The antidepressant tianeptine persistently modulates glutamate receptor currents of the hippocampal CA3 commissural-associational synapse in chronically stressed rats

MAARTEN H.P. KOLE [1,3], LAURA SWAN [2] AND EBERHARD FUCHS [1]

[1]Division of Neurobiology, German Primate Center and
[2]European Neuroscience Institute (ENI), Göttingen, Germany
[3]School of Behavioral and Cognitive Neurosciences, University of Groningen, The Netherlands

Summary

Recent hypotheses on the action of antidepressants imply a modulation of excitatory amino acid transmission. Here, the effects of long-term antidepressant application in rats with the drug tianeptine were examined at hippocampal CA3 commissural-associational (C/A) glutamate receptor ion channels, employing the whole-cell patch-clamp technique. The drug's impact was tested by subjecting rats to daily restraint stress for three weeks in combination with tianeptine treatment (10 mg/kg/day). Whereas stress increased the deactivation time-constant and amplitude of *N*-methyl-D-aspartate (NMDA) mediated excitatory postsynaptic currents (EPSCs), it did not affect the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) mediated EPSCs. Concomitant pharmacological treatment of stressed animals with tianeptine resulted in a normalized scaling of the amplitude ratio of NMDA receptor to AMPA receptor-mediated currents and prevented the stress-induced attenuation of NMDA-EPSCs deactivation. Paired-pulse-facilitation and the frequency-dependent plasticity remained unchanged. Both in control and stressed animals, however, tianeptine treatment strengthened the slope of the input-output relation of EPSCs. The latter was mimicked by exposing hippocampal slices *in vitro* with 10 μM tianeptine, which rapidly increased the amplitudes of NMDA- and AMPA EPSCs. The enhancement of EPSCs could be blocked by the intracellular presence of the kinase inhibitor staurosporine (1 μM), suggesting the involvement of a postsynaptic phosphorylation cascade rather than presynaptic release mechanisms at CA3 C/A synapses. These results indicate that tianeptine targets the phosphorylation-state of glutamate receptors at the CA3 C/A synapse. This novel signal transduction mechanism for tianeptine may provide a mechanistic resolution for its neuroprotective properties and, moreover, a pharmacological trajectory for its memory enhancing and/or antidepressant activity.

Acknowledgements

The presented study has been partially supported by I.R.I Servier, Courbevoie, France. We thank C. Muñoz for initiating the study and we are grateful to B. Czéh, B.S. McEwen and L. Reagan for helpful comments on previous versions of the manuscript.

Introduction

Research on the pharmacological mechanisms and effects of antidepressants is a key approach to resolution of the underlying factors of the pathophysiology of depression, a disorder for which fundamental biological criteria are still lacking (Wong & Licinio, 2001). During recent decades, research on antidepressants has been primarily oriented towards the concept of an imbalance in monoamine neurotransmission, such as noradrenaline or serotonin (Coppen & Doogan, 1988). Although considerable progress has been made, there are still serious gaps and limitations in the explanation of the clinical aspects, etiology and pathophysiology of major depression (Wong & Licinio, 2001). For example, the currently available data on the cellular and neurochemical effects of the antidepressant tianeptine is difficult to reconcile with a monoamine hypothesis of depression, although the drug possesses clear clinical efficacy (Piñeyro et al., 1995, Wagstaff et al., 2001).

Major stressful life events are recognized as risk factors in the etiopathology of depression (Manji et al., 2001, Wong & Licinio, 2001). Preclinical and clinical research suggests that the hippocampus may be relevant to research on the cellular mechanisms of antidepressants in the central nervous system, because of its integrative function during the stress response, an important constituent of the depressive syndrome (McEwen, 2000, Sheline et al., 1996). Several lines of evidence indicate that the NMDA receptor for glutamate, the principal excitatory neurotransmitter in the brain, mediates the major functional and cellular effects of stress. First, receptor binding and subunit expression for hippocampal NMDA-receptors is enhanced following stress (Bartanusz et al., 1995, Krugers et al., 1993). Second, via NMDA-receptor activation stress shifts hippocampal synaptic plasticity (Kim et al., 1996), the cellular correlate to learning and memory (Malenka & Nicoll, 1999), to a lower threshold for long-term depression (LTD). Third, the application of an NMDA-receptor antagonist prevents stress-induced dendritic remodeling of CA3 principal neurons, a highly replicable consequence of chronic stress (Magariños & McEwen, 1995a).

In line with these and other data, the recent hypothesis emerged that reduction of NMDA receptor-mediated functions may be implicated in antidepressant activity (Skolnick, 1999, Petrie et al., 2000, Krystal et al., 2002). Indeed, chronic application of antidepressants selectively reduces expression of hippocampal NMDA receptor subunits (reviewed by Skolnick, 1999), and in various animal models for depression the application of NMDA antagonists exhibit pharmacological antidepressant potency (reviewed by Petrie et al., 2000). Interestingly, a recent clinical trial (Berman et al., 2000) also provided evidence that treatment with the NMDA-antagonist ketamine produced a significant, albeit short, improvement of mood in patients suffering major depression.

In the present study, we hypothesized that the antidepressant tianeptine may exert direct effects on the hippocampal glutamate receptor system, and furthermore that this interaction may be important in treatment-relevant conditions. In one series of experiments involving repeated restraint stress, we established which stress-induced physiological modifications occur at whole-cell recorded NMDA and AMPA-receptor postsynaptic currents (EPSCs), mediated by the CA3 commissural-associational

synapses. Applying this model, we investigated the particular role tianeptine has in modifying the EPSCs of CA3 C/A synapses using concomitant drug application. In a separate set of experiments, the short-term modulation of tianeptine at CA3 C/A EPSCs was studied *in vitro* using hippocampal slices from animals without previous treatment. Here, we searched for the molecular route that tianeptine may possibly use when interacting with the hippocampal glutamate receptor-mediated currents.

Materials and Methods

Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; C/A, commissural-associational; CA3, cornu ammonis layer 3; EPSCs, excitatory postsynaptic currents; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PPF, paired-pulse facilitation; R_N, membrane input resistance; SSRI, serotonin reuptake inhibitor; V_M, membrane potential.

Experimental animals

Male Wistar rats (Winkelmann, Borchen, Germany) weighing 100–120 g at the beginning of the experiments were housed in groups of three with ad libitum access to food and tap water. Animals were maintained in a temperature-controlled room, with a light/dark cycle of 12/12 hrs (lights on at 0600 h). The rats were handled and weighed daily for two weeks and randomly assigned to the experimental groups. Experiments were performed during the light period of the cycle. All treatments, which included body weight measurement and injection, were performed during the light period. Animal experimentation was done in accordance with the European Council Directive of November 24th 1986 (86/EC), and was approved by the Government of Lower Saxony, Germany.

Restraint stress and tianeptine treatment

A 2 × 2 experimental procedure was applied, separating the effects of stress, drug administration and their interaction. The groups were Control; Control + Tianeptine; Stress; and Stress + Tianeptine, and the experimental phases are displayed in Fig. 1A. The first experimental phase ('Habituation') lasted for 14 days, during which body weight was recorded daily. The second phase of the experiment ('Treatment') lasted for a period of 21 days, during which the animals of the Stress, and Stress + Tianeptine groups were submitted to daily restraint stress for 6 hrs/day (1000–1600 h). Restraint stress was carried out according to an established paradigm (Magariños & McEwen, 1995a). Before the onset of the daily restraint period, animals of the Stress + Tianeptine group received tianeptine (Stablon®, S 01574-01, N-(8-chloro-10-dioxo-11-methyl-dibenzo(c,f)(1,2)-5-thiazepinyl) sodium heptanoate, Servier, France) in a concentration of 10mg/kg, i.p., dissolved in sterile 0.9% NaCl. Animals of the Stress group were injected daily with the vehicle only. During restraint stress, the rats were

placed in plastic tubes in their home cages and they had no access to food or water. Control rats were not subjected to any type of stress except daily injections. Animals of the Control + Tianeptine group received tianeptine (10 mg/kg; i.p.) daily at 1000 h, whereas animals of the Control group were injected with the vehicle only. Each experimental group consisted of six animals and body weights were recorded daily.

After the 21 days of treatment and a recovery period of 1.5 days, animals were briefly anesthetized with ether and decapitated (0900 h). Brains were rapidly removed and processed for slice preparation (see below), and trunk blood was collected in EDTA containing tubes. Blood samples were immediately centrifuged and plasma stored frozen at -20°C for subsequent corticosterone measurement.

In vitro effects of tianeptine

Rats not subjected to any type of treatment were briefly anesthetized with ether and decapitated (0900 h). Brains were rapidly removed and processed for slice preparation (see below).

Brain slice preparation

The brain was rapidly removed and immediately placed in oxygenated (95% O_2, 5% CO_2) ice-cold sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 198 Sucrose, 1 $MgCl_2$, 2.5 KCl, 2 $MgSO_4$, 1.25 Na_2HPO_4, 26 $NaHCO_3$, 14 D(+)-Glucose, 1 kynurenic acid, 1.5 $CaCl_2$, 2 L(+) ascorbic acid. After chilling for 1.5 min, the brain was glued at its dorsal side in a vibratome (Vibracut-2, FTB Feinwerkmechanik, Bensheim, Germany), and transverse slices of 400 µm nominal thickness were cut and stored in oxygenated ACSF (125 NaCl, 2.5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 1.5 $CaCl_2$, 1 L(+) ascorbic acid, 14 D(+)-Glucose, ~300 mOsm, pH 7.4. Slices were allowed to equilibrate at 33°C for 1 hr, and then kept at room temperature.

Whole-cell recording

Slices were hemisected and the hippocampal-enthorinal area isolated and transferred to a submerged type of recording chamber (flow rate: 1–2 mL/min) with continuously oxygenated ACSF. CA3 pyramidal neurons were visualized and approached using infrared-differential interference contrast microscopy (Zeiss, Göttingen, Germany) allowing pre-selection and recording standardization (Fig. 1B). As densities of C/A afferents vary in function according to their proximo-distal position in the longitudinal axis, we recorded through the CA3b region (Ishizuka et al., 1995). Patch-clamp borosilicate glass pipettes (Hilgenberg, Malsfeld, Germany) were pulled on a two-stage puller (PP-830, Narishige, London, UK), possessing a resistance of 2–5 MΩ in the bath, and filled with the intracellular solution composed of (in mM) 100 cesium gluconate, 17.5 $CsCl_2$, 2 $MgCl_2$, 8 NaCl, 10 HEPES, 1 EGTA, 3 ATP-Na_2, 10 phosphocreatine, 2.2 QX-314 (a Na^+ channel blocker) and 0.3 tris-GTP, added freshly from –80°C stock solutions. The pH was set to 7.3 with CsOH and 295 mOsm. All data were collected at 32 ± 0.5 °C. Whole-cell voltage-clamp recording was performed

with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA), low pass filtered at 2 KHz and digitized at 10 kHz with an ITC-16 computer interface (HEKA Elektronik, Lambrecht, Germany), allowing control of amplifier command potentials, step protocols and data collection with the PULSE software (HEKA). Pipette offset and capacitance were compensated for, using the appropriate controls at the amplifier. Series resistance (R_S) was routinely monitored in voltage-clamp using either test-pulses or R_S-cancellation amplifier readout. Whole-cell measurements typically were performed with 7 MΩ and aborted when > 20 MΩ. Stimulation of C/A afferents was performed using monopolar tungsten microelectrodes (0.1 MΩ, WPI Inc., Sarasota, FL, USA) placed in the stratum radiatum at ~300 µm lateral from the whole-cell recording site (Fig 1B). Brief 100 µs stimulation pulses with varying intensities (10–200 µA) were employed. EPSCs were always recorded under the presence of α-aminobutyric acid $(GABA)_A$ receptor antagonists (-)- bicuculline (10–20 µM) and picrotoxin (20–40 µM), respectively. Extracellular Ca^{2+} was raised to 3.5 mM and Mg^{2+} to 2.5 mM preventing epileptic events. While keeping the cell at –80 mV, leak always amounted less than –200 pA.

Chemicals

EGTA, HEPES, gluconic acid, cesium chloride, tris-GTP, ATP-disodium, phosphocreatine and kynurenic acid were obtained from Sigma-Aldrich (Steinheim, Germany). CNQX-disodium salt, QX-314, D-AP5 (D-[-]-2-Amino-5-phosphonovaleric acid), DCG-IV, (-)-bicuculline-methobromide and picrotoxin were obtained from Tocris (Biotrend, Köln, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Data analysis

Data analysis at current- and voltage-traces was performed with the software PULSEFIT (HEKA) using a minimum of 5 consecutive sweeps and then averaged. For graphical processing, Origin 6.1 (Microcal Software Inc., Northampton, MA, USA) was used. Statistical analysis of the *in vivo* experiments (restraint stress and tianeptine treatment) was conducted with ANOVA SPSS 7.5 (SPSS Inc., Chicago, IL, USA) monitoring the two-way interaction design, and isolating the effects of tianeptine, stress and their interaction. Data are presented as mean ± S.E.M. of recorded cells. All reported findings were statistically analyzed with animals as dependent variable as well. These analyses gave similar results both qualitatively as quantitatively, indicating that the inter animal differences were lower than the variation between groups, for the parameters that were found to be affected. The *in vitro* effects of tianeptine were tested with two-tailed unpaired and paired t-tests. A level of $P < 0.05$ was considered significant.

Results

Effects of stress and tianeptine treatment on body weight and corticosterone

The effectiveness of the stress procedure was assessed by monitoring body weight and plasma corticosterone, which are both associated with the intensity of stress exposure (McEwen, 2000). Chronic restraint reduced body weight gain throughout the entire stress period ($F[1,3] = 41.2$, $P < 0.001$). In both the Stress and Stress + Tianeptine group, body weight gain was reduced (34 ± 5%, compared with Control and Control + Tianeptine groups). There was no interaction detected with the drug treatment ($F[1, 3] = 0.9$, $P > 0.4$), demonstrating that tianeptine did not prevent the stress-induced reduction of body weight gain. Morning plasma corticosterone values were not affected by the three weeks of stress (Control: 12.5 ± 1.7 ng/mL, Stress: 12.3 ± 2.3 ng/mL; $P > 0.12$). Concomitant tianeptine treatment, however, raised these levels (Stress + Tianeptine: 21.2 ± 6.9 ng/mL and Control + Tianeptine: 43.0 ± 10.2 ng/mL; $P < 0.01$). There was no interaction between stress and tianeptine ($P > 0.1$).

Isolation of the commissural-associational excitatory postsynaptic currents

The commissural-associational (C/A) CA3 synaptic currents were studied by stimulation of afferent fibers in the stratum radiatum (Fig. 1B). The excitatory postsynaptic currents (EPSCs) were evoked at a rate of 0.05 Hz from a holding potential of –80 mV. The ionotropic glutamate receptors producing a dual-component EPSC can be divided into the kinetically fast AMPA receptors and the slow NMDA receptor (Hestrin et al., 1990). The application of 20 μM CNQX rapidly blocked the fast, inward EPSC, and at a holding potential of -80 mV a pure AMPA-receptor mediated EPSC was recorded (Fig. 3A). Preliminary experiments (data not shown) indicated that the paired-pulse facilitation (PPF) magnitude of C/A synapses were lower compared with PPF from mossy fiber EPSCs. Also their kinetics were considerably slower compared with mossy fiber synapses, and were thereby indicative of C/A EPSCs (Salin et al., 1996). To further evaluate the synaptic source of the EPSCs, we applied 1 μM DCG-IV, pharmacologically blocking the mossy fiber transmission (Kamiya et al., 1996). The amplitude and kinetics of the EPSCs were only marginally affected (2.3 ± 4%, n = 5), indicating that the currently applied scheme yielded ample pure C/A EPSCs.

Effects of stress and tianeptine treatment on CA3 glutamate receptor currents

Comparing the EPSCs between slices of the different experimental groups revealed that the various treatments did not change resting CA3 neuronal membrane properties (Table 1). Neither the cellular input resistance nor membrane potentials, nor the time kinetics of the increase in whole-cell AMPA-EPSC were changed by stress or antidepressant treatment. As our main objective was to study cross-slice treatment effects, we normalized the AMPA EPSC amplitude to 250–300 pA by adjustment of the stimulus intensities. Scaled EPSCs of the four groups were similar in latency, duration of increase and decay kinetics (Table 1).

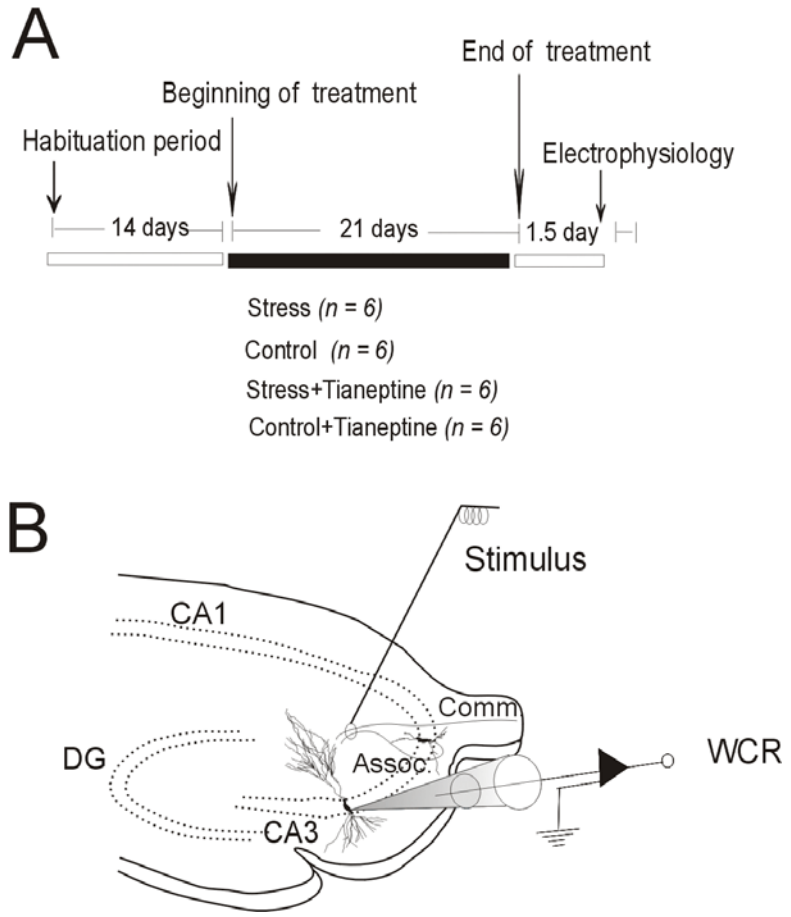

Fig. 1. (A) Temporal representation of the experimental design and the four experimental groups. A 14-day experimental phase consisted of a Habituation period. During the second phase, which lasted 21 days, the animals in the two stress groups (Stress, Stress + Tianeptine) were submitted to daily restraint stress (6 hrs/day). Stressed animals received a daily i.p. injection of tianeptine (Stress + Tianeptine; 10 mg/kg, n = 6) or vehicle (Stress, n = 6). Animals in the control group remained undisturbed except for a daily injection of tianeptine (Control + Tianeptine; 10 mg/kg; n = 6) or vehicle (Control, n = 6). Note that the animals were sacrificed 1.5 days after the last treatment, before brain slices were prepared for whole-cell recording. (B) Schematic diagram of the hippocampal slice preparation, arrangement of stimulus electrode and patch-clamp recording pipette. The patch-clamp electrode was positioned in the CA3b area and pyramidal shaped neurons selected for recording using IR-DIC video-microscopy. To activate the commissural-associational (C/A) pathway, a stimulus electrode was placed, under visual microscopic control, lateral from the CA3b pyramidal neuron in the stratum radiatum field. This stimulus protocol activates afferents both from the commissural [comm] fibers, originating from the contralateral CA3 region, and from the associational [assoc] fibers, from ipsilateral CA3 cells.

Table 1. Resting CA3 membrane and C/A EPSC properties

	Control	Stress	Control + Tianeptine	Stress + Tianeptine	P
R_N (MΩ)	104.1 ± 7.1 (27)	112.4 ± 4.6 (23)	93.6 ± 5.6 (19)	103.5±11.3 (19)	n.s.
V_M (mV)	-69.0 ± 1.6 (27)	-68.7 ± 2.2 (23)	-65.7 ± 4.8 (9)	-63.3 ± 2.2 (19)	n.s.
AMPA EPSC[a]					
Latency (ms)	2.8 ± 0.1 (18)	3.1 ± 0.1 (14)	2.9 ± 0.2 (10)	3.1 ± 0.2 (12)	n.s.
10-90% rise time (ms)	2.8 ± 0.2 (18)	3.0 ± 0.2 (14)	2.6 ± 0.3 (10)	2.8 ± 0.6 (12)	n.s.
Decay time (ms)	15.9 ± 1.2 (18)	15.7 ± 1.2 (14)	15.1 ± 1.0 (10)	16.9 ± 1.4 (12)	n.s.
NMDA- EPSC [b]					
Amplitude (pA)	72.9 ± 15.2 (15)	116.5 ± 24.4 (14)	62.9±12.5 (17) *	60.8 ±6.3 (12) *	0.03
10-90% rise time (ms)	4.9 ± 0.2 (15)	5.3 ± 0.2 (14)	4.8 ± 0.5 (15)	5.0 ± 0.4 (12)	n.s.
Decay rate (ms)	56.8 ± 3.7 (15)	82.6 ± 8.9 (14) *	61.0 ± 5.5 (17)	64.7 ± 4.8 (12)	0.003

Data represent mean ± S.E.M. (n = number of cells).[a]Determined with amplitudes normalized to 250–350 pA, at a holding potential of –80 mV, [b] Determined at a holding potential of +60 mV. *$P < 0.05$ main effect of two-way ANOVA.

Whole-cell recordings were then continued at depolarized voltages between +40 and +60 mV for a minimum of 1.5 s to fully relieve the voltage-dependent Mg^{2+} block of the NMDA-channel (Chen et al., 1999, Hestrin et al., 1990). An outward EPSC with slow rise and decay kinetics was obtained with pharmacological sensitivity to the specific NMDA receptor blocker D-AP5 (50 μM inhibited 87 ± 3%, n = 7, Fig. 2A). The NMDA-EPSC was then expressed as the percentage of the AMPA EPSC (Ito et al., 1997, 2000). Stress exposure significantly enhanced the ratio of NMDA/AMPA EPSC peak amplitudes ($F[1, 57] = 8.1$, $P < 0.01$; Fig. 2C and 2D). Stimulation intensities were not related to NMDA/AMPA ratio (r = –0.1 for Control, n = 18), indicating that the results of the NMDA/AMPA ratios were not contaminated by differential stimulus intensities. In fact, group comparisons of the absolute NMDA-receptor mediated currents (at +60 mV) were significantly affected by treatment ($F[2, 57] = 3.6$; $P < 0.03$), while tianeptine lowered amplitudes in the Stress + Tianeptine group ($F[1, 55] = 7.0$; $P < 0.01$). Furthermore, chronic restraint stress reduced the pace of decay kinetics by 146 ± 16% ($F[1, 49] = 6.9$; $P < 0.01$, Fig. 2B), and tianeptine treatment (Stress + Tianeptine) significantly counteracted this effect (two-way interaction; $F[1, 49] = 4.8$; $P < 0.03$, Table 1).

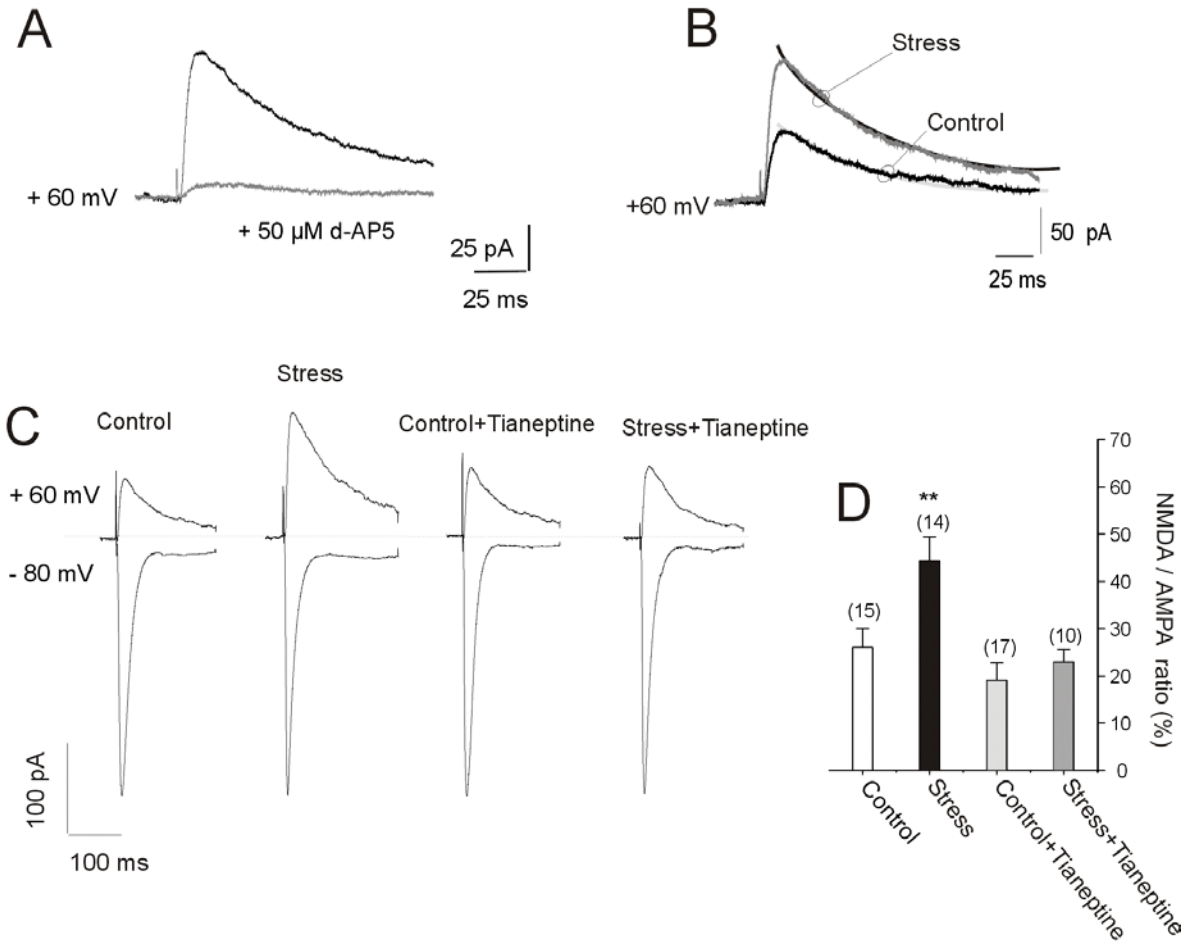

Fig. 2. (A) Isolation of the NMDA-receptor mediated component of the CA3 C/A EPSC (baseline trace) was obtained in whole-cell voltage-clamp configuration and in ACSF containing bicuculline (20 µM), picrotoxin (40 µM) and CNQX (20 µM), applying a depolarizing holding potential of +60 mV. The control current (baseline) was fully blocked by 50 µM of a NMDA-receptor blocker (D-AP5). (B) Chronic stress (Stress group, open dots) selectively resulted in a slower deactivation kinetic of the NMDA-receptor mediated EPSC (Stress: 82.6 ± 9 ms vs. Control: 56.8 ± 4 ms, $P < 0.001$). This effect of stress was not seen in NMDA-EPSC recordings in slices from animals subjected to concomitant tianeptine treatment (Stress+Tianeptine, see Table 1 for data). The lines through the current traces are the fits obtained with a single exponential function. (C) For each group, traces of AMPA EPSCs (–80 mV, lower traces) are compared with the NMDA-receptor mediated EPSCs (upper traces, recorded at +60 mV + 20 µM CNQX). In slices from animals subjected to repeated stress, the NMDA-component appeared enhanced. (D) Summarizes all data from the peak amplitudes, expressed as NMDA/AMPA ratios, for each treatment group. Chronic stress resulted in an enhanced ratio of NMDA/AMPA CA3 C/A EPSCs (44.3 ± 5.1% vs. 26.1 ± 3.9%, $P < 0.01$, two-way-ANOVA), but this effect was counteracted by long-term tianeptine treatment ($P < 0.03$). Data are the mean ± S.E.M., numbers indicate the cells obtained from 6 animals per group.

Stress is known to reduce the length and complexity of CA3 apical dendrites (Magariños et al., 1999), thereby warranting the interpretation of kinetic data of postsynaptic current from distal synapses. A reduced dendritic length would shorten the electrotonic distance and thus accelerate the rise-times, especially for rapidly occurring synaptic currents such as the fast AMPA currents (Spruston et al., 1993, Hestrin et al., 1990). However, when comparing the kinetics we could detect no alteration of EPSC latency or rise-time kinetics for either the NMDA- or AMPA receptor mediated EPSCs (Table 1). This suggests that the observed changes in NMDA-receptor EPSC cannot be directly attributed to different space-clamp conditions between treatment procedures. NMDA current-voltage (*I-V*) relationships

were additionally reconstructed by stepwise 10 mV hyperpolarizing of the membrane potential within a voltage range of +60 to –80 mV. The membrane potential was always maintained for several seconds before recording approximately five sweeps (Chen et al., 1999). Recordings from both Control and Stress treated animals showed I-V relationships with a similar shape and amplitude. The reversal potential, measured at the intersection on the voltage axis of a linear fit of the current, was unchanged by stress treatment (Control: 9 ± 3 mV, Stress: 14 ± 2 mV; $P > 0.7$), but shifted by +10 mV after chronic tianeptine treatment ($F[1, 35] = 11.4$; $P < 0.002$) (Stress + Tianeptine: 21.3 ± 4.8 mV). Also these data indicate that the whole-cell recordings of CA3 pyramidal neurons of animals exposed to stress, containing putative retracted dendrites, are not different with respect to voltage-control.

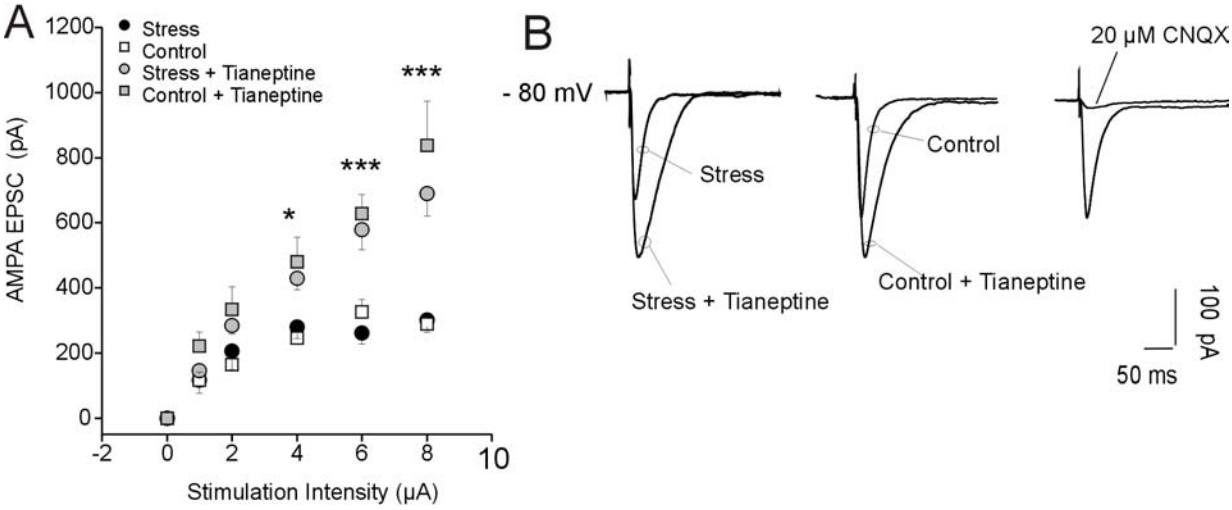

Fig. 3. (A) The stimulus-response curve for AMPA EPSCs shows that long-term treatment with tianeptine shifted the response curve to greater amplitudes at stimulation intensities of greater strength (132% increase at 4 µA, 234% at 8 µA). However, chronic stress had no effect on the AMPA EPSC peak amplitudes, at any of the stimulus intensities (P > 0.05). Mean ± S.E.M. are shown per treatment group. *P < 0.05, ***P < 0.001, for the main effect of tianeptine. (B) The graph shows typical EPSCs of CA3 C/A synapses recorded at a holding potential of –80 mV. These EPSCs were mediated by AMPA receptors since bath application of the specific blocker CNQX (20 µM) completely abolished the inward current. Slices of animals treated long-term with tianeptine (Control + Tianeptine and Stress + Tianeptine) expressed AMPA EPSCs with larger amplitude.

The final nominal intensities needed for eliciting 250 pA peak amplitude AMPA EPSC were ~60 % lower for the tianeptine-treated animals. The three-week treatment with tianeptine, in both the Stress + Tianeptine and Control + Tianeptine groups, had a significant effect on the peak amplitude of AMPA EPSC. This is shown by plotting the input-output relationship (e.g., 4 µA, main-effect $F[2, 51] = 3.55$, $P < 0.05$, Fig. 3A-B). Stimulation intensities larger than 50 % of the maximum amplitude increased EPSC peak amplitudes approximately twofold (e.g., 221 ± 29 % [n = 8], Stress + Tianeptine compared with Stress).

Given that tianeptine thus affects the conductance of AMPA EPSCs, we asked further whether stress and/or antidepressant treatments affect mechanisms involved with the generation of short-term plasticity mediated by glutamate-receptor activation. Two protocols were used to approach this question. We first tested paired-pulse-facilitation (PPF) of AMPA EPSCs with an inter-pulse duration of 80 ms. In this

schedule the pulse enhancement is traditionally interpreted as depending on accumulation of presynaptic bound and/or residual Ca^{2+} during the conditioning pulse, enhancing transmitter release within a time window of milliseconds (Zucker, 1999). Comparing slices between groups, the degree of PPF ($EPSC_2$:$EPSC_1$) appeared unchanged after chronic stress or long-term tianeptine treatment (mean facilitation in Control: 147.2 ± 4.1 %, n = 20, Stress: 144.5 ± 5.1 %, n = 16, Control + Tianeptine: 140.9 ± 11.2 %, n = 17, Stress + Tianeptine: 145.1 ± 5.2 %, n = 11). The second test protocol entailed a more slowly developing short-term plasticity of the CA3 C/A synapse, shown previously to depend on intra-terminal Ca^{2+} and Calmodulin kinase (Salin et al., 1996). Switching from a 0.05 to 2.0 Hz stimulus frequency scheme, the EPSC peak amplitudes of AMPA-R currents gradually increased and reached a plateau current within one minute, whereby upon a return to 0.05 Hz the baseline amplitudes were re-established (Fig. 4A). The mean magnitude of frequency potentiation for each group was very similar to the paired-pulse facilitation (Fig. 4B). In line with PPF comparison between groups, no differences in expression of the frequency-dependent EPSC short-term plasticity were detected (Fig. 4B).

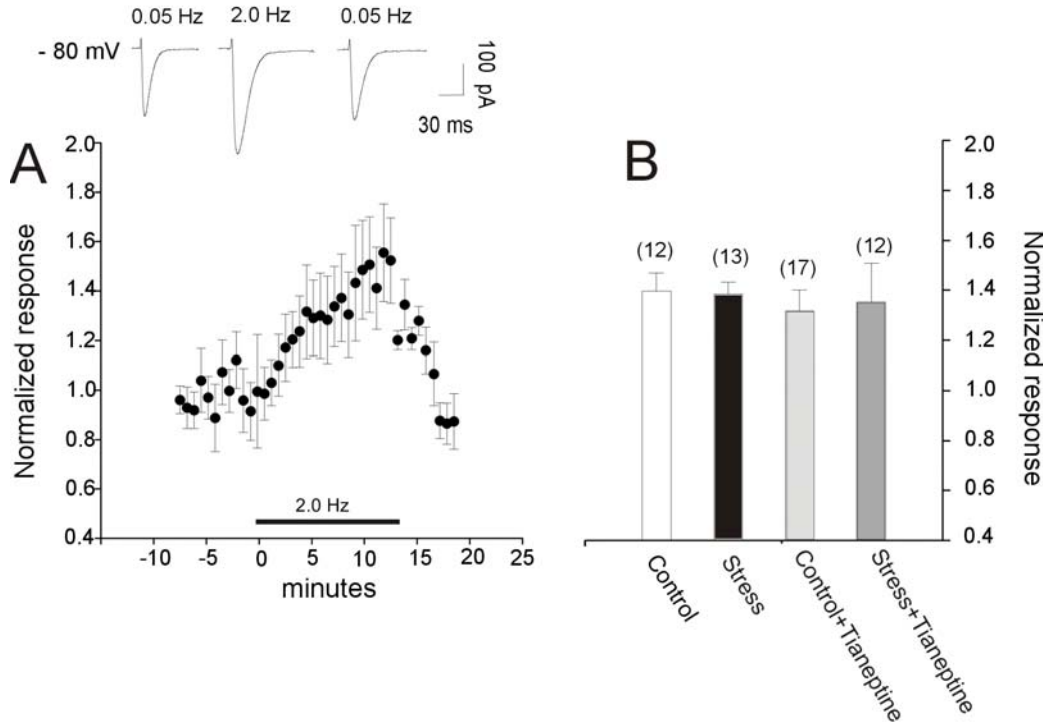

Fig. 4. Lack of effects of chronic stress or chronic tianeptine treatment on short-term frequency-facilitation. (A) CA3 AMPA EPSC recordings were initially made at 0.05 Hz for 8 minutes during the baseline period. A rapid and reversible enhancement of the peak amplitude of AMPA EPSCs was observed when the stimulation frequency was altered from 0.05 Hz to 2.0 Hz. (B) The short-term facilitation was quantified as the ratio of the mean of 0.05 Hz, for all cells per group (n = 12 - 17) and compared. Both types of treatment, repeated stress and chronic antidepressant, did not alter frequency-facilitation ($P > 0.1$). The group mean ± S.E.M. of frequency-facilitated amplitude is shown. The absolute magnitude of potentiation was very similar for paired-pulse-facilitation experiments.

Effects of in vitro tianeptine application

The results from the effects of tianeptine in the Stress + Tianeptine and Control + Tianeptine animals indicated drug-specific augmentation of glutamate receptor-mediated transmission. We aimed to obtain further insights into the cellular pathways or mechanisms by which tianeptine is able to facilitate EPSCs at the CA3 C/A synapses. Previous studies showed that antidepressants or mood stabilizers are able to enhance EPSC via pre-synaptic mechanisms such as phosphorylation of the transmitter release site (Bouron & Chaton, 1999) or action potential broadening (Colino et al., 1998). In *in vitro* experiments, tianeptine in a concentration of 10 µM, reflecting therapeutically relevant brain concentration (Couet et al., 1990), was applied to the bath. Within five to seven minutes after application, tianeptine enhanced the EPSC, reaching a plateau at which the magnitude was typically a twofold rise in peak amplitude (210 ± 30 %, n = 7, Fig. 5A). There was no effect on the latency or duration of current increase (-0.5 ± 0.4 ms, n = 5). However, the decay time of the EPSC increased slightly from 14 ± 3 ms to 22 ± 4 ms (n = 5), and the amplitude at 70–80 ms after stimulus produced an increase of 310 ± 30 % (n = 4).

Under the presence of 50 µM D-AP5, a specific NMDA-receptor blocker, the same amplitude increase was obtained, but without a broadening of the EPSC width. Thus, both the fast AMPA and the kinetically slower NMDA-receptor mediated currents are increased by the antidepressant. This was further tested in seven cells by determining the ratio of NMDA/AMPA after slices were incubated for a minimum of two hours with 10 µM tianeptine. The relative NMDA/AMPA ratio was 27 ± 6 %, which was not different from control. These *in vitro* effects thus corroborated the data on long-term treatment. The fact that NMDA and AMPA EPSC amplitudes were increased similarly may suggest a change in presynaptic glutamate transmitter release. However, this contrasts with the finding that chronic tianeptine treatment was found not to change the CA3 C/A PPF of EPSCs.

Nevertheless, we tested whether *in vitro* effects of tianeptine can be found at the presynaptic site of the CA3 C/A synapse. Using a $KMeSO_4$-based intracellular solution, acute exposure to tianeptine (10 µM) as measured in current clamp recordings did not change the threshold for CA3 somatic depolarization-induced spike amplitudes or width (2 ± 6 % and 1 ± 2 %, respectively, n = 4), and neither the intrinsic membrane input resistance nor the time constant was changed (data not shown). A more direct approach was adopted from Colino et al. (1998). By applying continuously paired pulses (50 ms interval, 0.05 Hz), the enhancement of the AMPA EPSC by tianeptine and its effect at PPF were monitored simultaneously. The tianeptine-induced EPSC augmentation was not accompanied by a modification of PPF (baseline: 139.3 ± 4 %, after tianeptine: 126.5 ± 7 %, n = 4, paired t-test, $P = 0.48$). Collectively, these data lead to the suggestion that tianeptine enhances the CA3 C/A EPSC by mechanisms located postsynaptically rather than presynaptically.

Tianeptine acts via intracellular mechanisms

Because both the NMDA and AMPA glutamate receptors may be internally modulated via Ca^{2+}-dependent mechanisms, we tested the dependence of the upregulation by inhibiting the increase of intracellular Ca^{2+}, using 20 mM of the Ca^{2+}-

chelator 1,2-bis (2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) added into the pipette solution. To allow diffusion throughout the neuron, application of tianeptine commenced 15 min after establishing the whole-cell configuration. The presence of BAPTA in this concentration slightly suppressed, but did not inhibit the enhancement of synaptic response in the presence of tianeptine (168 ± 40 %, n = 5).

Several different phosphorylation mechanisms have been shown to effect changes in hippocampal synaptic plasticity, and in particular, to regulate glutamate mediated synaptic transmission via both Ca^{2+}-dependent and Ca^{2+}-independent pathways (the latter largely via interactions with calcium/calmodulin-dependent protein kinase II), targeting both AMPA and NMDA type receptors (Winder & Sweatt, 2001). In the presence of 1 µM of the broad-spectrum kinase inhibitor staurosporine (Tamaoki et al., 1986) the effect of tianeptine on EPSC was nearly neutralized to an increase of 118 ± 12 %, which was significantly lower than the control response (n = 5, unpaired t-test; $P < 0.001$, Fig. 5A and 5B).

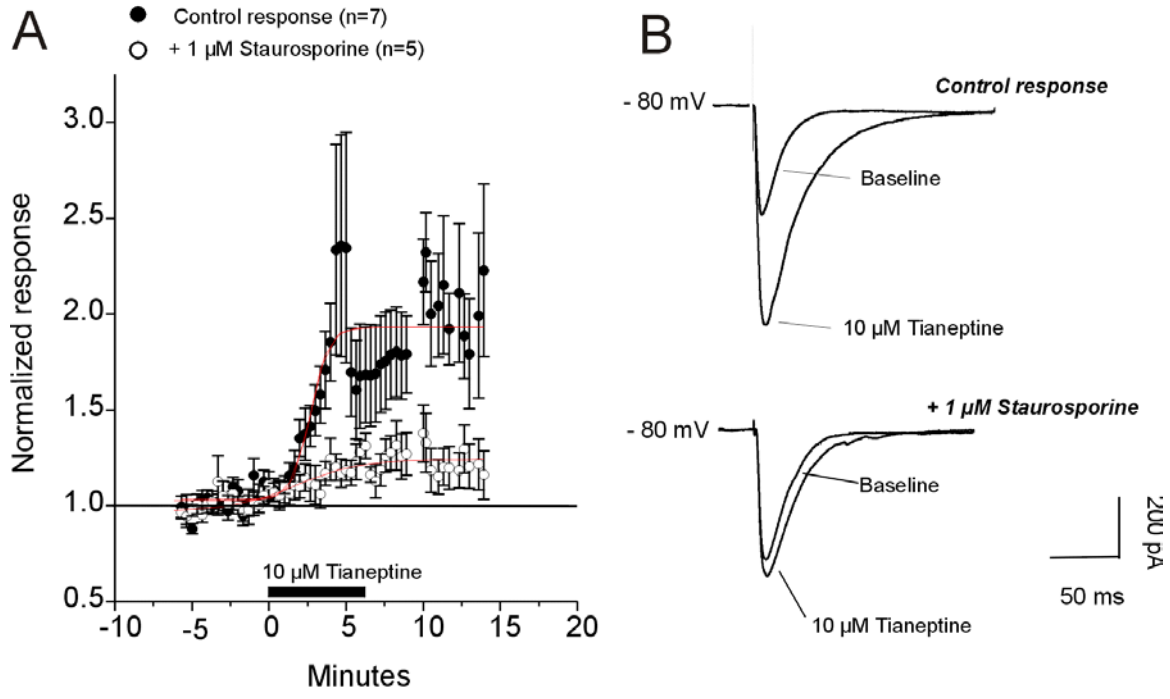

Fig. 5. (A) CA3 C/A AMPA EPSCs, adjusted to a baseline response of 200–300 pA, were recorded at a frequency of 0.05 Hz in hippocampal slices from treatment naïve animals. After a seven-minute baseline period, tianeptine-sodium (10 µM) was applied to the bath solution. Black bar indicates onset and end of tianeptine perfusion. Within minutes, a doubling of the peak amplitudes (*closed circles*) of the AMPA-R mediated EPSCs could be observed (210 ± 30 %). The postsynaptic glutamate-receptor enhancement resembled the effects of the chronic (21 days) treatment with tianeptine (see Fig. 2A). The *in vitro* tianeptine-induced EPSC amplitude increase is blocked by intracellular presence of the broad-spectrum kinase inhibitor staurosporine (Tamaoki et al., 1986). Staurosporine added into the patch solution (*open circles*) blocked the AMPA EPSC enhancement ($P < 0.001$). (B) Representative traces from the population data presented in (A). Upper traces show tianeptine effects on CA3 C/A EPSCs during baseline recordings. In the lower panel is an example shown that staurosporine blocks the effect of tianeptine on AMPA EPSCs.

Discussion

With a combined approach using an animal model for stress and electrophysiological measurements, we showed that repeated restraint stress imposed for 21 days persistently enhanced the NMDA receptor component of EPSCs at the C/A synapses onto CA3 pyramidal neurons. When stressed animals were treated simultaneously with the antidepressant tianeptine, the NMDA/AMPA receptor ratio and the decay kinetics of NMDA EPSCs were similar in slices from Control + Tianeptine and Control animals, suggesting that tianeptine successfully counteracted the stress-induced increase in the NMDA/AMPA ratio. The drug effect was selective in that both the NMDA-R and AMPA-R mediated EPSCs increased equally and stimulus-dependently in Stress + Tianeptine and Control + Tianeptine rats. The *in vitro* application of tianeptine to brain slices from naïve animals mimicked the conductance-enhancement of NMDA-R and AMPA-R mediated EPSCs, and required the phosphorylation of intracellular protein kinases. Although long-term drug and stress treatments clearly affected CA3 C/A glutamate receptors, the short-term plasticity of both the frequency and PP-facilitation of EPSCs remained similar between groups.

Stress affects synaptic glutamate currents

The restraint stress paradigm used in this study modulates neuroendocrine parameters and spatial memory associated with impairments of the hippocampal structure and function (reviewed by McEwen, 2000). In this study, we showed that chronic restraint stress selectively upregulates the NMDA-receptor-mediated currents of the CA3 C/A EPSC and slows the deactivation of the NMDA EPSC. Different ratios of NMDA/AMPA EPSCs may be related to the subunit composition of the NMDA receptors (Weiskopf & Nicoll, 1995; Ito et al., 1997, 2000). Our data corroborate the increases in NR2B- and NMDA-receptor binding specific to the CA3 area following a single stressor (Bartanusz et al., 1995; Krugers et al., 1991). Higher levels of NR2B subunits slow the deactivation kinetics of NMDA EPSCs (Tovar et al., 2000; Vicini et al., 1998), which is consistent with our observation. Whether the expression of NMDA subunits is altered by 21 days of stress remains to be tested. However, long-term corticosterone treatment, which mimics the stress-induced effects on CA3 neurons (McEwen, 2000), elevates the hippocampal mRNA levels of NR2A and NR2B subunits (Weiland et al., 1997).

The activation of NMDA receptors at CA3 C/A synapses is functionally involved in the regulation of LTP and LTD (Weisskopf & Nicoll, 1995; Salin et al., 1996; Debanne et al., 1998; Ito et al., 1997, 2000) and it has been shown that increased NMDA/AMPA ratios make synapses weaker and is associated to enhanced LTD (Thomas et al., 2001). Recent data provide evidence that chronic restraint stress diminishes the magnitude of LTP at the CA3 C/A synapses, but not at the mossy-fiber synapse (Pavlides et al., 2002). Therefore, our data on the re-scaling of the NMDA/AMPA-receptor-mediated currents at C/A synapses, obtained with a similar stress paradigm, might be involved in the regulation of stress-induced suppression of long-term potentiation.

Tianeptine blocks the effects of stress

Tianeptine is a novel antidepressant agent with an efficacy equivalent to that of classical antidepressants (Wagstaff et al., 2001). The principal finding of our experiments is that chronic tianeptine treatment prevents the stress-induced re-scaling of NMDA/AMPA-receptor-mediated EPSCs at the recurrent synapses. This is in line with previous investigations showing that tianeptine prevents/reverses the effects of stress on hippocampal cell cycling, metabolism, dendritic shortening and Schaffer-collateral LTP (Czéh et al., 2001, Magariños et al., 1999, Shakesby et al., 2002) and is consistent with the hypothesis that reducing NMDA receptor-mediated functions may play an important role in blocking stress-induced effects on hippocampal cellular properties. What kind of mechanism could account for this observation? The elevation of corticosteroids that accompanies chronic stress has been implicated in the mediation of stress-induced impairment of hippocampal neurons and also does affect NMDA receptor receptor expression (Magariños & McEwen, 1995b; Weiland et al., 1997, McEwen, 2000). In one study it was also reported that tianeptine treatment inhibited the plasma elevation of corticosterone after a single stressor (Delbende et al., 1994). In the present study, however, treatment with tianeptine caused a slight increase in plasma corticosterone levels, although they remained within the range of basal morning levels.

Together with the fact that tianeptine did not reverse stress-induced body weight reduction, these data are in agreement with recent reports demonstrating that tianeptine treatment does not counteract the stress-induced activation of the HPA axis (Czéh et al., 2001; McEwen, 2000; Broqua et al., 1994; Shakesby et al., 2002). Although we cannot fully exclude a direct block of tianeptine at the NMDA channel, as has been described for the antidepressant imipramine (Watanabe et al., 1993; Sernagor et al., 1989), it is unlikely that tianeptine was present at the time of recording. Our measurements were made about two days after the last tianeptine treatment, whereas the elimination of tianeptine is rapid and its metabolites disappear after several hours, even after long-term application (Couet et al., 1990; Wagstaff et al., 2001).

One possibility by which tianeptine might reverse stress-induced NMDA receptor activation is via a concurrent downregulation of selective NMDA receptor subunits. Several distinct classes of antidepressants, such as tricyclic compounds and selective serotonin reuptake inhibitors (SSRI), regionally reduce the transcript levels of the NR2B or NR2A subunits of NMDA (Boyer et al., 1998). Whether tianeptine regulates the expression of the NMDA subunits is not yet known, but it would be interesting to address this possibility in future research. More speculatively, tianeptine might prevent the stress-induced effects at the hippocampal CA3 synapses by acting at the level of the intracellular compartments, preventing the transcription of glucocorticoid regulated genes, as shown recently by Budziszewska et al. (2001).

Tianeptine directly modulates hippocampal EPSCs

Insights into a potential role for intracellular proteins are demonstrated with our *in vitro* experiments. The results strongly indicate that the tianeptine-induced increases in EPSC is obtained via a direct postsynaptic modulation at NMDA- or AMPA receptors; the effect of tianeptine could be blocked by simultaneously loading the postsynaptic cell with the protein kinase inhibitor staurosporine. Significant phosphorylation sites have been found at the intracellular domains of the AMPA and NMDA receptors

(Yakel et al., 1993; Winder & Sweatt, 2001), which regulate the single-channel conductance of the glutamate receptors. The rapid up-regulation of the basal conductance of glutamate-receptor-mediated EPSCs by tianeptine is consistent with the findings of Audinat et al. (2001). Importantly, we extend these observations by showing that also repeated treatment with tianeptine, in a therapeutically relevant concentration, results in a persistent increase in CA3 C/A EPSC amplitudes, resembling its rapidly induced changes.

The increased EPSCs that facilitate the propagation and enhanced summation of synaptic input, will reduce action potential thresholds. This is consistent with earlier findings that tianeptine increases CA1 and CA3 spike frequencies *in vivo* (Piñeyro et al., 1995; Dresse & Scuvée-Moreau, 1988). Interestingly, a recent study from Bouron and Chatton (1999) showed that the antidepressant desipramine requires a Ca^{2+}-independent phosphorylation factor to increase glutamate release, which was found to be blocked by the kinase inhibitor staurosporine as well. Furthermore, Budziszewska et al. (2001) showed that a many antidepressants, including tianeptine, act via protein kinase C to inhibit glucocorticoid-mediated gene expression in cultured hippocampal neurons; this fact supports the hypothesis that tianeptine has a direct effect intracellular on the activity of protein kinases. Identification of the kinase subtypes that might be involved in tianeptine's modulation of EPSCs (PKC, CaMK II, or PKA) requires further research.

Potential implications for tianeptine's antidepressant mechanism of action

The chemical structure of tianeptine contains a tricyclic ring system with a 3-chlorodibenzothiazepin nucleus and aminoheptanoic side chain (Labrid et al., 1988). Therefore, it deviates markedly from tricyclic antidepressants in structure, neurochemical profile and therapeutic use, giving it a favorable pharmacokinetic profile (Couet et al., 1990, reviewed by Wagstaff et al., 2001). We propose, and provide data to support, that a target downstream from receptors and ion channels might play an important role in the cellular effects of tianeptine in cortical neurons.

Upregulation of basal excitatory hippocampal synaptic transmission has been reported for a number of different SSRIs and tricyclic antidepressants, the mood stabilizer lithium, and electroconvulsive shock therapy (Stewart & Reid, 2000; Colino et al., 1998). This common property and the results of other studies have influenced current thinking on the cellular mechanisms of antidepressants, which emphasizes the importance of glutamate-mediated processes (Skolnick, 1999, Petrie et al., 2000, Krystal et al., 2002) and slow adaptive changes in post-receptor signaling pathways or gene expression (Thome et al., 2000, reviewed by Manji et al., 2001 and Popoli et al., 2001), that all might be critically important to an ultimate improvement in the symptoms of mood disorders.

Various antidepressant drugs directly affect protein phosphorylation and/or the translocation of protein kinases, which are highly enriched at the synaptic terminals and the postsynaptic density affect many important processes, including monoamine neurotransmitter release (Popoli et al., 2001). The phosphorylation of glutamate receptors has been implicated in cellular processes such as the regulation of basal signal transduction from the cell surface towards the intracellular compartment, the activation of mitogen-activated protein kinase (MAPK) signaling pathways, and the

transcription of brain-derived neurotrophic factor (BDNF) (Hayashi et al., 1999) or the induction of LTP (Malenka & Nicoll, 1999, Winder & Sweatt, 2001). Moreover, protein kinase activation delivers a priming signal for the activity-dependent structural shaping of dendrites, either by promoting dendritic outgrowth or providing enhanced structural stability (Wu and Cline, 1998).

Taken together, our results show that tianeptine facilitates glutamate-receptor-mediated signal transduction at the CA3 commissural–associational synapses, via a putative intracellular phosphorylation-dependent mechanism. Long-term treatment with tianeptine establishes a lasting increase in glutamate-receptor-mediated synaptic currents, but prevents a stress-induced increase in NMDA receptor currents. These data add physiological support to the hypothesis that kinase phosphorylation and regulation of NMDA-receptor-mediated processes are important targets for the therapeutic treatment of major depression (Manji et al., 2001; Popoli et al., 2001, Krystal et al., 2002).