Continuing bi-directional remodeling
of pyramidal neuron dendrites
following stress alleviation

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Summary

A long-standing hypothesis is that repetition of stressful experiences is a requirement for induction of structural adaptations of the apical dendritic branches of CA3 pyramidal neurons. In the present study we tested the role of the passage of time in the rearrangement of dendrites and changes in the functional aspects of the commissural–associational (C/A) synapses. Adult male rats were exposed to two social defeats followed by three weeks without further treatment. A control group was exposed to repetitive social defeats in the course of three weeks. Using somatic whole-cell recording of CA3 pyramidal neurons, the kinetics and activity-dependent plasticity of C/A excitatory postsynaptic potentials (EPSPs) were recorded and the cells intracellularly labeled. The results indicate that double defeat long-lastingly impaired the long-term potentiation of C/A synapses, and not differently compared to repeated defeat. Morphometric analysis of post-hoc reconstructions demonstrated that CA3 dendritic processes from repeatedly defeated rats were reduced in surface area and length selectively at the apical cone (70% of control, ~280 µm from the soma). Surprisingly, also double defeat produced a decrease in apical dendritic length, to similar degree (77% of control, 400 µm from the soma), but increased length and branch complexity at the basal cone (167% of control). These data indicate that even a brief stressor drives a dynamic and bi-directional reorganization of the patterns of synaptic connectivity of the CA3 pyramidal neuron: apical dendrites regress over the course of weeks, but de novo dendritogenesis occurs in the stratum oriens. We conclude that the course of time after stress might contribute as a critical variable in stress and stress-related disorders.
**Introduction**

A single stressful event induces lasting behavioral and physiological changes, developing over the course of days, weeks or even months afterwards (van Dijken et al., 1992; Buwalda et al., 2001; Martí et al., 2001; Dal-Zotto et al., 2002; for review, see Koolhaas et al., 1997 and Antelman et al., 2000). Stress-induced changes, for example, might reflect a time-dependent sensitization of the hypothalamic–pituitary–adrenal (HPA) axis towards novel mild challenges weeks later (Koolhaas et al., 1990; van Dijken et al., 1992; Schmidt et al., 2001) or conversely, result in a habituation response when animals are re-exposed to the same test stimulus (Martí et al., 2001; Dal-Zotto et al., 2002). It has now been shown in many biological systems and species that experience-dependent habituation and sensitization responses are supported by enduring large-scale modifications of the dendritic architecture of the neurons, establishing new connectivity and input–output characteristics of the neural circuit (Bailey and Chen, 1983, 1989; Robinson and Kolb, 1997; Wainwright et al., 2002; Gray and Weeks, 2003). Therefore, it can be hypothesized that the long-term effects of a brief stress experience will be accompanied by morphometric adaptations of key structures involved in the regulation of the stress response.

Being part of the limbic system controlling the stress response (Jacobson and Sapolsky, 1991), the hippocampal CA3 pyramidal cells regress in dendritic branch length and structure following a continuous hyperactivation of the HPA axis or repeated stress experience over the course of three weeks (Magariños and McEwen, 1995a; Sousa et al., 2000; Vyas et al., 2002; for review see McEwen, 1999). Structural changes in the CA3 neurons are traditionally considered to develop as a function of stressor repetition (McEwen, 1999), but no study has yet tested animals long after the stimulus.

Here, we addressed the possibility of structural plasticity after a short-lasting stressful episode. We investigated male rats that were subjected to a resident-intruder paradigm consisting of social defeat on two consecutive days followed by three weeks of no further treatment (Buwalda et al., 1999, 2001; Koolhaas et al., 1990). The results were compared with those obtained by repetitive social defeat over the course of three weeks. Somatic whole-cell recording of CA3 pyramidal cells in acute hippocampal slices allowed intracellular labeling and parallel voltage recording of the properties of the excitatory postsynaptic potentials of the recurrent commissural–associational (C/A) collaterals, which provide the major excitatory input to the CA3 pyramidal neurons (Urban et al., 2001). In this way, information was collected concerning the correlations between morphological structure and cellular physiology.

**Materials and methods**

*Animals and housing*

Male Wistar rats (Harlan-Winkelmann, Borchent, Germany) were housed in groups of four animals per cage (type V) with food and water ad libitum. Animals were acclimatized for two weeks before the start of the experiment and kept under reversed light–dark conditions (lights on 21.00–09.00 h) and at a room temperature of 21 ± 1°C.
Resident rats (wild-type strain, Haren, University of Groningen, The Netherlands) were housed in pairs of one male and one sterilized female in large plastic cages ($60 \times 40 \times 40$ cm = $l \times w \times h$) located in a separate room. All manipulations were performed during the rats’ active period, between 13.00 h and 15.00 h (during the dark phase), under supplementary dim red light ($< 1$ lux). Animal experiments were conducted in accordance with the European Council Directive of November 24, 1986 (86/609/ECC), and were approved by the Government of Lower Saxony, Germany. The minimum number of animals required to obtain consistent data was used.

**Stress procedure**

Social defeat was achieved as described previously (Buwalda et al., 1999). Before the start of the social defeat procedure, the female wild-type rat was removed from the cage. The experimental male Wistar rat was transferred from its group and introduced into the resident’s cage. In typically less than a minute, the animal was attacked by the resident, after which it adopted the freezing response and submissive postures. In all cases, the intruder animal was defeated by the resident male rat. For the remaining hour, the intruder was enclosed in a small wire-mesh compartment ($25 \times 15 \times 8$ cm$^3$) within the resident’s cage. Thus, the intruder animals were protected from direct physical contact, but remained in olfactory, visual, and auditory contact with the resident male rat. Afterwards, the intruder animal was housed singly until the electrophysiological experiments. Control and defeated animals were housed in the same room to rule out differential spatial clues (Lee and Kesner, 2002).

Two different defeat paradigms were applied (see Fig. 1A–B). One group of rats was defeated on two consecutive days and left singly housed for a total of 21 days (*double defeat*, $N = 9$). Control animals ($N = 10$) were subjected to similar handling procedures and transferred to a novel cage with sawdust bedding for 1 h on two consecutive days. In a second series of experiments, rats were exposed to social defeats on every second day, for a period of 21 days, equivalent to 11 defeats (*repeated defeat*, $N = 4$). Control animals ($N = 4$) were subjected to similar handling procedures and transferred to a novel cage with sawdust.

![Fig. 1.](image)

**Fig. 1.** Graphical representation of time and treatment frequency for the two experimental paradigms. A. Double defeat was performed by exposing rats ($n = 9$) to a standard social defeat paradigm (1 h) on two consecutive days. After the initial social defeat, animals were left isolated and were not handled until they were killed on day 22. Control animals ($n = 10$) were handled in the same manner but transferred into a similar cage with sawdust only. B. The repeated-defeat paradigm consisted of a protocol in which rats ($n = 4$) were exposed to the social defeat paradigm on an intermittent basis, by 48 hrs, for 11 defeats. As in the double-defeat protocol, the animals were killed on day 22 after the first stress experience and standard hippocampal slices were prepared. Control animals ($n = 4$) were handled in the same manner but transferred into similar large cages with sawdust.
**Hippocampal slice preparation**

In the morning of day 22, between 10.00 and 11.00 h, animals were weighed, then deeply anesthetized (with a mixture of 50 mg/mL ketamine, 10 mg/mL xylazine, and 0.1 mg/mL atropine, by intraperitoneal injection), and subsequently transcardially perfused with ice-cold carbogenated (95% O₂/5% CO₂) sucrose-based artificial cerebrospinal fluid (ACSF) solution. Sucrose–CSF contained (in mmol/L) 206 sucrose, 1 MgCl₂, 2.5 KCl, 1.25 Na₂HPO₄, 26 NaHCO₃, 14 d-glucose, 1 kynurenic acid, 1.5 CaCl₂, 1 L(+)-ascorbic acid. This pre-perfusion (i) removes red blood cells that interfere with the histological peroxidase reaction and (ii) enhances the viability of CA3 neurons (Aghajanian and Rasmussen, 1989; Kapur et al., 1998). The brain was rapidly removed and transverse 400-µm hippocampal slices were cut with a vibroslicer (Vibracut 2, FTB, Bensheim, Germany). Slices were stored in a carbogenated-ACSF-containing chamber at 33°C for 1 h and afterwards maintained at room temperature.

ACSF for slice storing consisted of (in mmol/L) 125 NaCl, 2.5 KCl, 1.25 Na₂HPO₄, 2 MgSO₄, 26 NaHCO₃, 1.5 CaCl₂, 1 L(+)-ascorbic acid, 14 d(+)-glucose, at 300 mOsm (all chemicals from Merck, Darmstadt, Germany). The adrenal glands were weighed immediately after the animals were killed, using an analytical balance.

**Patch-clamp recording**

The entorhinal–hippocampal area was dissected from the slice and transferred to a submerged type of recording chamber with continuously oxygenated ACSF (flow rate: 1–2 mL/min). Cell bodies and their proximal segments were visualized by infrared–differential interference contrast (IR–DIC) video microscopy with an upright microscope (Axioskop 2 FS, Zeiss, Göttingen, Germany) equipped with an ×40/0.80 W objective (Zeiss IR-Acroplan). To reduce any location-dependent variation in morphology (Ishizuka et al., 1995), only pyramidal-shaped somata inside the CA3 stratum pyramidale and located slightly distant from the tip of the hippocampal fissure (~CA3b) were used for recording. Post-hoc visualization of labeled neurons verified this location. Patch-clamp recording was performed using borosilicate glass pipettes with 3–5 MΩ resistance, connected to an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), and PULSE software (HEKA, Lambrecht, Germany). The intracellular patch solution contained (in mM) 120 KMeSO₄ (ICN, Eschwege, Germany), 20 KCl, 10 HEPES, 0.2 EGTA, 2 Mg²⁺-ATP, 10 phosphocreatine, and 0.3 Tris-GTP (Sigma-Aldrich, Steinheim, Germany), adjusted to pH 7.3 with KOH and to 290 mOsm. Data were collected by low-pass Bessel filtering at 5 kHz and further digitized and stored at 50 kHz using an ITC-16 computer interface (Instrutech Corp., Port Washington, NY, USA) and PULSE software (v. 8.11, HEKA). In whole-cell current-clamp mode, the ‘fast clamp’ amplifier circuit was used, and both bridge voltage and series resistance were continuously monitored and corrected when appropriate.

Tungsten electrodes (0.1 MΩ) were used to electrically stimulate C/A fibers. Because the distance between C/A axon stimulation and the cell-body recording site determines the rise-time kinetics of the excitatory postsynaptic potential (EPSP) (4.1 ms at < 200 µm, and 5.6 ms at > 200 µm; Kapur et al., 1998), we standardized the placing of the electrode at ~300 µm lateral from the recording site. In current-clamp, the cells were held close to –75 mV, approximating the type A α-aminobutyric acid.
(GABA) reversal potential, and C/A EPSPs were collected at a basal frequency of 0.05 Hz. EPSPs were isolated by blocking GABA_A-mediated activity with 10 µM (-)-bicuculline methobromide and 50 µM picrotoxin (Tocris, Bristol, UK). To test for plasticity, EPSPs were evoked and recorded at a basal frequency of 0.05 Hz for 8 min and then shortly potentiated by applying a mild low-frequency paradigm of 3 Hz for 3 min. All data were collected at 32 ± 2°C.

Labeling of identified neurons

All neurons were intracellularly labeled (Horikawa and Armstrong, 1988) with 3 mg/mL Neurobiotin (Vector Laboratories Inc., Wertheim, Germany), added to the patch solution, and neurons were reconstructed post-hoc. This approach facilitates visualization of the entire dendritic tree, including the fine natural endings proceeding into the lacunosum-moleculare (Pyapali et al., 1998). After recording, the patch-pipettes were carefully withdrawn from the membrane and the slices were fixed in 0.1 M phosphate buffer (PB) with 4% paraformaldehyde (pH 7.4) and stored at 4°C for two days. Whole slices were processed, floating in wells, by first removing endogenous peroxidase activity in a 0.1 M PB solution containing 2% H₂O₂. They were subsequently rinsed with an avidin–biotin peroxidase solution (diluted 1:100; ABC, Vector Laboratories, Linaris, Wertheim, Germany) with 1% bovine serum albumin (Sigma) and 0.3% Triton X-100, and stored for 24 h at 4°C. Slices were washed several times in fresh 0.1 M PB and then treated with a PB solution containing 0.04% 3’,3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) and 0.002% NiH₄SO₄ for 20 min. This was immediately followed by incubation in a second freshly prepared DAB solution with 0.001% H₂O₂ until dark brown staining appeared, in typically less than 10 min. The reaction was terminated by several washings in fresh 0.1 M PB and distilled water. Tissue sections were dehydrated in an ascending series of ethanol, cleared in xylene, and flat-embedded in Eukitt (Kindler, Freiburg, Germany) on glass slides. Slices from stressed and control animals were always processed simultaneously.

Neuronal reconstruction and morphometric analysis

Labeled cells were visualized with light microscopy and evaluated for staining-quality criteria, which included visibility up to the most distal apical dendrites and clear dense labeling of the processes. In a number of cases, cell coupling was observed (~3%). These were omitted from the analysis because the dendrites could not be assigned unequivocally to a single cell. The IR–DIC patch-clamp technique provides an excellent high signal-to-noise ratio for electrophysiological recordings, but restricts the collection of neurons to within 100 µm from the slice surface, where some cells are defective in anatomical structure, with, for instance, severed main apical or basal dendrites. Obviously compromised cells were omitted from the analysis. Complete and optimally labeled pyramidal neurons meeting all criteria were photographed and quantified for dendritic morphometry using NeuroLucida software (Microbrightfield Inc., Colchester, VT, USA) in combination with an automated stage and focus control connected to a microscope (Zeiss II RS). The data were collected as line drawings consisting of X, Y, and Z coordinates, together with quantitative information on the dendritic diameter, by superimposing a circular cursor to the size of the dendrite.
Dendritic length and surface measurements were made by tracing dendrites with a ×40 (N.A. 0.75) objective, with a final magnification of ×40.000 at the monitor. Here, the step sizes of the circular cursor were 0.16 µm, sufficiently below the limits of light-microscopy resolution (~0.25 µm). Numerical analysis and graphical processing of the neurons were performed with NeuroExplorer (v. 3.21, Microbrightfield). Sholl plots (Sholl, 1953) were constructed by plotting the summed dendritic length as a function of distance from the middle of the soma, set at zero, and dendrites summed in each subsequent radial bin of 20 µm.

Ethanol dehydration and xylene clearance causes tissue shrinkage (Pyapali et al., 1998). To estimate shrinkage in the Z plane, the slice thickness was determined with the microscope micrometer, and by carefully monitoring the slice edges. A correction factor of 1.35 was applied to each plane, which is within the range of previously determined factors (Pyapali et al., 1998). It should be noted that, for the ultimate comparison of between-subject effects, the linear shrinkage correction will have no direct effect on the outcome or conclusions. The use of uncut 400-µm slices greatly facilitates the reconstruction of the complete dendritic structure and length, but did not allow us to resolve the anatomical fine structures, such as dendritic spines (see also Ishizuka et al., 1995; Henze et al., 1996). Therefore, we made no attempt to quantitatively determine spine distribution or density, so the present measurements of surface area and volume should be accepted tentatively as estimates. When comparing basic numerical data with previously described CA3 morphology (Henze et al., 1996; Ishizuka et al., 1995) the total dendritic length was on average only slightly less (9.7 mm in this study versus ~11 mm).

Data analysis

Voltage recordings were only included for analysis when showing (i) a membrane potential of –55 mV or lower and (ii) a stable series resistance was measured for longer than 30 min. In LTP experiments, the data were expressed as the percentage change in EPSP amplitude normalized to the average EPSP during the initial baseline period (100%). In the cumulative probability plots, all data points between 20 and 30 min after LTP establishment were averaged between animals and binned. Monoexponential fitting of EPSP decay was performed with PULSE-FIT (HEKA). Preliminary analysis of variance (ANOVA) of data collapsed across neurons between control animals that were repeatedly handled or handled twice with a three-week delay indicated that repeated handling had no effect per se on either body weight or adrenal weight (P > 0.06), dendritic morphometry (P > 0.1), or EPSP kinetics (P > 0.4), which justifies the pooling of these data. Throughout this article, therefore, three groups are used: control, doubly defeated, and repeatedly defeated animals. The null hypothesis was explored by one-way ANOVA (SPSS v10.0, SPSS Inc., Chicago, IL, USA) followed by least significant difference (LSD) post-hoc comparisons. Correlation analysis was performed with bivariate Pearson’s correlation test. All significance levels were set to P < 0.05. Graphical processing was done with Origin (v. 6.1, Microcal Software, Northampton, MA, USA). Data are given as means ± SEM.
Results

Effects of stress on body and adrenal weights

Stress significantly affected the mean body and adrenal weights (\(F_{2, 26} = 3.90, P < 0.034\), and \(F_{2, 26} = 12.50, P < 0.001\), respectively). The rats subjected to the repeated-defeat paradigm (Fig. 1B) had a final mean body weight of 88.2 ± 1.8% that of the controls (control: 317.5 ± 6.0 g; stress: 280 ± 5.8 g; post-hoc test \(P < 0.015\)) and their adrenal weights were significantly increased to 131.3 ± 8.4% of the control weight (stressed, 11.8 ± 1.5 g/100 g; control, 9.0 ± 0.3 g/100 g; \(N = 4\); \(P < 0.05\)).

When rats were exposed to the double-defeat protocol with a 21-day delay (see Fig. 1A), there was a similar increase in average adrenal weight (126.8 ± 4.5% of the control; \(P < 0.0001\), \(N = 9\)) indicating a hyperactivated HPA axis. Body weight, however, was less reduced, at 91.5 ± 3.1% of the control value (\(N = 9\), \(P < 0.10\)). Therefore, the endpoint adrenal-to-body-weight ratio changed to a similar degree after the repetitive- and double-defeat exposures, but with a slight additional effect insofar as there was a more pronounced reduction in body weight when the exposure to stress was repetitive.

Resting CA3 membrane properties and EPSP kinetics

The resting membrane properties of 59 CA3 pyramidal neurons were determined immediately after breakthrough in somatic whole-cell configurations, using current- or voltage-clamp recording within the linear range of the I–V curve. Table 1 lists the average resting membrane properties for each group. The results show that neither the resting membrane potential (\(V_M\)) nor the input resistance (\(R_N\)) differed in variance (\(F_{2, 23} = 1.16, P < 0.33\), and \(F_{2, 23} = 2.16, P < 0.14\), respectively).

Upon stimulation of the C/A fibers and with inhibitory receptor blockers in the bath, somatically recorded EPSPs in control animals were quantified to establish their kinetics (Fig. 2A–B, Table 1). In control recordings, the C/A EPSP responses, which are a composite of \(N\)-methyl-d-aspartate (NMDA) and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor-mediated potentials (Debanne et al., 1998), were characterized by 10–90% rise times of, on average, 4.7 ± 0.19 ms (range 3.3–7.0 ms, \(n = 36\)) which is consistent with a C/A origin, and is frequently used as a major indicator to distinguish mossy-fiber and C/A input (Kapur et al., 1998). Furthermore, the onset latency for the C/A EPSP was on average ~2.6 ms (range 1.9–3.8 ms), in agreement with previous data (2.7 ms in Kapur et al., 1998). Using a similar spatial stimulus-recording configuration within all hippocampal slices, we compared the average rise and decay kinetics of C/A EPSPs between the three groups.

The results indicate that only exposure to repeated defeat altered the C/A EPSP kinetics (Table 1). Here, the latency of the EPSP was reduced by 17% (\(F_{2, 23} = 4.78, P < 0.019\)) and paralleled the rise-time kinetics, which were faster (\(F_{2, 23} = 3.32, P < 0.056\)). Furthermore, across the range of the EPSP responses to varying stimulus strength, there was a clear effect on the input–output (IO) relation in the increased slope of the EPSPs (Fig. 2A–B; \(F_{2, 17} = 4.69, P < 0.03\)). The repeatedly defeated animals had larger IO slope values than those of the control or doubly defeated animals (post-hoc test, \(P < 0.05\) and \(P < 0.001\), respectively).
Bi-directional remodeling after stress

Fig. 2. Defeat differentially affects compound commissural–associational EPSPs. A. Examples of a family of EPSP responses elicited by varying stimulus intensities (1 µA steps) for each experimental group, from left to right; (open circles) control, (closed triangles) double defeat and (gray squares) repeated defeat. B. Plot of the input–output curves constructed for the C/A EPSP amplitudes. Repeated defeat significantly increased the slope of the linear fit through the data points (repeated defeat, ~3.1 mV µA relative to the control, 1.1 mV µA; F2,17 = 4.69, P < 0.026). Data are given as mean ± SEM.

Table 1. Resting membrane and CA3 C/A EPSP kinetics of control, doubly and repeatedly defeated animals

<table>
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<th></th>
<th>Control</th>
<th>Double defeat</th>
<th>Repeated defeat</th>
<th>P</th>
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<tbody>
<tr>
<td>N (n)</td>
<td>12 (27)</td>
<td>8 (22)</td>
<td>4 (10)</td>
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<tr>
<td>Vm (mV)</td>
<td>-73.6 ± 1.52</td>
<td>-76.2 ± 2.00</td>
<td>-78.0 ± 3.1</td>
<td>n.s.</td>
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<tr>
<td>Rm (MΩ)</td>
<td>70.6 ± 4.62</td>
<td>79.3 ± 4.85</td>
<td>88.0 ± 7.9</td>
<td>n.s.</td>
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C/A-EPSP

<table>
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<th></th>
<th>Control</th>
<th>Double defeat</th>
<th>Repeated defeat</th>
<th>P</th>
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<tr>
<td>Latency¹ (ms)</td>
<td>2.46 ± 0.01</td>
<td>2.64 ± 0.13</td>
<td>2.05 ± 0.24</td>
<td>* §§ 0.019</td>
</tr>
<tr>
<td>10-90% rise time (ms)</td>
<td>4.63 ± 0.26</td>
<td>5.45 ± 0.40</td>
<td>4.04 ± 0.36</td>
<td>½ 0.056</td>
</tr>
<tr>
<td>Exponential decay (ms)</td>
<td>34.2 ± 4.07</td>
<td>46.2 ± 8.41</td>
<td>37.3 ± 10.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>I-O slope (mV µA)</td>
<td>1.70 ± 0.26</td>
<td>1.13 ± 0.18</td>
<td>3.23 ± 1.08</td>
<td>** 4 0.026</td>
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¹Latency was between beginning of the stimulus artifact and onset of the EPSP. P indicates significance of the group comparison with one-way ANOVA. The significance levels from LSD post-hoc analysis are indicated as follows: *P < 0.05 and ***P < 0.001 from control. ¹P < 0.05, ½P < 0.01 from double defeat. n.s. = not significant. N indicates the number of animals, n number of cells. Data are given as mean ± SEM.
Stress effects at commissural–associational LTP

We tested further the prediction that social defeat would affect the LTP of the C/A CA3 synapses. During baseline EPSP, recording amplitudes were elicited from a potential of ~75 mV and scaled to ~6.5 mV (not different between groups, \( P > 0.9 \)) to obtain similar depolarization levels. As shown in Fig. 3A–C, in control animals, LTP was elicited by a low-frequency stimulation of 3 Hz for 3 min, which led to a gradual and progressive increase in the EPSP amplitudes, reaching 150% potentiation 20 min after frequency stimulation with high probability (Fig. 3B–C).

In recordings from repeatedly defeated animals, low-frequency potentiation led to a reversal of the EPSP amplitude, resembling long-term depression (LTD; 56% probability of a 70% EPSP amplitude, Fig. 3B). In slices from doubly defeated animals after a 21-day time delay, LTP could not be evoked (98.4 ± 14.6%, \( n = 7 \), \( P < 0.05 \) compared with control LTP; Fig 3B–C) and the EPSPs showed a probability distribution similar to that of repeatedly defeated rats (Fig. 3C). These data show that the repetition of the stressor does not significantly suppress low-frequency LTP more at C/A synapses.

Fig. 3. Long-lasting bimodal regulation of social-defeat stress on tetanic potentiation of the C/A synapse. A. Example traces of EPSPs before and after low-frequency stimulation. Left is an example from the control group; in the right panel, from the repeated-defeat group. B. Scatter plots show the time development of EPSP potentiation, recorded at a frequency of 0.05 Hz. Data were averaged for three successive sweeps. The low-frequency stimulation of 3.0 Hz (3.5 min) induced a robust LTP-like potentiation at the C/A synapse in control animals (open circles) with a mean amplitude change of 153.9 ± 8.8%, \( n = 12 \) (see inset). Doubly defeated animals, in contrast, showed significant changes in the probability and direction of synaptic potentiation (\( F_{2, 22} = 7.81, P < 0.003 \)). In repeatedly defeated animals (\( n = 4 \)), a robust LTD-like EPSP depression (63.8 ± 8.9%, \( P < 0.05 \)) was observed. Animals exposed to a double-defeat paradigm with 3 weeks of single housing showed a mixed response to mild synaptic potentiation with a mean of 98.1 ± 17.2 % (\( n = 8 \)). C. The bimodal modification of synaptic plasticity after defeat was also evident in the cumulative probability. The dotted line summarizes the probability of EPSP amplitudes during baseline recording. Within the 20–25 min post-frequency potentiation, the cumulative probability for 130% LTP in control animals (open circles) was 40%, but for synaptic depression (70% amplitude) was only 1%. For the defeated animals, however, there was a 56% probability of a 70% EPSP amplitude in repeatedly defeated animals (gray squares) and 47% in doubly defeated animals (black pyramids).
Morphology of CA3 pyramidal neurons

For 36 of the 79 (47%) CA3 pyramidal cells examined in this study, the morphology could be recovered, allowing quantitative analysis of essential aspects of their dendritic structures (Fig. 4A–D). All CA3 neurons included in the analysis (the criteria are described in Methods) showed dendritic patterns characteristic of CA3b pyramidal neurons, as previously described (Ishizuka et al., 1995; Henze et al., 1996). The CA3 dendritic architecture consists of a low number of side branches, a maximum branch order of 6–11, dense thorny excrescences in the stratum lucidum (Fig. 4C), spines (Fig. 4D), and a low level of sub-branching of dendrites in the lacunosum-moleculare (Fig. 4B). Dendritic arborization was analyzed for standard numerical parameters. Table 2 lists the average values for each group. Repeated defeat had a significant effect on CA3 morphology. There was a great reduction in total volume and surface area (~40% reduction; Table 2, Fig. 5), although not in the number of dendritic nodes. The changes occurred selectively on the apical side of the dendritic tree, where the dendritic branches were reduced in volume, surface area, and length (~30–50% reduction, n = 8). Repeated defeat did not affect basal dendrites.

Fig. 4. A. Photomicrograph of an intracellularly labeled CA3 pyramidal neuron from a doubly defeated rat. Note the highly polarized branch distribution between the apical and basal cones of the cell. B. Line-drawing of the same neuron obtained by reconstruction with NeuroLucida and used for morphometric analysis. The arrow marks an axon running through the stratum oriens. The relative position of the CA3 pyramidal cell is given by lines indicating the various hippocampal layers. DG, dentate gyrus; o, stratum oriens; p, stratum pyramidale; luc, stratum lucidum; r, stratum radiatum; m, stratum moleculare; l-m, lacunosum moleculare. C. High-magnification photomicrograph of the thorny excrescences (indicated by arrows) of the same neuron. Scale bar indicates 5 µm. D. High-magnification photomicrograph of spines (arrows) located on an oblique branch within the stratum radiatum. Scale bar indicates 4 µm.
Fig. 5. Brief and repetitive defeat affect the CA3 neuronal dendrite structures. Shown are representative Neurolucida reconstructions of CA3 pyramidal neurons for the control, the doubly defeated, and the repeatedly defeated rats. Note the reduction in dendritic length on the apical side in both groups exposed to defeat ($P < 0.05$), but the selective sprouting of basal branches in the neurons of doubly defeated rats ($P < 0.005$). For further numerical details, see Table 2.

The length of the apical dendrites was also reduced in the neurons of animals exposed to a double defeat, with a magnitude similar to that observed after repeated stress ($–23\%, P < 0.05$; Fig 5 and Table 2). Surprisingly, in contrast to the apical tree, the branches from the basal dendrites were increased in all aspects, including length, volume, and nodes (65–95% increase; LSD post-hoc test, $P < 0.05$ for all; see Table 2). Moreover, branch complexity, evaluated by the highest branch order, was significantly increased ($P < 0.05$). In addition, we also detected an increase in apical trunk diameter (Table 2). Importantly, despite the marked opposite changes in dendritic elements between the different cones, the summed dendritic length, surface area, and volume were conserved relative to control values (Table 2). To evaluate the detailed distribution of dendritic processes across the various sublayers of the CA3 region, we created Sholl plots (Sholl, 1953) in which summed dendritic length is expressed as a function of distance from the soma in radial bins of 20 µm (Fig. 6A–B). It appeared that the reduction of dendritic elements after repeated defeat was restricted to the middle part of the apical tree, between ~280 and 340 µm from the soma (Fig.
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6A). Double defeat followed by single housing led to a branch-length decrease that occurred more distal from the apical tree, 400–600 µm from the middle of the soma. Basal dendrites were increased in length between 80–160 µm (Fig. 6B). Similar conclusions were made when comparing the density of dendritic intersections as a function of radial distance (data not shown). To yield an estimation of the absolute modification change induced by stress, we subtracted within each ring the mean length data from the treated and control animals. The differences were pooled to identify changes with a negative sign (putative retractions) and a positive sign (additions) and the sum of these is plotted in Fig. 6C. The data show that, with repeated stress, there was a clear net loss of branches, whereas three weeks after double defeat, the distributed additions and retractions did not induce a net difference, but were instead balanced.

### Table 2. Morphometric data of CA3 pyramidal neuron dendrites of control, doubly and repeatedly defeated animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Double defeat</th>
<th>Repeated defeat</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>12</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Soma perimeter (µm)</td>
<td>97.4 ± 8.85</td>
<td>91.8 ± 7.46</td>
<td>78.0 ± 6.13</td>
<td>n.s.</td>
</tr>
<tr>
<td>Soma surface (µm²)</td>
<td>216 ± 11.2</td>
<td>264 ± 20.4</td>
<td>236 ± 11.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total tree</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (mm)</td>
<td>9.69 ± 0.45</td>
<td>9.44 ± 0.76</td>
<td>7.68 ± 0.38</td>
<td>n.s.</td>
</tr>
<tr>
<td>surface (mm²)</td>
<td>25.9 ± 1.90</td>
<td>26.8 ± 2.62</td>
<td>17.6 ± 1.51 * §</td>
<td>0.03</td>
</tr>
<tr>
<td>volume (mm³)</td>
<td>8.61 ± 0.96</td>
<td>9.19 ± 0.10</td>
<td>5.12 ± 0.73 * §</td>
<td>0.04</td>
</tr>
<tr>
<td>Apical dendrites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk diameter (µm)</td>
<td>3.83 ± 0.21</td>
<td>4.43 ± 0.18 *</td>
<td>3.49 ± 0.27</td>
<td>0.04</td>
</tr>
<tr>
<td>length (mm)</td>
<td>7.47 ± 0.50</td>
<td>5.73 ± 0.72 *</td>
<td>5.13 ± 0.72 *</td>
<td>0.03</td>
</tr>
<tr>
<td>surface (mm²)</td>
<td>21.2 ± 1.85</td>
<td>17.7 ± 2.23</td>
<td>12.6 ± 1.72 *</td>
<td>0.03</td>
</tr>
<tr>
<td>volume (mm³)</td>
<td>7.26 ± 0.83</td>
<td>6.72 ± 0.90</td>
<td>3.94 ± 0.52 * §</td>
<td>0.04</td>
</tr>
<tr>
<td>nodes (#)</td>
<td>25.1 ± 1.80</td>
<td>21.4 ± 3.43</td>
<td>19.8 ± 2.75</td>
<td>n.s.</td>
</tr>
<tr>
<td>endings (#)</td>
<td>27.1 ± 1.80</td>
<td>23.5 ± 3.84</td>
<td>21.3 ± 2.66</td>
<td>n.s.</td>
</tr>
<tr>
<td>max. branch order</td>
<td>8.88 ± 0.47</td>
<td>8.67 ± 0.67</td>
<td>8.63 ± 0.71</td>
<td>n.s.</td>
</tr>
<tr>
<td>Basal dendrites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (mm)</td>
<td>2.21 ± 0.28</td>
<td>3.71 ± 0.36 *</td>
<td>2.56 ± 0.31</td>
<td>0.005</td>
</tr>
<tr>
<td>surface (mm²)</td>
<td>4.70 ± 0.61</td>
<td>9.05 ± 1.04 ***</td>
<td>4.94 ± 0.84 §§</td>
<td>0.001</td>
</tr>
<tr>
<td>volume (mm³)</td>
<td>1.35 ± 0.21</td>
<td>2.47 ± 0.33 **</td>
<td>1.26 ± 0.25 §§</td>
<td>0.006</td>
</tr>
<tr>
<td>nodes (#)</td>
<td>10.8 ± 1.50</td>
<td>19.0 ± 2.01 **</td>
<td>12.8 ± 1.10 §</td>
<td>0.003</td>
</tr>
<tr>
<td>endings (#)</td>
<td>13.8 ± 1.58</td>
<td>22.8 ± 2.22 **</td>
<td>16.6 ± 1.36 §</td>
<td>0.003</td>
</tr>
<tr>
<td>max. branch order</td>
<td>5.27 ± 0.36</td>
<td>6.33 ± 0.26 *</td>
<td>4.63 ± 0.32 §</td>
<td>0.009</td>
</tr>
</tbody>
</table>

The numerical average (mean ± SEM) of the morphometric parameters of cells from different experimental groups. n indicates the number of cells. P designates the significance level of one-way ANOVA, which was followed by LSD post-hoc analysis. The significance levels are denoted by *P < 0.05, **P < 0.01, and ***P < 0.001 relative to controls and §P < 0.05, §§P < 0.01 relative to doubly defeated rats. n.s. = not significant.
Fig. 6. Sholl analysis was used to evaluate the distance-dependent distribution of the apical and basilar dendrites. Dendritic length was summed in radii at 20 µm distance, with the middle of the soma set at zero. Apical dendrites are plotted to the right and basilar dendrites to the left as a function of distance from the soma center. A. Comparing control with repeatedly defeated animals shows that there is a reduction in dendritic length in the stratum radiatum for the rings 280, 300 and 380–440 µm from the soma (unpaired t-tests: P < 0.05). No changes occurred in the basal dendrites. B. Neurons from doubly defeated rats had shorter dendritic length within 400, 540, 560, and 600 µm from the soma (unpaired t-tests: P < 0.05). Marked differences were detected in the increased length of basal dendrites in the stratum oriens at the rings between 80 and 160 µm distance (unpaired t-tests: P < 0.01). Asterisks indicate significant difference (P < 0.05) within each ring of 20 µm. C. The plot summarizes the sum of differences between stressed and control rats, by dendritic subtraction within each ring from the Sholl analysis shown in a) and b). The sign of change was used to calculate the total amount of dendritic additions and retractions. Note the markedly balanced change in CA3 neurons from doubly defeated rats.

Morphological correlates of physiological parameters

Because the rise and decay kinetics of remote synaptic events depend on dendritic structure and electronic filtering (Henze et al., 1996; Golding et al., 2001; Vetter et al., 2001; reviewed in Spruston et al., 1999), we explored whole-cell patch-clamp data from neurons that were morphologically reconstructed to test whether the defeat-induced dendritic changes are involved in shaping the kinetics of the C/A EPSPs. Linear association analysis showed that the apical surface and length correlated negatively with the value of the resting input resistance ($R_N$) ($r = -0.48$, $P < 0.01$, n = 32). A scatter plot is provided in Fig. 7A. As expected, the $R_N$ was also associated with the duration of EPSP decay kinetics ($r = 0.43$, $P < 0.01$, n = 32; data not shown). The latency of onset of the C/A EPSP was weak, but correlated significantly with the geometrical structure of the apical tree, in that a greater number of nodes or greater
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Apical length resulted in a longer latency time ($r = 0.45, P < 0.006$, and $r = 0.33, P < 0.05$, respectively; $n = 37$; Fig. 7B). This partly corresponds to the difference in physiology of the three groups: although the apical nodes were not significantly reduced in the repeatedly defeated animals, CA3 neurons from this group did have significantly less apical surface (Table 2) and also a smaller latency of EPSP (Tab. 1). Aspects of the basal dendrites did not correlate with any evaluated property.

**Discussion**

We examined the structural and functional properties of the CA3 pyramidal neurons of rats exposed to two distinct social defeat paradigms. CA3 cells from doubly defeated animals were significantly different in their increased complexity and the size of their CA3 basal dendrites, but were similar to the CA3 cells of repetitively defeated rats in the regression of their dendrites at the apical cone. These data demonstrate that CA3 pyramidal neuron dendrites and C/A synaptic potentiation are not exclusively regulated by the repetition of a stressor. Aspects of this plasticity are established even after a short episode of stress, probably developing dynamically with time.

**Repeated stress induces regression only and facilitation in the C/A glutamate-receptor potentials**

We firstly demonstrated that in our model the repetition of a psychosocial stressor led to a net branch regression specific to the apical tree, without affecting basal dendrites (Fig. 5). This experience-dependent change in the neuron structure is in line with previous chronic stress paradigms (Magariños and McEwen, 1995a; McEwen, 1999; Sousa et al., 2000; Vyas et al., 2002). We markedly extended these observations by showing that a reduction in the size and structure of the CA3 apical tree (in length and number of nodes) was involved in (i) an increased somatic resting input resistance, and (ii) shortened latency of the evoked C/A EPSP (Fig. 6). Such relation is also predicted...
from compartmental modeling studies that isolated the biophysical effects of apical dendritic morphological changes (Krichmar et al., 2002; Vetter et al., 2001; Golding et al, 2001). In the present study we show that whereas the geometry-related effects on membrane input resistance was not apparent in the variance between groups, the predicted shortening of the onset latency of compound C/A EPSPs was indeed observed after repeated defeat (Table 1). This provides empirical evidence of how stress-induced geometrical changes affects excitatory signaling: the net reduction in the length of dendrites after repeated defeat leads to a facilitation of excitatory transmission at the recurrent synapses (Fig. 2). EPSP amplitude changes, however, were not related to structure, but might be related to the chronic stress-increased NMDA conductance (Kole et al., 2002).

The activity-dependent increase of synaptic strength was markedly blocked by repetitive social defeats, observed as a shift from LTP towards LTD (Fig. 3). The applied low-frequency 3-Hz tetanization of C/A synapses in control animals positively regulated the synaptic strength of the glutamatergic EPSPs, instead of inducing LTD (Chattarji et al., 1989; Debanne et al., 1998). This might have been caused by the fact that under current-clamp conditions the application of blockers for GABA A-mediated receptor currents facilitates frequency-dependent disinhibition, by a reduction of the driving force of chloride and the presynaptic inhibition of GABA release by GABA B receptor activation (Wigström and Gustafsson, 1983; Hsu et al., 1999; Kuenzi et al., 2000). Second, the isolation housing of control animals might have additionally contributed to a reduced threshold for LTP induction (Kehoe and Bronzino, 1999). Independent of the exact source of the low-frequency LTP, we recently observed that 100-Hz-induced LTP is also blocked three weeks after double defeat (M. Kole, unpublished observations). Furthermore, the observed shift from LTP to LTD induced by defeat stress is common in that both acute and chronic stressful experiences suppress LTP or facilitate hippocampal LTD (Xu et al., 1997; Pavlides et al., 2002; von Frijtag et al., 2001; Alfarez et al., 2002; for review see Kim and Diamond, 2002).

**Brief stress results in bi-directional morphological dendritic changes with homeostasis in structure and input**

Three weeks after a double defeat, a complex shift in the branching pattern of the CA3 pyramidal neurons was detected. The patterns were similar to repeated defeat in the dendritic regression at the apical dendrites, and in the impairment of low-frequency LTP at C/A afferents at CA3 cells (Fig. 3).

Numerical and Sholl analyses confirmed, however, that specific to the double defeat, more dendritic branches were detected at the basal cone. This suggest de novo dendrite development within the stratum oriens of the CA3. Importantly, despite the obvious profound structural redistribution, the total surface area and dendritic length were maintained at control level (Fig. 5, Table 2). Also the amplitude and kinetics of EPSPs where not different compared to control. Although this suggests a homeostatic maintenance of the total available postsynaptic area, more detailed investigations including spine measurements are required to confirm this. Presently, we do not know the extent to which the excitatory synaptic input that derives directly from synapses of the basal dendrites is changed. The basal segments receive synaptic input from the fimbria and lateral septum (Amaral and de Witter, 1990; Li et al., 1994) and act
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physiologically independent of the apical excitatory input (Bradler and Barrionuevo, 1989).

The finding that even brief stress, and not necessarily its recurrence, induces large-scale dendritic rearrangements has significant implications. These include the question of its persistence, the mechanism of its regulation, and its possible functional role. Our data support the formulation that the repetition of stress temporary suppresses the addition of new branches, and consistent with previous evidence, show that the growth of dendrites is facilitated after stress (see also Vyas et al., 2002). In our paradigm, however, such de novo branches were confined to the basal cone and we did not observe structural normalization, not even three weeks after the alleviation of stress, suggesting a rather permanently modified circuit. Although the plasticity of the CA3 dendrites is reported to occur within hours under conditions of torpor (Popov et al., 1992), their modification by stress requires at least two weeks (McEwen, 1999). Conversely, the re-establishment of normal branch lengths also requires a 10-day recovery period after the termination of the stress (Conrad et al., 1999; Sousa et al., 2000).

A role in corticosteroid feedback?

It is generally thought that repeated stress-induced dendritic regression is under the synergistic control of prolonged endogenously elevated corticosteroids and glutamate (Magariños and McEwen, 1995b; McEwen, 1999). Previous studies have shown that a social-defeat episode increases free corticosterone during the first 5 h only, after which normal levels are maintained for at least three weeks, both during the peak and through the diurnal cycle (Buwalda et al., 1999, 2001). Therefore, it is difficult to reconcile the bi-directional structural changes with a basal hyperactivation of the HPA axis. Despite preservation of resting corticosterone levels there is cumulative evidence that, corticosteroid-feedback regulation is altered over time after a single episode of stress (van Dijken et al., 1993; Buwalda et al., 1999; Libezon et al., 1999). Three weeks after the application of a single stressor, adrenocorticotropic hormone (ACTH) release is hypersensitive to corticosteroid-releasing-factor (CRF) activation (Buwalda et al., 1999), as well as to novel stressors (van Dijken et al., 1993), whereas enhanced corticosteroid feedback has been demonstrated with exposure to a homotypical stressor (Martí et al., 2001; Dal-Zotto et al., 2002). A role for the hippocampus within an adaptation of the HPA feedback is supported by the reduced binding and expression of mineralocorticoid receptors three weeks, but not one week, after a single episode of stress (Buwalda et al., 1999, 2001; Libezon et al., 1999). Therefore, the rearrangements of the CA3 dendrites demonstrated here correlate temporally with a different tuning of the HPA axis, but do not necessarily follow from a hyperactivated basal increase.

The extent to which the altered dendritic patterns contribute to feedback regulation remains to be determined, but the dense excitatory CA3 network is in a strategic position. Activity of the CA3 subfield is implicated in HPA-axis regulation, through putative trans-synaptic inhibitory connections with paraventricular-nucleus (PVN) neurons (Jacobson and Sapolsky, 1991; Roozendaal et al., 2001). Furthermore, the CA3 recurrent synapses have the ability to recall neuronal representations of single events largely independently of external input, and their NMDA-receptors support the
reorganization required for novel cognitive demands (Lee and Kesner, 2002; Nakazawa et al., 2003). Because we did not observe normalization of the postsynaptic dendritic segments long after defeat, it is tempting to assume that the CA3 subfield retains information concerning the stress experience, and functions as a neural component through which future adrenocortical feedback responses might be inhibited or disinhibited (Martí et al., 2001).

**Conclusions**

We conclude from our data, taken together, that the passage of time is critically involved in the long-term adaptations in form and function of the CA3 pyramidal neurons after stress. This provides evidence and a neuroanatomical basis for the progressive and time-dependent nature of the dynamic changes in neural systems induced by stress after the actual experience (Koolhaas et al., 1997; Antelman et al., 2000; Dal-Zotto et al., 2002). The features of neuronal changes that persist long after the stress is alleviated might contribute to our understanding of the development of stress-related disorders that are typically characterized by a delay in onset, such as post-traumatic stress disorder (Yehuda and Antelman, 1993).