Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2014

doi: 10.1111/jnc.12685

ORIGINAL ARTICLE

A new semisynthetic derivative of sauroine induces LTP in hippocampal slices and improves learning performance in the Morris Water Maze

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Abstract

Two semisynthetic acetyl derivatives of the alkaloid sauroine from *Huperzia saururus,* monoacetyl sauroine, and diacetyl sauroine (DAS) were obtained and their chemical structures were analyzed by NMR. While monoacetyl sauroine is the typical product of acetylation, DAS is an unexpected derivative related to the keto-enol formation of sauroine. Recordings of field excitatory post-synaptic potentials from the CA1 region of rat hippocampal slices showed that only DAS acutely applied induced chemical long-term potentiation (LTP) in a dose-dependent manner with an EC_{50} of 1.15 ± 0.09 μM. This effect was blocked by 10 μM D(-)-2-amino-5-phosphonopentanoic acid (AP5), suggesting dependence on the NMDA receptor. DAS significantly increased NMDA receptor-dependent excitatory post-synaptic currents without affecting α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor-dependent currents. Repetitive administration of DAS improved visuo-spatial learning in the Morris Water Maze. In slices from rats tested in the Morris Water Maze, LTP resulting from electrical synaptic stimulation was 2.5 times larger than in controls. Concentration of DAS measured in the brain after repetitive administration was 29.5 μM. We conclude that slices perfused with DAS display a robust NMDA receptor-dependent chemical LTP. During chronic treatment, DAS enhances

Received May 22, 2013; revised manuscript received January 10, 2014; accepted February 11, 2014.

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Abbreviations used: ^{1}H-^{1}H COSY, proton–proton correlated spectroscopy; ACSF, artificial cerebrospinal fluid; AP5, D(-)-2-amino-5-phosphonopentanoic acid; br d, broad doublet; CA, *cornu ammonis* area of the hippocampus; DAS, diacetyl sauroine; dd, doublet of doublets; d, doublet; EIMS, electron ionization mass spectroscopy; fEPSPs, field excitatory post-synaptic potentials; GLC-MS, gas liquid chromatography–mass spectrometry; HMBC, heteronuclear multiple bond correlation; HSQC–DEPT, heteronuclear single quantum correlation–distortionless enhancement by polarization transfer; J, coupling constant; LTP, long-term potentiation; MAS, monoacetyl sauroine; m, multiplet; MWM, Morris Water Maze; AMPA, a-amino-3-hydroxy-5-ethylisoxazole-4-propionate; NMDA, *N*-methyl-D-aspartate; NMR, nuclear magnetic resonance; PPF, paired-pulse facilitation; ppm, parts per million; s, singlet; TBS, theta burst stimulation; td, triplet of doublets; TLC, thin layer chromatography; t, triplet; δ, chemical shift.

learning abilities through a metaplastic mechanism as revealed by the augmentation of LTP in slices. DAS, therefore, may be a promising compound as a nootropic therapeutic drug.

Keywords: learning and memory, long-term potentiation, lycopodium alkaloids, metaplasticity, sauroine acetylation, synaptic plasticity.
J. Neurochem. (2014) 10.1111/jnc.12685

There is an urgent need to develop new clinical drugs for numerous pathologies resulting from malfunctions of the CNS, such as Alzheimer's disease (Phillipson 2001). The drugs presently prescribed for the treatment of cognitive deficits are regarded as symptomatic drugs. On the other hand, cognitive decline is a common complication of aging that impacts a variety of brain functions including processing speed, inductive reasoning, and spatial learning and memory (Hedden and Gabrieli 2004).

Natural products already have a proven track record for CNS activities (Phillipson 2001) offering a rich source in the search for both pathological and natural neurodegeneration therapies. *Huperzia saururus* (Lam.) Trevis. (Lycopodeaceae) is an autochthonous species in Argentina. Its common name is 'cola de quirquincho'. Ethnomedicine attributes aphrodisiac properties to the species (Amorín 1974). Furthermore, it is mentioned because of its memory improving properties (Martinez Crovetto 1981).

Ten alkaloids belonging to the Lycopodium group have been identified (Ortega *et al.* 2004a,b; Vallejo PhD Thesis, 2009), sauroine (7α,8-*endo*-dihydroxylycopodine) being the predominant one. A previous study *in vitro* as well as *in vivo* has shown that the alkaloid extract and purified sauroine exert a facilitating effect on learning and memory processes (Ortega *et al.* 2006; Vallejo *et al.* 2007). Sauroine is the most active compound isolated from this species (Vallejo *et al.* 2009). It has been demonstrated that sauroine (3.5 μM) strongly enhances hippocampal long-term potentiation (LTP) on the perforant path-dentate granule cell synapses as well as memory retention as shown in the step-down test (Vallejo *et al.* 2009).

We created two new semisynthetic derivatives of sauroine: monoacetyl sauroine and diacetyl sauroine (MAS and DAS). In this study, we investigated the effects of either compound on synaptic plasticity recording from rat hippocampal CA3-CA1 synapses and performing behavioral tests for visuo-spatial learning using the Morris Water Maze (MWM). We found that the doubly acetylated sauroine derivative DAS, applied acutely on hippocampal slices, dose-dependently induced a NMDA receptor-dependent chemical LTP. In chronical conditions, DAS not only improves learning performance in the MWM, but also facilitates LTP induced by high-frequency electrical stimulation. Furthermore, we determined the concentrations of DAS reaching the brain and found that they coincided with the dose–response relationship for its LTP-inducing effects.

Material and methods

Materials

Column chromatography was performed on Sephadex LH-20 (Sigma-Aldrich Inc., St. Louis, MO, USA) and G-10 (Sigma Chemical Co., St. Louis, MO, USA). Thin layer chromatography was carried out on pre-coated silica gel GF_{254} plates (Merck, Darmstadt, Germany), and alkaloids were revealed under UV light after spraying the plates with Dragendorff's reagent. All the reagents used in the chemical modification and purification were of analytical grade.

Plant material was collected and specified and extraction of sauroine performed as described in Vallejo *et al.* (2009).

Derivatization of sauroine

An acetic anhydride (0.1 mL) solution of sauroine (40 mg) catalyzed by pyridine (0.1 mL) was stirred at 25°C for 48 h. After usual work-up, the crude mixture was subjected to thin layer chromatography using ciclohexane/diethylamine (1 : 1) as mobile phase and two compounds were obtained as a result (Fig. 1).

Identification of sauroine and its derivatives

The identification of the compounds was carried out using gas liquid chromatography–mass spectrometry (GLC-MS) and NMR. GLC-MS analyses were performed in a Shimadzu QP5050A equipment (Shimadzu Co., Kyoto, Japan) using a VF-5 ms (5% phenyl and 95% dimethyl polisiloxane) column. One- and two-dimensional ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance II 400 NMR spectrometer (400.16 MHz for ^{1}H and 100.6 MHz for ^{13}C; Bruker Co., Karlsruhe, Germany), using $CDCl_3$ as solvent. WIN-NMR software was used to process the NMR data.

Animals

All procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, Vicerrectoría de Investigación y Desarrollo, University of Santiago de Chile.

Sprague–Dawley rats (3–4 weeks postnatal) were purchased from the animal care unit at the University of Santiago of Chile and housed two per cage with food and water available *ad libitum* under a 12:12-h light–dark cycle.

Hippocampal slices and electrophysiology

Hippocampal slices were prepared, maintained, and recorded from as described before (Rozas *et al.* 2012). Briefly, slices of 300–400 μM thickness were maintained by 1 h before to be transfer to a recording chamber contained artificial cerebrospinal fluid (ACSF): in mM, 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26

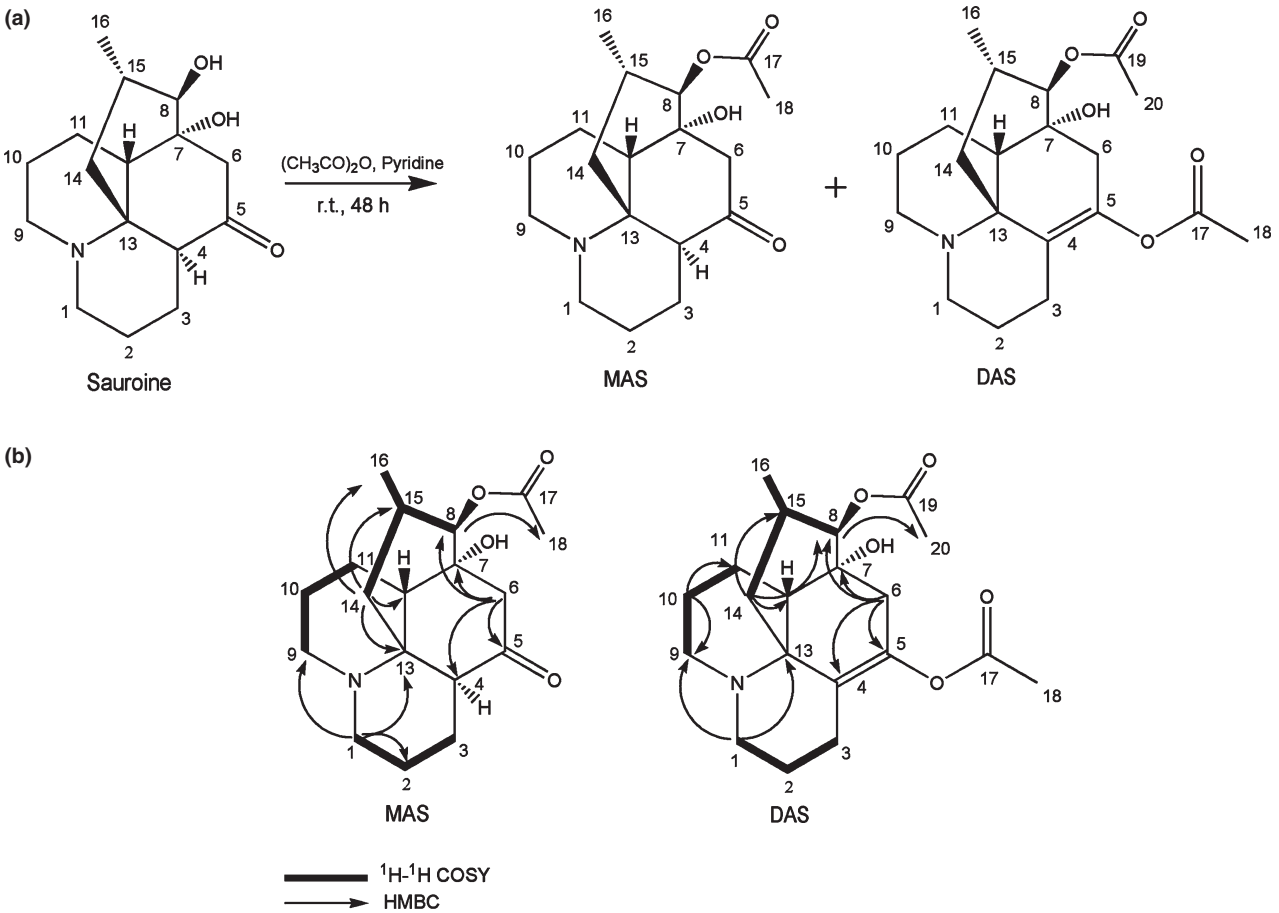

Fig. 1 Synthesis and structure of monoacetyl sauroine (MAS) and diacetyl sauroine (DAS). (a) Semisynthesis of MAS and DAS from sauroine. Reaction of sauroine with acetic anhydride led to the expected product MAS, where the hydroxyl group in C8 was acetylated. Owing to the low reactivity of the tertiary alcohol, the hydroxyl group in C7 remained unaltered. Instead, DAS was also generated and an additional acetyl group in C5 was introduced. (b) Selected 2D NMR correlations for MAS and DAS. A COSY experiment provided information about proton-to-proton interaction. Vicinal proton coupling allows the construction of the marked skeleton. On the other hand, heteronuclear multiple bond correlation (HMBC) spectrum discloses the heteronuclear correlation between protons bound to a specific carbon atom. Similar HMBC correlations were found for MAS and DAS.

$NaHCO_3$ and 10 Glucose (pH 7.4, in 95% O_2/5% CO_2). Hippocampal slices were superfused with ACSF at a rate of 1 mL/min at 30°C.

Field excitatory post-synaptic potentials (fEPSPs) were evoked by square current pulses (0.2 ms) delivered with a bipolar stimulation electrode (200 μm diameter; FHC Inc., Bowdoinham, ME, USA) localized in the Schaeffer collateral–commissural fibers and recorded using glass microelectrodes (1-2 MΩ) filled with ACSF from the *stratum radiatum* of the CA1 region. Test pulse stimulation intensity was adjusted initially to evoke 50% of the maximal response. After recording a stable baseline for at least 20 min (testing stimulation applied every 15 s) LTP was induced with theta burst stimulation (TBS; consisting of 5 trains of 10 bursts at 5 Hz each, 1 burst = 4 pulses at 100 Hz). In all experiments, the fEPSP recordings were continued for 60 min after initiating TBS. Recordings were filtered at 10 kHz and digitized at 5 kHz using Igor Pro (WaveMetrics Inc., Lake Oswego, OR, USA). MAS or DAS as hydrochloride was diluted in ACSF and applied by superfusion during the whole experiment, starting 10 min before TBS. The synaptic responses were quantified as the peak of the initial slope of evoked fEPSPs and plotted as absolute value or as percentage change referred to the slope measured during baseline recording.

To differentiate between pre- and post-synaptic components of the synaptic responses, we used the following paired-pulse stimulation protocol: two pulses were applied every 15 s with interstimulus intervals starting with 20 ms and ending with 2560 ms, doubling the interval after each trial. This protocol was applied 20 min before and 30 min after TBS. The results are presented as the ratio between the initial slopes of fEPSPs evoked by the second and the first stimulus. This measure reflects the quantal release of neurotransmitter from pre-synaptic components. If a compound modifies the paired-pulse facilitation (PPF) curve, it can be concluded that it acts at a pre-synaptic level. In slices obtained from rats treated with sauroine derivatives during behavioral experiments, paired pulses of 50-ms intervals were

applied during the entire TBS-induced LTP protocol (Schulz *et al.* 1994).

MAS or DAS as hydrochloride was diluted in ACSF and applied 10 min before TBS and continued for another 10 min. Average slopes recorded during 20 min before TBS were compared to data obtained between 40 and 60 min after TBS.

For whole-cell voltage clamp recording (Morales *et al.* 2002), the cells were visually identified with an infrared differential interference contrast microscope Zeiss (Oberkochem, Germany). Patch pipettes (2–4 MΩ) were filled with internal solution consisting of (in mM): 130 K-gluconate, 8 KCl, 10 EGTA, 10 HEPES, and 1 QX-314, pH 7.4 (CsOH, 275–285 mOsm). The junction potential (typically < 5 mV) was compensated. Only cells with membrane potentials more negative than −65 mV, access resistance < 20 MΩ (8–18 MΩ, compensated at 80%), and input resistance > 100 MΩ (130–410 MΩ) were studied. Cells were discarded if the input or the access resistance changed by more than 15%. Synaptic responses were evoked by stimulation of Schaeffer collaterals and recorded from CA1 neurons. The AMPA and NMDA receptor-mediated excitatory post-synaptic currents (AMPA or NMDA EPSCs) were recorded at holding potentials of −65 and +40 mV, respectively. Bathing solution was ACSF with 10-μM picrotoxin; a blocker of $GABA_A$ receptors. AMPA receptor-dependent currents were measured at maximal amplitude and NMDA receptor-dependent currents 200 ms after peak to avoid contamination by AMPA currents.

Morris Water Maze

Over 2 days, the animals were acclimatized to the holding room, maintained at a temperature of 22–23°C. DAS as hydrochloride or vehicle (isotonic saline) was administered 1 h before conducting experiments similar to that described in Arias-Cavieres *et al.* (2010). Two groups of eight animals received 2 mg/kg of DAS intraperitoneally, or saline, respectively, in the training sessions. No injections were given before the first two sessions and the last one. Behavioral training and testing were conducted in a circular pool (diameter 180 cm, depth 60 cm) painted white and, half-way filled with water rendered opaque with white latex paint, and maintained at a temperature of 24 ± 2°C. Four geometrical figures in black and white (approx. 20 cm wide) attached to the upper rim of the pool and situated at an angle of 90° to each other provided the optical cues, thus forming four quadrants. A white curtain surrounded the pool to conceal external cues. Behavioral data were recorded and analyzed using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA). In the first two sessions (a session consisted of only one trial) the rats were allowed to swim for 2 min in the pool. In the subsequent four sessions a circular white platform (12 cm wide) was present, 1 cm above water level at a distance of 20 cm from the pool's edge in the middle of a quadrant whose position was changed arbitrarily from session to session. During the following eight sessions the escape platform was rendered invisible by placing it 2 cm below the water surface without changing its position. Subjects were allowed to swim until they had placed all four paws on the platform, or until 120 s had elapsed. Animals that did not get to the platform in 2 min were placed on it. The animals were left on the platform for 30 s after the end of each trial. Finally, one more session was performed with the platform removed. For all groups, experimental sessions were run twice daily beginning at 10 a.m. and 15 p.m. for 6 days. Animal movements were monitored with a video camera (Microsoft LifeCam Studio™ Webcam, Sandyford Industrial Estate Dublin, Ireland) mounted above the pool.

The day after the last session in the maze, the rats were killed by decapitation under halothane anesthesia and their brains removed. Next, electrophysiological procedures were carried out following the same steps as described above for the perfusion experiments, but using ACSF only.

DAS quantification in brain and plasma

Two groups of rats were administered i.p. with DAS (2 mg/kg), the first with a single dose (n = 6), and the second during 7 days, twice a day (n = 6). Animals were killed by decapitation under halothane anesthesia, keeping their brains and blood, the first 45 min after injection and the second 24 h after the last injection, as in the MWM experiments.

Plasma was obtained from the blood by centrifuging (2500 g, 10 min). Then, it was acidified until pH 2 by adding HCl 0.1N and extracted five times with dichloromethane to remove interference material. NaOH 0.1N was added to the aqueous phase until pH 10, and the solution was extracted 10 times with ethyl acetate. Fractions from ethyl acetate were reunited and concentrated to dryness.

Brain was homogenized in phosphate-buffered saline and then, HCl 0.1N was added until pH 2. After centrifugation, the supernatant was kept and extracted as described above for the plasma.

Later, all samples were analyzed by using a GLC-MS equipment and the internal standard method. A calibration curve was developed with freshly prepared mixed solutions of DAS (from 0.05 μg/mL to 10 μg/mL) and caffeine (3 μg/mL) as internal standard, in 100 μL of ethanol-acetone (1 : 1). Each DAS concentration was injected by triplicate. Thus, the areas ratio (DAS/caffeine) was calculated to construct the curve. Plasma and brain purified samples containing the internal standard (3 μg/mL) were reconstituted in 25 μL of ethanol-acetone (1 : 1) and immediately injected. DAS concentration in both type of samples was determined from the curve.

Statistics

Data are presented as mean ± SEM displaying the percentage of the baseline value (average slope of fEPSPs measured before the TBS protocol). LTP was measured during the final 20 min of the recording and presented as the averaged percentage of baseline.

The LTP data of the different groups were evaluated using the Mann–Whitney U-test, while the Wilcoxon signed rank test was applied for comparing values before and after TBS in the same group. A value of $p < 0.05$ was considered significant.

Results

Identification of sauroine and derivatives

Sauroine of 40 mg (0.08% yield) was isolated and purified from 5 kg of aerial parts of *H. saururus*. Its identification was performed by spectroscopic techniques and compared with an authentic sample previously obtained as described before (Ortega *et al.* 2004b).

Two alkaloid sauroine derivatives were semisynthesized: 7α-hydroxy,8-*endo*-acetyl-lycopodine (MAS) and 7α-hydroxy-5,

8-diacetyl-lycopodine (diacetyl sauroine, DAS; Fig. 1a). Both compounds were obtained as colorless oil in 47% (20 mg) and 42% (18 mg) yield, respectively. The EIMS data were m/z = 321 and m/z = 363, respectively. ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) data are shown in Table 1. Eighteen carbon signals were observed for MAS, whereas DAS spectrum showed 20 carbon signals. The gross structures of MAS and DAS were validated by analyzing 2D NMR [proton–proton correlated spectroscopy (^{1}H-^{1}H COSY), heteronuclear single quantum correlation (HSQC)] and heteronuclear multiple bond correlation (HMBC) data.

The HSQC-distortionless enhancement by polarization transfer spectrum of MAS (see Figure S1) revealed the presence of two methyl, eight methylene, and four methine groups. The ^{1}H-^{1}H COSY spectrum of this derivative (Fig. 1b) disclosed the following connectivities: C-1 to C-2, C-9 and C-13, C-9 to C-10 and C-13, and C-8 to C-6, C-12, C-15 and C-17. The HMBC correlations (Fig. 1b) led us to connect these three partial units. H-1 is connected to C-2, C-9, and C-13; H-6 to C-3, C-4, C-5, C-7, C-8, and C-12; H-14 to C-8, C-12, C-13, C-15, and C-16; H-10 to C-12, C-13, C-15, and C-16.

Regarding DAS, the HSQC-distortionless enhancement by polarization transfer spectrum (see Figure S2) showed the presence of three methyl, eight methylene, and three methine groups. The ^{1}H-^{1}H COSY (Fig. 1b) revealed the connections of C-1 to C-3 and C-9, C-9 to C-12, and C-8 to C-14, C-15, C-16, and C-18. The important HMBC correlations (Fig. 1b) led us to connect these three partial units. H-1 is connected to C-10, C-13, and C-20; H-6 to C-4, C-5, C-7, C-8, C-12,

Table 1 ^{1}H and ^{13}C NMR data of MAS and DAS in $CDCl_3$

	MAS		DAS	
Position	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	a.3.16 (br d 12) b.3.56 (m)	47.23	a. 2.57 (br d 12.0, 2.38) b. 2.93 (td 12.4)	48.82
2	a.1.55–1.73(m) b. 1.82–1.94 (m)	18.05	a.1.89 (m) b. 2.41 (m)	19.88
3	a. 1.55–1.73 (m) b. 2.23 (br d, 14.2)	17.83	a. 1.28 (m b. 1.96 (m)	16.65
4	2.97 (br d, 12)	42.75[a]	–[b]	119.73[a]
5	–	205.28[c]	–	140.21[c]
6	a.2.43 (d, 16.7) b.2.84 (dd, 16.7, 1)	44.19[d]	a. 2.19 (d, 17.5 b. 2.41 (bd, 17.4)	32.51[d]
7	–	73.93	–	73.44
8	4.71 (d, 10.7)	82.11	4.56 (d, 10.7)	84.6
9	a.2.92 (br d 12.6) b.3.40 (td, 12.9, 3)	47.27	a. 2.72 (d 14) b. 3.40 (td, 13.7, 3.39)	47.20
10	a.2.08 (br d, 12.7) b.2.33 (br d, 14.3)	22.71	a. 1.59 (td 12.8, 3.4), b. 1.79 (td 12.8, 3)	25.28
11	a. 1.55–1.73(m) b.1.82–1.94 (m)	17.83	a. 1.30 (m) b. 1.95 (m)	21.10
12	2.45 (d, 11.9)	47.97	1.53 (dd, 12.5, 3.5)	49.68
13	–	62.35	–	59.19
14	a.2.05 (d 12.9 b.2.52 (dd, 13.7, 4.7)	37.45	a. 1.22 (m) b. 2.47 (d, 8.28)	42.16
15	1.44 (m)	30.39	2.05 (m)	30.20
16	0.95 (d, 6.2)	18.66	0.93(d, 6.4)	18.39
17		172.20	–	172.85
18		20.89	2.14 (s)	20.62
19	–	–	–	168.59
20	–	–	2.16 (s)[e]	20.98

[a]Differences in chemical shifts for C4 in MAS and DAS were observed.

[b]The lack of H signal correlating to C4 in DAS also supports the double bond generation.

[c]C5 chemical shift was modified as well in DAS.

[d]Position of C6 was also modified.

[e]Signal of the second acetyl group is shown in C20 of DAS.

Main differences can be seen in C4, C5, C6, and C20.

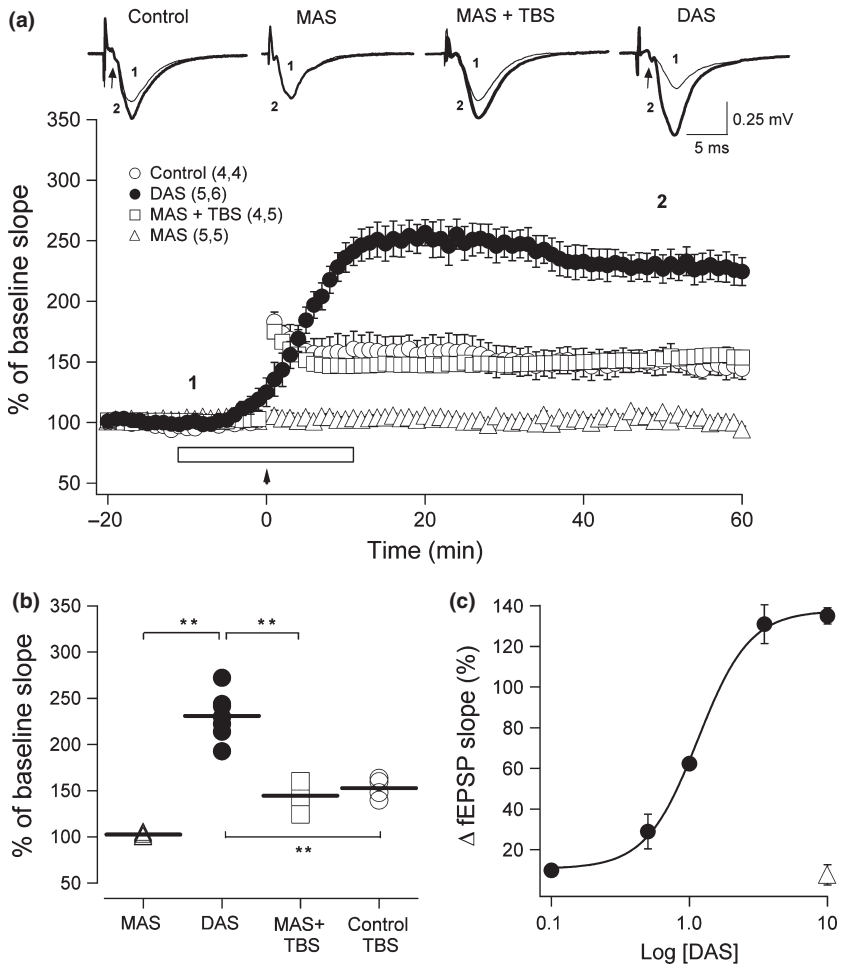

Fig. 2 Diacetyl sauroine (DAS) induces chemical long-term potentiation (LTP) in the CA1 region of the hippocampus *in vitro*, whereas monoacetyl sauroine (MAS) has no effect. (a) Time course of the LTP in rat hippocampal slices after perfusion (bar) with 3.5 μM of DAS during 20 min (solid circles), after addition of 3.5 μM of MAS (open triangle), and LTP induced by theta burst stimulation (TBS) (arrow) under control condition (open circles) and in presence of MAS (open squares). Numbers in parentheses: number of rats, number of slices. Insets above – Representative recordings; average of 10 traces under the four conditions at time marked 1 (thin) and 2 (thick) in the main panel. (b) Quantification of the chemical LTP induced by DAS (means of 10 min); average MAS: 102.61 ± 1.09% (n = 5.5); average DAS: 230.92 ± 9.52% (n = 6.7) (**p < 0.01); average control (TBS-induced LTP): 152.73 ± 5.49%, (n = 4.4); MAS+TBS: 144.53 ± 6.81%, (n = 4.5) (**p < 0.01). (c) Concentration–response relationship of the chemical LTP induced by DAS. Each symbol represents the slope of field excitatory post-synaptic potential (fEPSP) measured 50 min after inducing LTP, presented as percentage of the response obtained in control conditions (0.1 μM: **p = 0.008, n = 3; 0.5 μM: **p = 0.008, n = 4; 1.0 μM: **p = 0.008, n = 3; 3.5 μM: **p = 0.003, n = 7; 10 μM: **p = 0.001, n = 3). Increasing the concentration of MAS to 10 μM did not cause a significant difference either (open triangle, n = 3, p = 0.26; average ± SEM).

and C-13; H-14 to C-4, C-8, C-12, C-13, C-15, and C-16; H-10 to C-1, C-9, C-11, and C-12. It is important to point out that vinyl carbon signals were observed for C-4 and C-5 rather than methine and carbonyl signals, respectively. These facts support the presence of the double bond between C-4 and C-5.

DAS induces LTP at CA3-CA1 synapses of the hippocampus

To determine the effects of MAS and DAS on synaptic plasticity, we performed field potential recordings in CA1 of rat hippocampal slices. Figure 2 illustrates the effects of acute applications (3.5 μM, 20 min) of MAS and DAS on the LTP. MAS (n = 5.5) failed to cause any significant difference in the TBS-dependent LTPs compared to controls (n = 4.4) or in the basal synaptic response (n = 4, p > 0.05). However, acutely applied DAS strongly increased the synaptic response without TBS stimulation from baseline (100%) to 230.92 ± 9.52% with DAS (n = 5.7; p < 0.01; Fig. 2a, b). The synaptic response remained increased for at least 40 min thus fulfilling the requirements of LTP. The DAS effect on LTP was concentration dependent since concentrations of 0.1, 0.5, 1.0, and 10 μM DAS increased

the magnitude of LTP accordingly (Fig. 2c). The experimental data were fitting a Hill curve with an EC_{50} of 1.15 ± 0.09 μM and a maximal response of 137.23 ± 3.49% above base line level.

The phenomenon has been called 'Chemical LTP' since it is induced solely by the application of a substance rather than by high-frequency electric stimulation. Application of TBS after DAS (3.5 μM) did not increase further the magnitude of the chemical LTP (DAS: 224.99 ± 3.07%; DAS + TBS: 225.17 ± 3.30%; $p = 0.5$), indicating that at the concentration used, the LTP had reached saturation (see Figure S3). Moreover, when DAS was applied after TBS potentiation, the resulting increase was similar to the one reached with DAS alone (TBS+DAS: 237.01 ± 5.99%; DAS: 224.99 ± 3.07%; $p > 0.05$).

The magnitude of LTP increase induced by DAS was significantly larger than the TBS-dependent LTP in control and with MAS (DAS: 249.07 ± 9.05, $n = 5.6$; Control: 152.73 ± 5.48, $n = 4.4$; MAS + TBS: 144.53 ± 6.81, $n = 4.5$; $^{**}p < 0.01$; Fig. 2b). A higher concentration of MAS (10 μM) was also unable to induce a significant effect (Fig. 2c; $n = 3$; $p = 0.25$).

The enhancement of LTP caused by DAS was not attributable to changes in the recruitment of pre-synaptic fibers, since no change was observed in the magnitude of the pre-synaptic fiber volley under any condition (arrow in traces, Fig. 3).

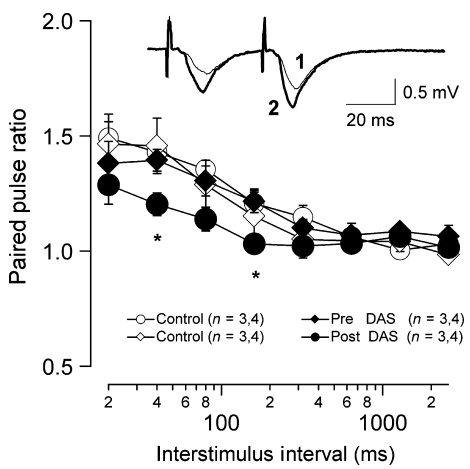

Fig. 3 Diacetyl sauroine (DAS)-dependent chemical long-term potentiation (LTP) involves pre-synaptic action. Synaptic responses evoked by paired-pulse protocols (pre- and post-DAS) in the presence and absence of DAS. Two synaptic stimulations were applied every 15 s; interstimulus intervals from 20 to 2560 ms, doubling the interval after each paired stimulation. Symbols represent ratios of the field excitatory post-synaptic potential (fEPSP) slope of the second versus the first pulse ± SEM, for each interval. Insets – representative traces of three mean field responses at 40-ms intervals (1 (thin) before and 2 (thick) after LTP). *Significant difference with $p < 0.05$.

Chemical LTP induced by DAS involves modification of pre-synaptic components

To determine whether DAS acts on pre-synaptic or post-synaptic components of CA3-CA1 pyramidal synapses, we analyzed the contribution of pre-synaptic terminals on the DAS-dependent increase of hippocampal LTP employing the protocol PPF. Figure 3 shows that the PPF curve changed after perfusion of DAS; a significant change of this ratio was obtained at 40-ms (1.41 ± 0.04 before LTP and 1.20 ± 0.05 during chemical LTP, $n = 4$, $^{*}p = 0.0286$) and 160-ms (1.21 ± 0.05 before LTP and 1.03 ± 0.03 during chemical LTP, $n = 4$, $^{*}p = 0.0143$) intervals comparing pre- and post-DAS (Fig. 3, $^{*}p < 0.05$). In contrast, no significant difference was observed in the paired-pulse ratios for all the interstimulus intervals tested in controls and MAS-treated slices (Fig. 3, $n = 4$, $p > 0.04$).

Taken together, these results suggest that the chemical hippocampal LTP induced by DAS involves, at least in part, modifications of pre-synaptic components at the CA3-CA1 synapse.

Chemical LTP induced by DAS is mediated by NMDA receptors

To evaluate if the chemical LTP induced by DAS is NMDA receptor dependent, we applied 100 μM D(-)-2-amino-5-phosphonopentanoic acid (AP5), an established inhibitor of the NMDA receptor. As illustrated in Fig. 4, AP5 inhibited almost completely the facilitation induced by DAS; from 220.07 ± 13.49% (DAS) to 108.20 ± 2.85% (DAS + AP5) (Fig. 4a; $^{*}p < 0.05$; $n = 3.3$). To test the possibility that DAS exerted its action directly on the NMDA receptors, we studied its effect on NMDA receptor-mediated synaptic currents employing whole-cell recording. Significant difference was observed in the amplitudes of NMDA receptor-dependent currents measured 200 ms after of peak (ACSF: 70.52 ± 6.27 pA, $n = 4$; DAS: 104.63 ± 6.21 pA, $n = 4$; $^{**}p = 0.004$). However, not significant difference was observed between the AMPA receptor-dependent currents from hippocampal slice superfused with ACSF and 3.5 μM DAS (ACSF: 306.32 ± 35.30 pA, $n = 4$; DAS: 298.83 ± 13.40 pA, $n = 4$; $p > 0.05$). The NMDA receptor-mediated, but not the AMPA receptor-mediated current was inhibited with 100 μM of AP5 from 104.63 ± 6.21pA (ACSF, $n = 4$) to 4.09 ± 1.45 pA (AP5, $n = 3$, $^{**}p < 0.004$) and from 298.93 ± 13.40 pA (ACSF, $n = 4$) to 262.38 ± 4.06 pA (AP5, $n = 3$, $p > 0.05$), respectively, indicating the viability of the neuron. To exclude the influence of neuron size in the responses, we normalized the NMDA EPSCs with the AMPA EPSCs. Under these conditions we also found significant differences between hippocampal slice superfused with ACSF and DAS (Fig. 4b, $^{**}p = 0.004$).

These results suggest that the chemical LTP induced by DAS is at least partly by a direct action on the NMDA receptor, increasing the NMDA current.

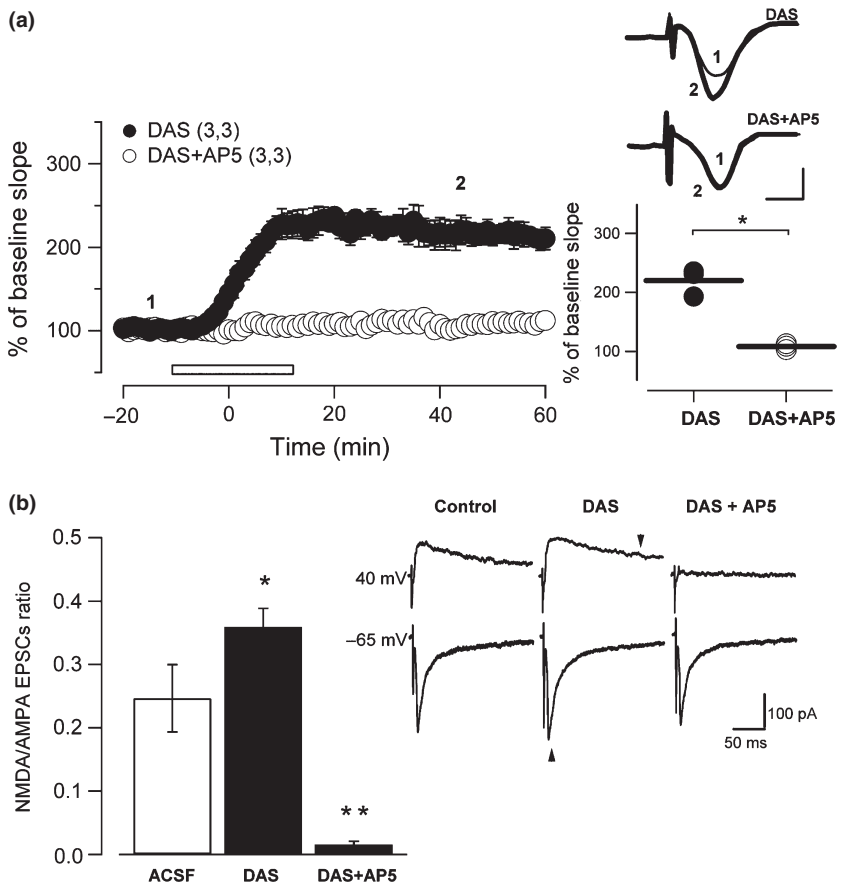

Fig. 4 Chemical long-term potentiation (LTP) induced by diacetyl sauroine (DAS) is NMDA receptor dependent. (a) Time course of the LTP in rat hippocampal slices after perfusion (bar) with 3.5 μM of DAS (solid circles) and after coapplication of 3.5 μM DAS with 100 μM AP5, a NMDA receptor blocker (open circles). Traces to the right: Representative recordings before (1, thin) and 50 min after application of drugs (2, thick). Lower right: Quantification of the effect of DAS and DAS + AP5 (average increase 220.07 ± 13.49% (DAS); 108.20 ± 2.85% (DAS + AP5) (*$p < 0.05$; $n = 3.3$). (b) Quantification of the DAS effect on NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) excitatory post-synaptic currents (EPSCs). Significant difference was observed in the NMDA/AMPA EPSCs ratio between hippocampus slices with 3.5 μM DAS (black bar) and ACSF (open bar) (0.36 ± 0.03, $n = 4$; 0.25 ± 0.05, $n = 4$; respectively. *$p < 0.05$). Inhibition of the NMDA EPSC with 100 μM AP5 (gray bar) significantly reduced the NMDA/AMPA EPSCs ratio from 0.36 ± 0.03 (DAS, $n = 4$) to 0.02 ± 0.01, (AP5, $n = 3$, **$p < 0.05$) (average ± SEM). Insets: representative recordings of average currents of five traces under the three conditions (ACSF, DAS and DAS+AP5) obtained in the same CA1 neuron. The current amplitudes were measured at the times indicated by arrowheads.

DAS improves visuo-spatial learning assessed in the MWM

Next, we studied the effect of chronic applications of DAS on behavioral parameters in the MWM (Morris 1984). In the sessions with the visible platform no difference was detected between controls and DAS-treated animals (2 mg/Kg). An overall significant decrease in latency was seen from session one to four in the treated group as well as in controls (1st = 68.51 ± 11.43 s and 4th = 22.63 ± 4.55 in saline, 1st = 68.64 ± 13.27 and 4th = 25.43 ± 4.95 in DAS, for $n = 8$ and $p < 0.01$) (Fig. 5a), displaying a similar time course of habituation to the experimental setup in treated rats as in controls. This indicates that DAS does not affect parameters such as swimming speed, vision, alertness or attention.

When visuo-spatial learning was tested, the DAS-treated group decreased its latencies for reaching the escape platform faster than the animals treated with saline only. Significant differences in the average times to reach the platform between both groups were observed: from the 5th up to 8th session (saline: 5th = 30.21 ± 5.37 and 8th = 23.40± 6.47; DAS: 5th = 15.51 ± 2.75 and 8th = 9.07 ± 1.92 $n = 8$; *$p < 0.05$ and **$p < 0.01$; Fig. 5b). These results suggest that DAS improved the acquisition of the task assigned in the MWM, *i.e.,* DAS facilitated visuo-spatial learning.

When the platform was removed, the average time of permanence in the quadrant where the platform had been situated before was significantly longer in DAS-treated rats

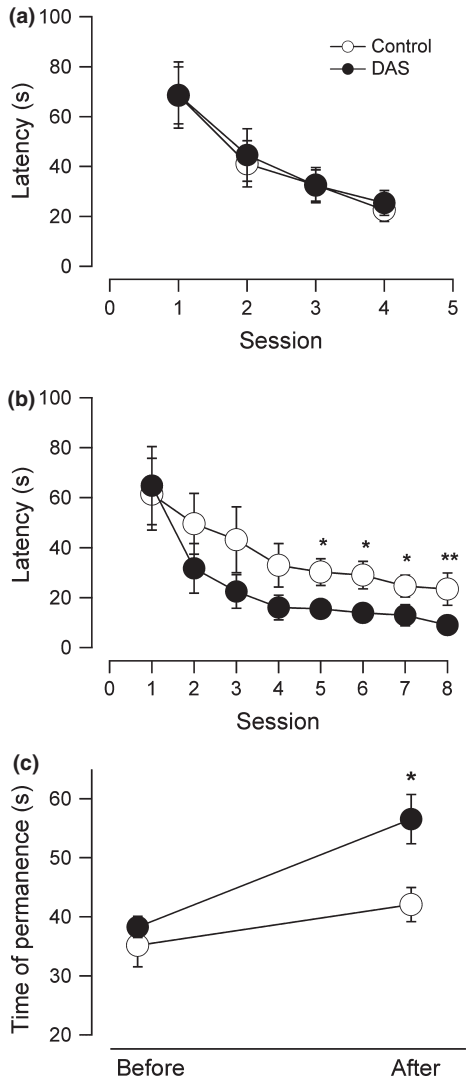

Fig. 5 Diacetyl sauroine (DAS) improves learning in the Morris Water Maze (MWM). (a) Cued trials: Average times to reach the visible platform diminished in a similar way for animals with and without treatment. Average times for the first session compared with the last session were significantly different in both groups ($n = 8$; $^{**}p < 0.01$). (b) Training trials: Visuo-spatial learning with the invisible platform improved in rats that had received DAS. Significant differences were observed from the 5th trial onward ($^{*}p < 0.05$) reaching the maximal difference in the 8th trial ($^{**}p < 0.01$). However, both groups showed a significant learning effect ($n = 8$). (c) Average time that animals stayed before ($n = 8$) and after training ($n = 8$) in the quadrant where the hidden platform had been situated during training trials. ($^{*}p < 0.05$; error bars: SEM).

(56.56 ± 4.17 s; $n = 8$) as compared to saline-treated controls (42.09 ± 3.04 s; $n = 8$; $p < 0.05$; Fig. 5c). These results indicate that DAS improved the recalling of the learned position in the MWM.

DAS applied chronically increases the magnitude of the TBS-dependent LTP in hippocampus slices

To investigate the relationship between behavior and synaptic plasticity, we recorded fEPSPs from hippocampus slices obtained from the same rats as used in the behavioral studies in the MWM 24 h after the last session (see Arias-Cavieres *et al.* 2010). As shown in Fig. 6, the magnitude of TBS-dependent LTP was significantly larger in the slices obtained from DAS-treated rats (312.15 ± 13.58%; $n = 7.8$) than in slices obtained from rats injected with saline (173.04 ± 14.71%; $n = 5.5$; $p < 0.001$). To evaluate whether this facilitation is owing to a repetitive application of DAS for several days or rather an immediate effect, we recorded LTP induced by TBS in slices from rats injected only once with DAS subject to only one training session in MWM. As illustrated in the Fig. 6a and b, no significant difference was observed between the TBS-triggered LTP from control slices (149.27 ± 4.95%; $n = 5.6$) compared to one time DAS-treated rats (160.31 ± 9.52%, $n = 3.5$; $p = 0.12$). These results suggest that prolonged treatment of DAS is required to increase the synaptic efficacy in the hippocampus.

In addition, the paired-pulse ratios for interstimulus interval of 40 ms for all the tested conditions in the last session remained unchanged (Fig. 6c). No significant difference was observed between untreated and treated rats with DAS (Control: 1.54 ± 0.11, $n = 6$; DAS: 1.52 ± 0.11, $n = 8$; $p > 0.05$), nor in control conditions pre- and post-TBS (1.52 ± 0.08 and 1.54 ± 0.11, $n = 6$, $p > 0.05$, Fig. 6c). Moreover, for the 40-ms interval in DAS presence, ratio values were not significantly modified: 1.36 ± 0.18 before LTP versus 1.52 ± 0.12 after LTP ($n = 8$, $p > 0.05$), suggesting that the DAS-dependent increase in electrically induced LTP does not involve modifications of pre-synaptic components at the CA3-CA1 synapse.

Taken together, these results imply that repetitive DAS administrations have a metaplastic effect rendering the mechanisms underlying the LTP more susceptible to synaptic stimulation. This increased susceptibility may underly the improved learning performance as observed in the experiments with the MWM. Furthermore, our data suggest that the DAS-dependent increase of hippocampal LTP only involves modifications of post-synaptic components in the CA3-CA1 synapse.

Concentrations of DAS in the brain determined simulating the conditions for acute and chronic administration

Analysis from the plasma and the brain of the first group of animals (single dose; 45 min delay) revealed the presence of DAS, in 1.5 ± 0.4 μg/mL in plasma and 7.0 ± 0.2 μg/g; 19 μM) in brain. Regarding the second group (7-day administration) DAS was not detected in plasma under the present conditions. On the other hand, DAS was determined at a level of 10.7 ± 1.1 μg/g; 29.5 μM in brain. Brain concentrations at both times were statistically significant (*t*-test, $p < 0.05$).

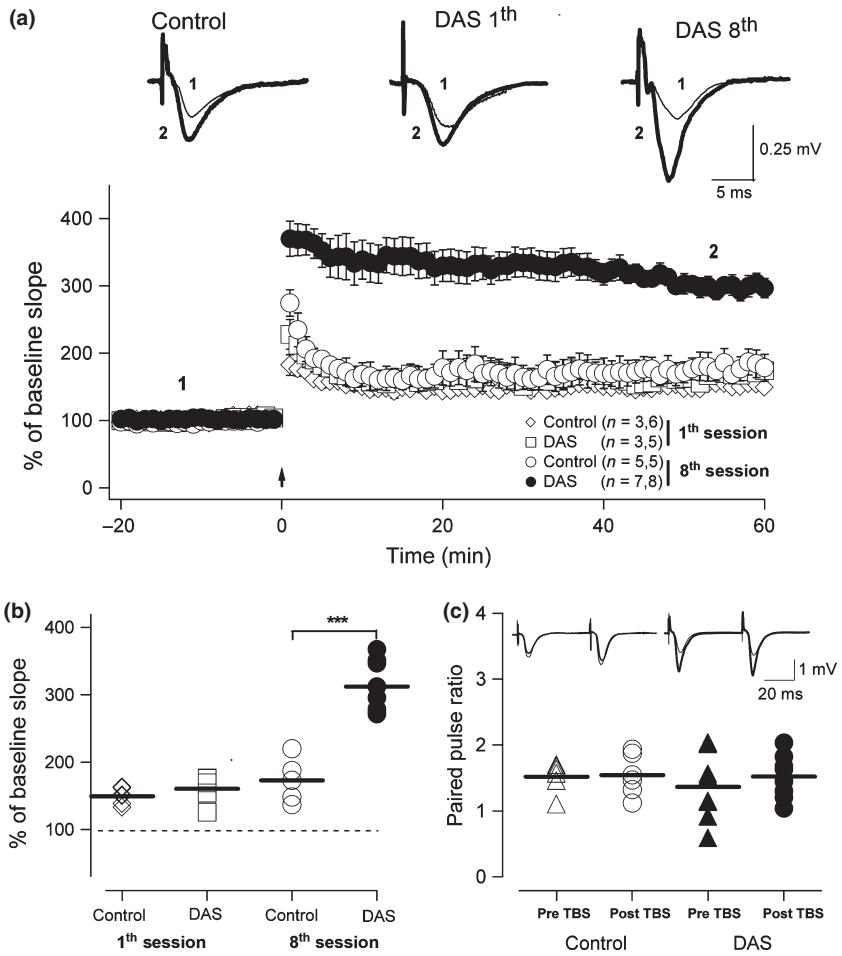

Fig. 6 Diacetyl sauroine (DAS), applied chronically, increases the theta burst stimulation (TBS)-induced long-term potentiation (LTP). Field excitatory post-synaptic potentials (fEPSPs) were recorded from hippocampal slices of the same rats used in the Morris Water Maze (MWM) behavioral studies, 24 h after the sessions in the MWM. (a) Time course of the TBS-dependent LTP from DAS-treated and untreated rats after one and eight sessions. DAS significantly increases the LTP (solid circles) compared to control (open circles) in the 8th session, but not after only one session; DAS (open square) versus ACSF (open diamond). Insets above: Traces for either condition at 40 min post-TBS (thick) compared to recordings before TBS (thin). Numbers in parentheses: number of rats, number of slices. (b) Quantification of the facilitations of TBS-dependent LTP induced by DAS 24 h after one single and after eight sessions. The four groups display significant potentiation after TBS. However, no significant difference was observed between slices obtained from ACSF-treated rats (149.27 ± 4.95%; $n = 5.6$; open circle) and rats treated only once (160.31 ± 9.52%, $n = 3.5$; open square) and after one MWM session ($p = 0.12$). In contrast, the increase was significantly different between the groups after eight MWM sessions ($p < 0.01$ throughout). The magnitude of TBS-dependent LTP was significantly larger in the slices obtained from repetitively DAS-treated rats (312.15 ± 13.58%; $n = 7.8$; solid circles) compared to slices obtained from saline-treated rats (173.04 ± 14.71%, $n = 5.5$; open circles) (***$p < 0.001$). (c) Accumulative plot of the paired-pulse ratios measured before and after the TBS protocol in eight different LTP recordings from rats treated with DAS and controls. Control (open symbols): ratio pre-TBS = 1.52 ± 0.08; ratio post-TBS = 1.54 ± 0.11 ($n = 6$, $p > 0.05$). DAS (closed symbols): ratio pre-TBS = 1.36 ± 0.18; ratio post-TBS 1.52 ± 0.12 ($n = 8$, $p > 0.05$). Inset above shows representative recordings obtained from the average of five traces for each condition; 40-ms interval.

Discussion

Chemistry of the sauroine derivatives used

To act as nootropic agent, a substance has to cross the blood–brain barrier. In general, an increase of lipophilicity facilitates the entry of substances into the brain (Greig 1992). To increase the lipophilicity, sauroine was isolated and subjected to chemical modifications targeted at its hydroxyl groups, evaluating the potential changes in the activity of the derivatives. Two acetyl derivatives of sauroine were obtained, MAS and DAS, the former was the expected product of a single derivatization and the latter was an unusual novel structure. According to the literature, acetylation of the secondary alcohol in C-8 was achieved, and

MAS was generated through a well-known, one-step, and high-yielding reaction. Owing to the low reactivity of tertiary alcohols, the hydroxyl group in C-7 remained unaltered. DAS was found to be acetylated in C-8 as well, but another acetyl group was detected in the NMR experiments. An explanation of its origin is related to a keto-enol formation that involves the proton of C-4 and the carbonyl group of C-5. It is known that under neutral conditions, the rate of conversion of the pure keto form into the enol form is slow, but is greatly accelerated by either acid or base catalysis (Wade 2009). In this case, pyridine was used as catalyzer. The enol form of sauroine was stabilized because of the reaction and two products were generated.

The main finding of the present work is that the doubly acetylated sauroine derivative DAS enhances synaptic plasticity as well as learning in a behavioral test.

DAS-induced vs electrically induced LTP

We have shown that acutely applied DAS (3.5 μM) augments the synaptic response reaching a level of approximately 150% above baseline, without electrical stimulation (Fig. 2). This is about three times the increase reached in controls with electrical tetanic stimulation. The chemically induced potentiation lasted at least half an hour after the application of DAS, thus fulfilling the requirements of chemical LTP (Sarvey *et al.* 1989; Otmakhov *et al.* 2004). In addition, this effect was dose-dependent and the experimental data were fitting a Hill function with an EC_{50} of 1.15 ± 0.09 μM and a maximal response of 137.23 ± 3.49%.

Since electrically induced LTP at the CA3-CA1 synapse depends on NMDA receptors (Bliss and Collingridge 1993), and DAS-induced LTP was almost completely suppressed in our experiments with the NMDA receptor blocker AP5 (Fig. 4a), we propose that the mechanisms involved in this LTP could be the same.

Furthermore, DAS application apparently saturated the mechanism of LTP, since tetanic stimulation as routinely used to induce LTP failed to further magnify the synaptic response. Conversely, when 3.5 μM DAS was added to a slice where LTP had been induced electrically, the saturation level reached was no different from the one with DAS application alone (see Figure S3). This finding also indicates that the chemical LTP observed with DAS and the one induced electrically are based on the same mechanisms.

Possible mechanisms of DAS action

Using PPF protocols before and after acute DAS application, we found the paired-pulse ratios for the 40-ms and 160-ms intervals to differ significantly, suggesting that at least part of the acute effect of DAS is pre-synaptic. In contrast, we have found no difference for any interstimulus interval after chronic treatments with DAS (see below), implying that the action of DAS under this modality involved only post-synaptic mechanisms.

Interestingly, slices obtained from rats, injected for 6 days with DAS (2 mg/Kg) during the behavioral experiments, displayed a strong facilitation for the electrical induction of LTP without DAS being present (Fig. 6) reaching similar, probably saturating levels, as observed in the acute experiments. One might believe that the concentrations reached in the brain during the behavioral experiments (2 mg/Kg) were significantly lower than the ones applied in the acute experiments (3.5 μM). However, the determinations of brain concentrations imitating the situation in the behavioral experiments and the one in slice recordings after chronic exposure (45 min after single injection vs. 24 h after repeated injection) reveal that the content of DAS in the brain was even considerably higher than our EC_{50} (see Fig. 3c). Considering that a big part of DAS, because of its lipophilicity, might be getting attached to lipid structures in the brain, the figures fit quite well (EC_{50} for the LTP effect: 1.15 vs. 19 μM for the acute injection).

In the behavioral experiments, DAS will probably have augmented the synaptic plasticity only a little, but repeated administrations may have changed the sensitivity for LTP induction. This view is supported by the observation that, unlike what was observed in the TBS-dependent LTP after the last session, we did not find differences in the TBS-dependent LTP in slices obtained from rats treated with DAS only once after just one trial in the MWM (Fig. 6). Furthermore, DAS seems to accumulate in the brain: After repetitive injections during the MWM training, a higher concentration (29.5 mM) is observed 24 h after the last injection compared to 15 min after just one injection (19 mM).

The metaplastic potential of DAS may be of special interest. It has been shown that in the hippocampus the sensitivity for plastic changes is itself dependent on the history of the synapse(s) involved (see Citri and Malenka 2008 for review). Thus, Wang and Wagner (1999) demonstrated that NMDA receptors are involved at least in some form of sensitivity changes for the induction of LTP and long-term depression. As for the behavioral tests, DAS robustly improves the learning process in the MWM while not revealing changes when the platform was visible, thus excluding swimming speed, vision or other processes different from visuo-spatial learning that might influence the time to reach the platform.

Although our results do not permit to clearly determine the molecular mechanisms involved in the plastic and metaplastic effects of DAS, some considerations may be in order. Since the facilitation induced by DAS was NMDA receptor dependent, one possibility is that DAS exerts its action directly on NMDA and/or AMPA receptors. Our results examining NMDA receptor-mediated currents are compatible with such a direct action. Significant differences were found between the NMDA receptor-dependent EPSCs,

but not in the AMPA receptor-dependent EPSCs from pyramidal neurons exposed to DAS *vs.* controls (Fig 4b). These results suggest that DAS exerts its effect, at least in part, directly on NMDA receptors. However, we cannot discard other possibilities. Another possibility is that DAS increases the membrane excitability fostering the removal of Mg^{2+} that blocks the NMDA receptor at voltages around the resting potential. Getting rid of the Mg^{2+} ion then allows the entry of Ca^{2+} activating Calmodulin-dependent protein kinase II, leading in turn to the insertion of additional AMPA receptors into the membrane (Pockett *et al.* 1993; Rozas *et al.* 2012). A third possibility is that DAS contributes pre-synaptically to this process by increasing the neurotransmitter release or inhibiting reuptake, thus increasing the activation of AMPA receptors (Otmakhov *et al.* 2004). In fact, in the experiments where the acute action of DAS was tested, we found a significant difference in the PPF, indicating an involvement of pre-synaptic mechanisms (Fig 4). On the other hand, DAS may indirectly facilitate NMDA channel function through cAMP-dependent processes as has been described previously (Cerne *et al.* 1993; Huang and Gean 1995; Raman *et al.* 1996; Westphal *et al.* 1999). Finally, we cannot discard a combination of these mechanisms either. Future studies will likely shed more light on these other possibilities.

The compound with only one acetyl group, MAS, was clearly devoid of effects on synaptic plasticity. Results obtained after the application of MAS were not distinguishable from controls. We conclude that changes in lipophilicity do not contribute to the difference observed between the effects of DAS and MAS, because the reaction leading to the acetylation of one hydroxyl group made the resulting substance more apolar but had no physiological effect, as it happens with MAS. However, the second acetylation that occurs on the keto group (C5, see Fig. 1) of the enolic sauroine (At C5; see Fig. 1) having almost no effect on polarity induced dramatic changes in the physiological action. Therefore, we suggest that the difference between DAS and MAS action is because of the conformational differences, and DAS may interfere with post-synaptic targets in the chain of events involved in the NMDA receptor-dependent LTP. An allosteric site at NMDA receptors would be a possibility for such a mechanism of action. However, pre-synaptic sites must also be considered because in the acute action we found evidence for pre-synaptic mechanisms.

In summary, in this study, we show that slices perfused with DAS, a semisynthetic derivate of sauroine, display a robust NMDA receptor-dependent chemical LTP. Furthermore, following chronic treatment, DAS increased the TBS-dependent LTP. In addition, DAS facilitated visuo-spatial learning in the behavioral task of the MWM.

The converging results for acute and chronic application as well as in the behavioral experiments together with its apparent metaplastic properties make DAS a strong candidate as a nootropic agent with potential use against the natural memory decline as well as in the treatment of neurodegenerative disorders. However, further tests involving other forms of learning and memory are necessary before preclinical experiments can be started.

Acknowledgments and conflicts of interest disclosure

This work was supported in Argentina by SeCyt-Universidad Nacional de Córdoba (05/C548), CONICET (PIP 2010/2012-00607 and 2009/2011-00628), ANPCyT (FONCyT; PICT 2010-1576 and 2007-352) and the Universidad Nacional de San Luis (22-Q508/7301); in Chile by FONDECYT and CONICYT, (grants 1120580 and ACT1113, respectively), and the University of Santiago de Chile (DICYT 020993Z). Dra. Vallejo owes thanks to the SPU, the Ministerio de Educación de la Nación Argentina for the Inter-U fellowship and PPUA grant Nro. 01-10-324 that allowed her to visit the Área de Química Orgánica, Universidad Nacional de San Luis and the Laboratorio de Neurociencia, Universidad de Santiago de Chile, to carry out the experiments.

All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. HSQC-DEPT NMR spectrum of MAS.

Figure S2. HSQC-DEPT NMR spectrum of Das.

Figure S3. LTP induced by DAS reaches levels of saturation.

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