100
200
500
Inter-stimulus interval (ms)

FIGURE 2

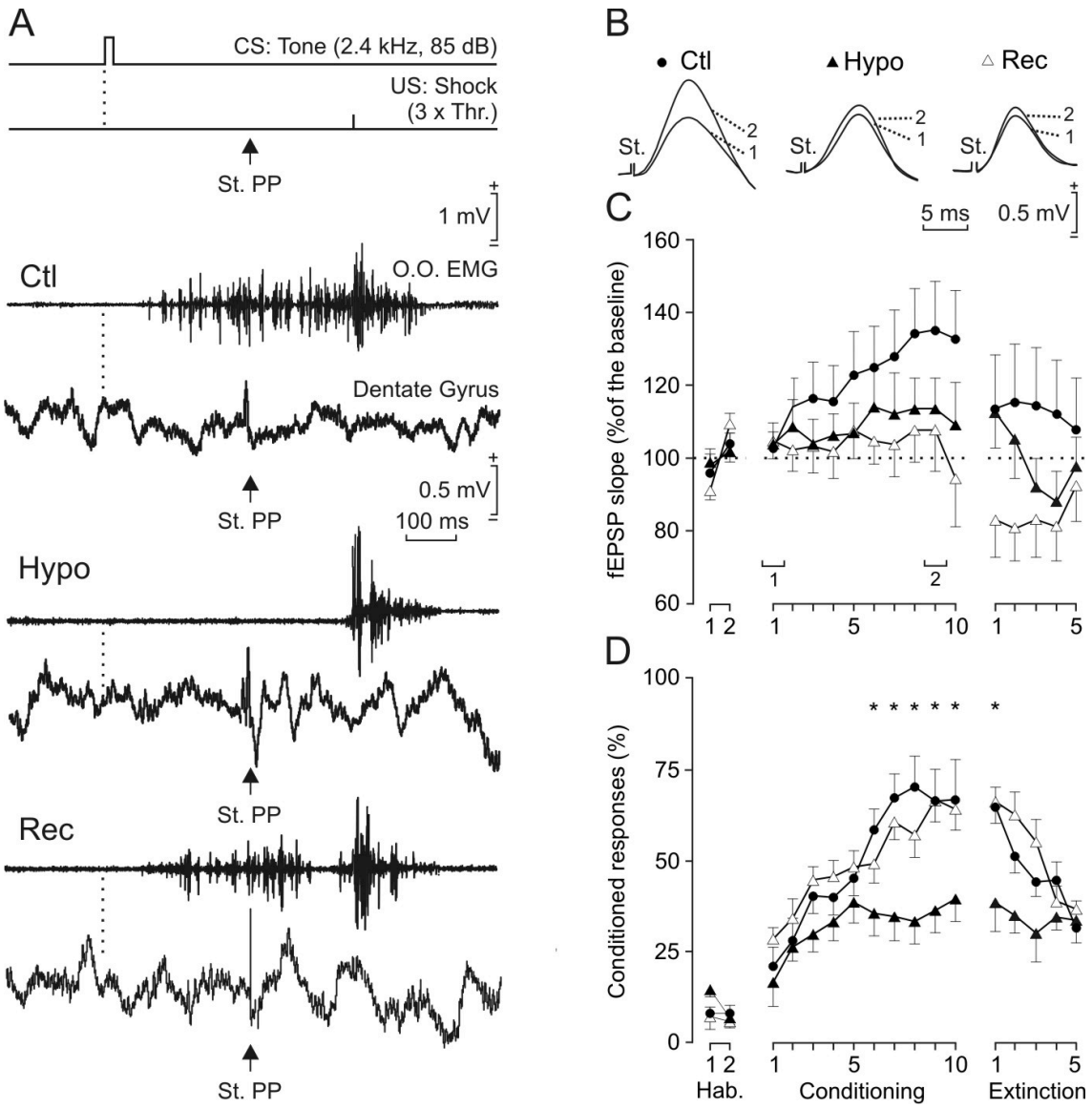
A
CS: Tone (2.4 kHz, 85 dB)
US: Shock
(3 x Thr.)
St. PP
1 mV
Ctl
O.O. EMG
Dentate Gyrus
St. PP
0.5 mV
100 ms
Hypo
St. PP
Rec
St. PP
B
Ctl
Hypo
Rec
St.
2
1
5 ms
0.5 mV
C
fEPSP slope (%of the baseline)
160
140
120
100
80
60
1 2
1
5
10
2
D
Conditioned responses (%)
100
75
50
25
0
Hab.
Conditioning
Extinction

FIGURE 3

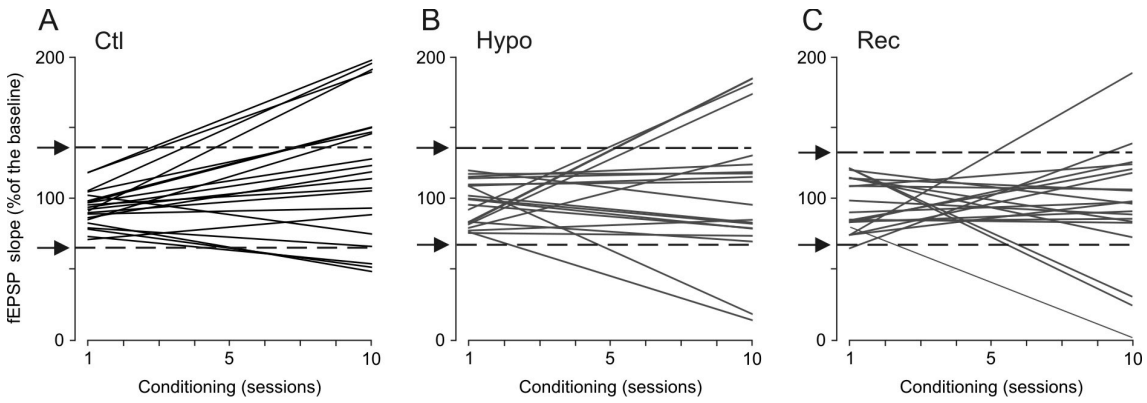
A
Ctl
B
Hypo
C
Rec
fEPSP slope (%of the baseline)
200
100
0
1
5
10
Conditioning (sessions)

FIGURE 4

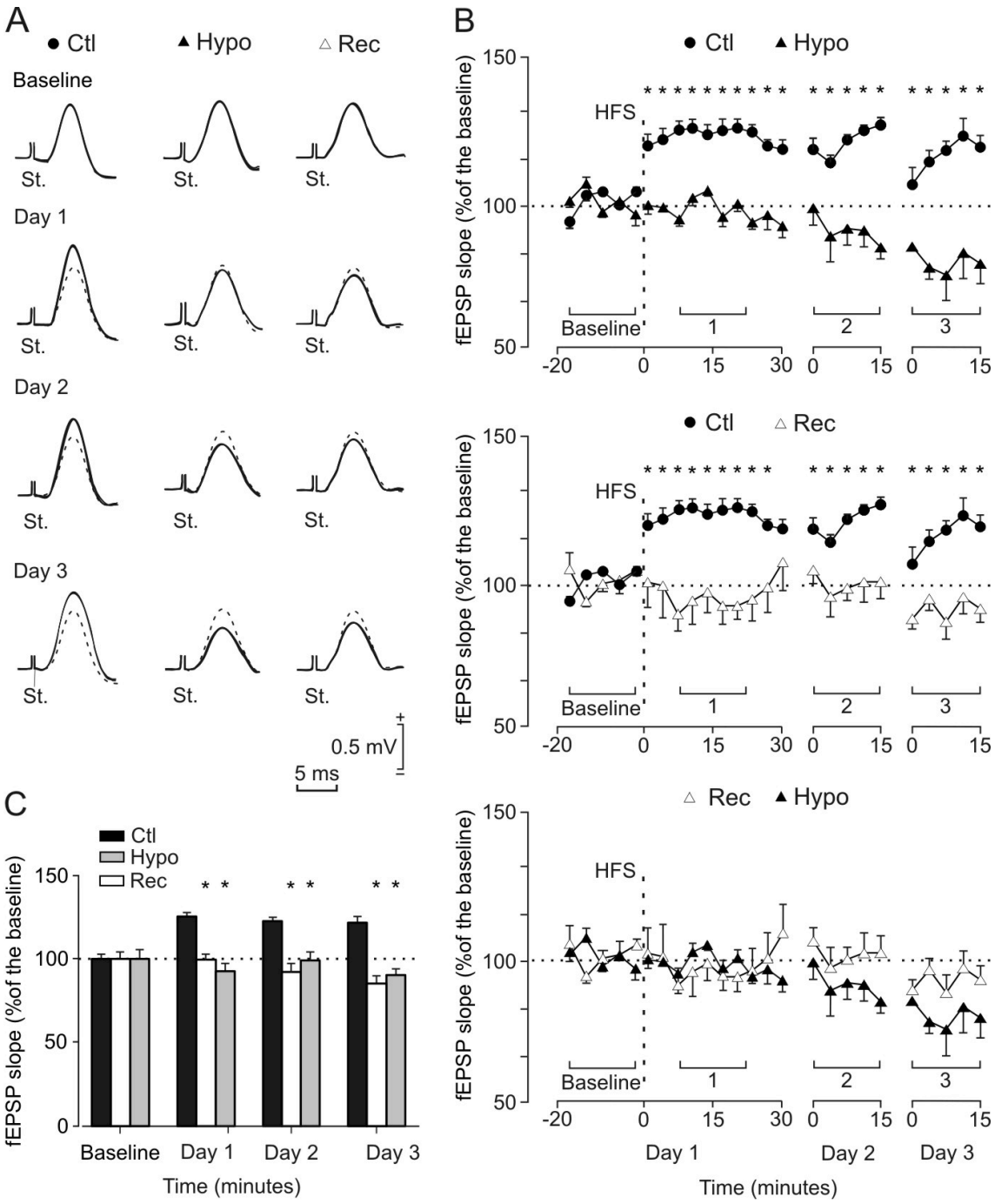
A
Ctl
Hypo
Rec
Baseline
Day 1
Day 2
Day 3
St.
0.5 mV
5 ms
B
HFS
fEPSP slope (%of the baseline)
Baseline
1
2
3
Time (minutes)
C
Ctl
Hypo
Rec
Baseline
Day 1
Day 2
Day 3

FIGURE 5

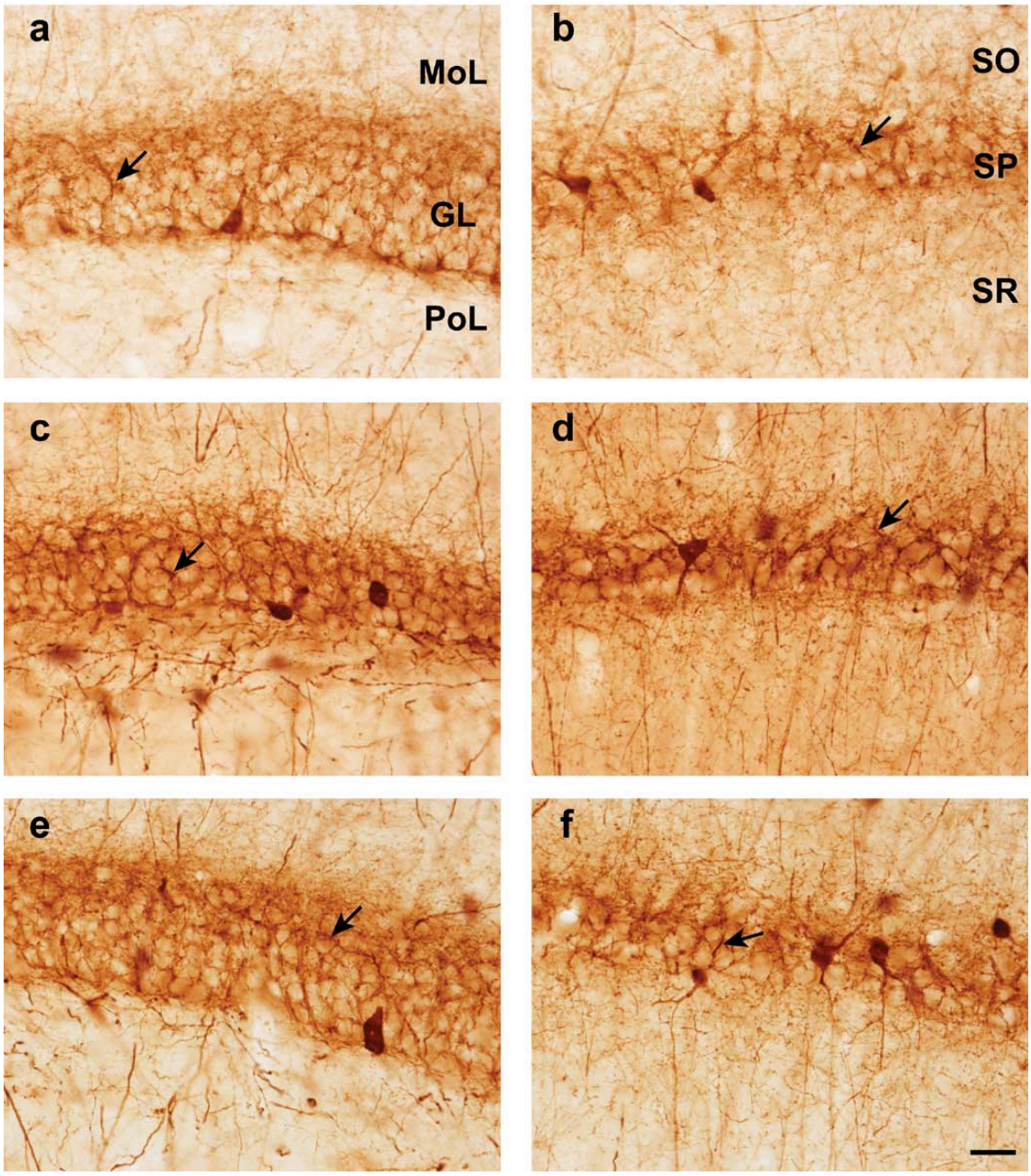

FIGURE 6