Effects of stress and corticosterone on the hippocampus: linking gene transcription to physiology
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Chapter 4

Effect of chronic stress and mifepristone treatment on voltage-dependent calcium currents in rat hippocampal dentate gyrus

Effect of chronic stress and mifepristone treatment on voltage-dependent calcium currents in rat hippocampal dentate gyrus

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Abstract
Chronic unpredictable stress affects many properties in rat brain. In the dentate gyrus, among other things, increased mRNA expression of the calcium channel α1C subunit has been found after 21 days of unpredictable stress in combination with acute corticosterone application (100 nM). In the present study, we examined (i) whether these changes in expression are accompanied by altered calcium currents in rat dentate granule cells recorded on day 22, and (ii) whether treatment with the glucocorticoid receptor antagonist mifepristone during the last 4 days of the stress protocol normalizes the putative stress-induced effects. Three weeks of unpredictable stress did not affect calcium current amplitude in dentate granule cells under basal conditions (i.e. after incubation with vehicle solution). However, the sustained calcium current component (which largely depends on the α1C subunit) was significantly increased in amplitude after chronic stress when slices had been treated with corticosterone 1-4 h before recording. These findings suggest that dentate granule cells are exposed to an increased calcium load after exposure to an acute stressor when they have a history of chronic stress, potentially leading to increased vulnerability of the cells. The present results are in line with the molecular data on calcium channel α1C subunit expression. A significant three-way interaction between chronic stress, corticosterone application and mifepristone treatment was found, indicating that the combined effect of stress and corticosterone depends on mifepristone cotreatment. Interestingly, current density (defined as total current divided by capacitance) did not differ between the groups. This indicates that the observed changes in calcium current amplitude could be attributable to changes in cell size.

Acknowledgements
Dr Henk Karst is acknowledged for his practical assistance and helpful discussions. Maaike van der Mark is acknowledged for the performance of the radioimmunoassay.
**Introduction**

In response to stress, the hypothalamo-pituitary-adrenal (HPA) axis is activated, resulting in the release of corticosteroid hormones from the adrenal glands (de Kloet et al. 2005; McEwen and Wingfield 2003). Corticosterone acts on peripheral organs but also enters the brain where it can bind to two types of receptors. The high affinity mineralocorticoid receptor is occupied to a large extent with basal corticosterone levels, whereas the lower affinity glucocorticoid receptor (GR) is only occupied when corticosteroid levels are high (e.g. during stress) (Reul and de Kloet 1985). Both receptors are highly expressed in principal neurones of the hippocampal CA1 area and the dentate gyrus (de Kloet 1991). Acute stress or corticosterone application have previously been shown to affect hippocampal cell and network functionality (Joels 2001; Kim and Diamond 2002). Also, chronic unpredictable stress largely alters properties of rat hippocampal cells (Cullinan and Wolfe 2000; Joels et al. 2004; Vyas et al. 2002).

Changes that occur after short-term exposure to stress are proposed to promote adaptation to the environment (Joels 1997; McEwen 2001), whereas chronic stress may eventually result in mal-adaptation and various physical and psychiatric diseases, such as depression (Brown et al. 2004; Holsboer 2000; Manji et al. 2001; McEwen 2001). In a subset of patients suffering from major depression, hyperactivity of the HPA axis is found (Dinan 2001; Heuser et al. 1994; Rybakowski and Twardowska 1999). Normalization of hormone levels upon treatment is an indicator for relapse probability (Greden et al. 1983; Holsboer et al. 1982; Ribeiro et al. 1993; Zobel et al. 1999). Interestingly, in patients suffering from bipolar or psychotic depression, symptoms were found to be alleviated within a week by treatment with mifepristone, a GR antagonist, suggesting that this compound can normalize disturbed neuronal function after only a few days (Belanoff et al. 2001a; Belanoff et al. 2002; Flores et al. 2006; Simpson et al. 2005; Young et al. 2004a).

Cellular effects of chronic stress on hippocampal structure and function have been studied extensively in the rodent CA3 area, where pyramidal neurones showed dendritic atrophy (Galea et al. 1997; Magarinos and McEwen 1995; Magarinos et al. 1996; Magarinos et al. 1997; Watanabe et al. 1992) and decreased long-term potentiation (LTP) (Pavlidis et al. 2002) after chronic stress. Also in other hippocampal areas structural and functional changes have been found in response to chronic stress. In the dentate gyrus, chronic stress was shown to affect among other things neurogenesis (Gould and Tanapat 1999; Heine et al. 2004), LTP (Alfarez et al. 2003), excitability of granule cells (Karst and Joels 2003), and mRNA expression of neuropeptide Y (Sergeyev et al. 2005) as well as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and γ-aminobutyric acid (GABA) receptor subunits (Qin et al. 2004).

Calcium channel subunits are also sensitive to effects of acute and chronic stress. In the rat CA1 area, α1C subunit mRNA expression was previously found to be increased by acute stress (Joels et al. 2003). This channel subunit forms part of L-type calcium channels, which give rise to a sustained calcium current at high voltages (Catterall et al.
2003). In accordance, increased calcium current amplitude particularly of the sustained component has been repeatedly found in response to incubation with a high dose of corticosterone in the CA1 area (Joels et al. 2003; Karst et al. 2000; Karst et al. 1994; Kerr et al. 1992). By contrast, in dentate granule cells, acute corticosterone application had no dramatic effect on calcium channel expression in cells from handled control animals; however, when slices from chronically stressed animals were incubated in vitro with a high dose of corticosterone, significantly increased (relative) calcium channel α1C mRNA expression was found compared to the expression in corticosterone-treated cells of handled control rats (Qin et al. 2004). To test whether the observed changes in calcium channel α1C mRNA expression after chronic stress are reflected in physiological changes, the present study investigated the effect of 21 days of chronic unpredictable stress in combination with acute corticosterone application on calcium currents in dentate granule cells, paying particular attention to the sustained (and α1C dependent) component. To examine the potential of a GR antagonist to normalize possible stress-induced effects, half of the animals were treated with mifepristone for the last 4 days of the chronic stress protocol.

Materials and methods

Animals

Male Wistar rats (Harlan, Horst, the Netherlands) weighing 215 ± 3 g at the start of the experiment were used in this study. Animals were housed in pairs on a 12:12 h light/dark cycle (lights on 08.00 h), with food and water available ad libitum. All experiments were approved by the local Animal Experiment Committee (protocol number DED115).

Stress paradigm and mifepristone treatment

Animals were stressed according to a chronic unpredictable stress paradigm adapted from Herman et al. (Herman et al. 1995). This protocol was preferred over, for example, repeated restraint stress because the unpredictable stress protocol has been shown to result in corticosterone hyper-secretion, increased adrenal weight, and reduced body weight gain (Cullinan and Wolfe 2000; Herman et al. 1995; Joels et al. 2004), indicating that this protocol indeed results in chronic stress in these animals. Briefly, rats (n = 48) were subjected to different stressors twice daily for 21 days: day 1, cold immobilization for 1 h at 4 °C and forced swim for 30 min at 25 °C; day 2, immobilization for 1 h and crowding for 24 h (overnight); day 3, cold forced swim for 5 min at 10-15 °C and isolation for 24 h (overnight); day 4, immobilization for 1 h and vibration for 1 h; day 5, forced swim for 30 min at 25 °C and cold immobilization for 1 h at 4 °C; day 6, cold forced swim for 5 min at 10-15 °C and crowding for 24 h (overnight); and day 7, vibration for 1 h and isolation for 24 h (overnight). This schedule was repeated two more times, so that rats were subjected to chronic unpredictable stress for 21 days. Control rats (n = 48) were weighed and briefly handled daily.
Half of the animals in the chronic stress group (n = 24) as well as half of the control animals (n = 24) received treatment with the GR antagonist mifepristone (Sigma, St Louis, MO, USA) during the last 4 days of the protocol (Figure 1A). The duration of mifepristone treatment was based on successful treatment regimens in humans (22-26). Mifepristone (5 mg/100 g body weight) was dissolved in 1.5 ml coffee milk and administered to the animals twice daily via a gastro-oesophageal tube directly in the stomach, prior to stress exposure or handling (Karst et al. 1997a). The other half of the animals received an equal amount of milk.

Slice preparation
Hippocampal slices were studied one day after the last stressor, so that only effects of chronic and not acute stress were studied. In the morning (09.30 h) of day 22, animals (i.e. two pair-housed rats; one of each pair was used for electrophysiological investigations like the present, the other was used for histological survey and is not reported on here) were taken out of their home cage and quickly decapitated (Figure 1A). Trunk blood was collected and centrifuged at 5000 r.p.m. for 20 min at room temperature. Plasma was stored at -20 °C until use in a radioimmunoassay (ICN Biomedicals Inc., Costa Mesa, CA, USA) to determine corticosterone levels. Both adrenals and the thymus were removed from the body and weighed on an analytical balance (Explore, Ohaus, France). Basal corticosterone level, adrenal weight and thymus weight could together indicate whether or not the animal had indeed experienced chronic stress.

The brain was rapidly dissected out of the skull and put in ice-cold carbogenated (95 % O₂/5 % CO₂) dissection buffer containing (in mM): NaCl (120), KCl (3.5), MgSO₄ (5), NaH₂PO₄ (1.25), CaCl₂ (0.2), glucose (10), NaHCO₃ (25). Next, 400 μm thick coronal slices containing the hippocampus were cut using a vibroslicer (Leica VT 1000S, Heidelberg, Germany). Slices were kept in carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (120), KCl (3.5), MgSO₄ (1.3), NaH₂PO₄ (1.25), CaCl₂ (2.5), glucose (10), NaHCO₃ (25) at room temperature. After an equilibration period of 1 h, slices were incubated in vitro with 100 nM corticosterone (dissolved in 0.01% ethanol; Sigma) or vehicle solution for 20 min at 32 °C to study the effect of acute high corticosterone levels in combination with chronic stress, a protocol that was applied earlier (see e.g. (Karst et al. 2000)) and shown to activate GRs in vitro. After incubation, slices were left in ACSF at room temperature for at least 1 h to allow genomic effects of corticosterone to develop (Figure 1A). Slices were transferred one at a time to the recording chamber, which was continuously perfused with warm (31-33 °C) carbogenated ACSF (pH = 7.4).

Recording
Calcium currents were recorded from dentate granule cells with the in situ whole-cell patch clamp technique during perfusion with ACSF containing the following compounds to block sodium and potassium channels: TTX (0.5 μM), TEA-Cl (10 mM), 4-aminopyridin (5
Chapter 4

A experimental design

Figure 1: (A) Schematic overview of the experimental design. Animals were either exposed to the chronic stress paradigm or control handling from days 1–21. On days 18–21, animals received mifepristone (mife) or milk treatment. On day 22, animals were decapitated and hippocampal slices were made. After 1 h, these slices were incubated with either 100 nM corticosterone (cort) or vehicle solution (veh). Whole-cell recordings of calcium currents in dentate granule cells were
made 1–4 h later. (B–E) Voltage protocols and typical examples of calcium currents evoked in dentate granule cells. (B) Total calcium current was evoked with the voltage protocol shown on the left. On the right, a typical example of the total calcium current is shown. For reasons of clarity, only the current traces associated with voltage steps to -60, -40, -20 and 0 mV are depicted. (C) When stepping directly from holding potential (voltage protocol shown on the left), only part of the currents are activated. This is shown in the example on the right. (D) Steady-state inactivation properties of the low-voltage activated current component were determined by varying the voltage of the hyperpolarizing prepulse, prior to a fixed depolarization step of -40 mV. Current amplitudes for the various prepotentials were normalized to the maximal current and fitted with a Boltzmann’s equation to determine the voltage of half-maximal inactivation ($V_{1/2}$). (E) In the double pulse protocol, two identical voltage steps to -10 mV were applied with varying interpulse intervals. With short intervals, the second pulse is smaller than the first pulse. Percentage of inactivation was plotted for each interval, and this curve was fitted with a standard exponential equation to determine the tau of the paired pulse inactivation curve ($\tau_{pp}$).

mM), and CsCl (5 mM). An upright microscope (Nikon Eclipse E600FN, Tokyo, Japan) with x 40 water-immersion objective and x 10 ocular was used to visualize the neurons after first cleaning the surface of the dentate gyrus with a large-tip patch pipette. Patch pipettes for recording (1.5 mm outer diameter, borosilicate glass; impedance approximately 5 MΩ) were pulled on a horizontal Sutter puller and filled with a pipette solution containing (in mM): Cs-methane sulphonate (141), HEPES (10), BAPTA (5), MgATP (2), NaGTP (0.3); pH 7.3; 300 mOsm. The patch electrode was placed on the surface of a dentate granule cell while applying positive pressure to keep the tip of the electrode clean. By removing the pressure followed by light suction, a tight seal was established (> 1 GΩ). Further suction disrupted the membrane, enabling whole-cell recording.

Whole-cell calcium currents were recorded under voltage clamp conditions, with an Axopatch 200B patch clamp amplifier (Axon Instruments, Union City, CA, USA). Series resistance was compensated for approximately 70 %. Data were collected using PClamp software version 8.2 (Axon Instruments). Each cell was subjected to the same voltage protocol: 5 min after the whole-cell configuration was established, a first protocol was run in which a 3-s hyperpolarizing prepulse to -130 mV was followed by a 200-ms pulse to increasing voltage levels (varying between -80 and 0 mV; Figure 1B). Immediately after this, a second protocol was run in which the same 200-ms hyper- and depolarizing pulses (voltages varying between -80 and 0 mV) were given directly from holding potential (-65 mV; Figure 1C). The current evoked after a prepulse to -130 mV is considered to represent the total calcium current of the cell, whereas currents induced when stepping directly from holding potential are less likely to contain the component liable to steady-state inactivation (Joels and Karst 1995). These first two voltage protocols were repeated starting 10 min after obtaining the whole-cell configuration in order to check the stability of the signal; the latter recordings were included in the results. Five minutes later a protocol was run in which the prepotential was varied between -130 and -30 mV, in all cases followed by a voltage step to -40 mV (Figure 1D). This protocol revealed the steady-state inactivation properties mostly of the low-threshold calcium current (Karst et al. 1997b) and was used to define the voltage of half-
maximal inactivation ($V_{H}$). Finally, a paired pulse protocol was run in which two voltage steps (200 ms duration) to -10 mV from holding potential were applied, with a decreasing interpulse interval (550, 450, 350, 250, 150 and 50 ms; Figure 1E).

In all protocols, there was a period of 10 seconds between successive pulses to allow full extrusion of calcium ions which had entered upon depolarization. If during the recording > 20% rundown of the signal was observed, recording of the cell was terminated and the cell was not incorporated in the analysis. Correction for leak current, as estimated from sequential depolarizing and hyperpolarizing voltage steps of 5 mV and 50 ms in duration at holding potential, was applied offline (Joels and Karst 1995). Capacitance was automatically measured by the PClamp program when 5 mV hyper- and depolarizing pulses of 50 ms duration were applied from holding potential. Moreover, membrane capacitance was read directly off the capacitance compensation potentiometer on the patch amplifier. This was repeated at the end of the recording, to exclude cells whose capacitance or input resistance had changed during recording. In our hands this gives a very reproducible value. It has been shown by others that capacitance readings with this method are in good agreement with capacitance measurements derived from integrating the current response to a small depolarizing step (Huguenard et al. 1991; Oh et al. 1995). All data were corrected and analysed using Clampfit software version 8.2 (Axon Instruments).

**Statistical analysis**

All data are presented as mean ± SEM. Data on the neuroendocrine parameters (body weight, adrenal weight, thymus weight, and plasma corticosterone level) were tested with a 2-way ANOVA, with chronic stress and mifepristone treatment as the between-subjects factors. If overall effects were observed, post-hoc comparisons between the appropriate groups were performed. For cell parameters (capacitance, input resistance, voltage of halfmaximal inactivation, and tau of paired pulse inhibition), significance was tested with a between subjects univariate analysis, with chronic stress, mifepristone treatment, and corticosterone incubation as the independent variables. Data on the calcium current amplitude were analyzed with a general linear model approach for repeated measures, with the same independent factors. If a significant effect of treatment was observed, data were subjected to a post-hoc multiple comparison of the mean. Correlations were tested with Pearson’s statistics. In all cases, $P < 0.05$ was considered to be statistically significant.

**Results**

**Neuroendocrine parameters**

At the start of the experiment, body weight of the rats did not differ between the experimental groups. Chronic unpredictable stress caused a significant attenuation of body weight gain, resulting in reduced body weight at the time of decapitation (Table 1). Thymus weight (when corrected for body weight) did not differ between the groups,
while adrenal weight (when corrected for body weight) was found to be increased in chronically stressed animals when compared to controls, both in milk- and mifepristone-treated animals. Basal plasma corticosterone level was not significantly affected by the chronic stress protocol.

Mifepristone treatment did not significantly affect body weight, thymus weight or morning plasma corticosterone level (Table 1). Also, the effects of chronic stress on body weight gain were not reversed by 4 days of treatment with mifepristone. However, a significant overall effect of mifepristone on adrenal weight was observed; adrenal glands from stressed animals treated with mifepristone were significantly larger than those obtained from stressed animals that received milk.

Cell parameters
A total of 98 cells divided over eight experimental groups (Figure 1A) were recorded in this study (Table 2). For membrane capacitance, a significant interaction effect of chronic stress and mifepristone treatment was found [F(1,97) = 4.57, P < 0.05], indicating that the effect of chronic stress on capacitance depended on the cotreatment with mifepristone. Post-hoc analysis revealed that an increased membrane capacitance was found in chronically stressed compared to control handled animals when slices had been subjected to corticosterone 1-4 h before recording (Figure 2A). This enhancement was no longer seen in cells from chronically stressed animals that had received mifepristone. Interestingly, mifepristone treatment of handled controls also yielded a larger capacitance 1-4 hours after incubation of the slices with corticosterone (Figure 2A). In slices incubated with vehicle solution, no effect of chronic stress nor of mifepristone treatment on membrane capacitance was found.

<table>
<thead>
<tr>
<th></th>
<th>Control Milk</th>
<th>Control Mifepristone</th>
<th>Stress Milk</th>
<th>Stress Mifepristone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>118 ± 5</td>
<td>106 ± 6</td>
<td>79 ± 4 **</td>
<td>81 ± 5 **</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>336 ± 8</td>
<td>323 ± 8</td>
<td>293 ± 7 **</td>
<td>294 ± 7 **</td>
</tr>
<tr>
<td>Thymus weight (mg) per 100 g body weight</td>
<td>199 ± 14</td>
<td>201 ± 7</td>
<td>201 ± 10</td>
<td>186 ± 9</td>
</tr>
<tr>
<td>Adrenal weight (mg) per 100 g body weight</td>
<td>11.8 ± 0.6</td>
<td>13.4 ± 0.7</td>
<td>15.0 ± 0.5 **</td>
<td>18.2 ± 1.1 § **</td>
</tr>
<tr>
<td>Basal plasma corticosterone level (μg/dl)</td>
<td>0.44 ± 0.11</td>
<td>0.74 ± 0.44</td>
<td>1.03 ± 0.35</td>
<td>1.61 ± 1.21</td>
</tr>
</tbody>
</table>

Table 1: Effects of chronic stress and mifepristone treatment on body, adrenal and thymus weight and plasma corticosterone levels. Body weight gain and body weight at the time of decapitation were significantly reduced in animals subjected to the chronic stress paradigm. Adrenal weight (corrected for body weight) was increased in chronically stressed animals. Also, mifepristone treatment resulted in larger adrenals in chronically stressed animals. Thymus weight was not affected by chronic stress or mifepristone treatment. Basal plasma corticosterone level did not differ significantly between the groups. N = 12 for all groups. 2-way GLM analysis showed: ¹ Significant effect of stress; ² Significant effect of mifepristone; No interaction effects were found. Post hoc test showed: ¹ Different from corresponding control handled group P < 0.05; ² Different from corresponding milk-treated group P < 0.05; § Different from corresponding milk-treated group P < 0.05.
Table 2: Cell characteristics. Voltage of half-maximal inactivation ($V_{\text{H}}$), tau of the paired pulse inactivation curve ($\tau_{\text{pp}}$), and number of animals (N) and recorded cells (n) are shown for each group. No significant differences in voltage of half-maximal inactivation or tau of the paired pulse inactivation curve were found between the groups.

<table>
<thead>
<tr>
<th></th>
<th>Control Milk</th>
<th>Control Mifepristone</th>
<th>Stress Milk</th>
<th>Stress Mifepristone</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{H}}$</td>
<td>-69.2 ± 0.6</td>
<td>-75.1 ± 3.5</td>
<td>-76.2 ± 2.2</td>
<td>-75.9 ± 4.6</td>
</tr>
<tr>
<td>(mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_{\text{pp}}$</td>
<td>81.6 ± 8.5</td>
<td>99.3 ± 11.9</td>
<td>96.5 ± 13.8</td>
<td>88.5 ± 6.7</td>
</tr>
<tr>
<td>(ms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (n)</td>
<td>10 (12)</td>
<td>8 (13)</td>
<td>11 (13)</td>
<td>9 (15)</td>
</tr>
</tbody>
</table>

Figure 2: (A) Membrane capacitance of dentate granule cells incubated with vehicle solution (left) or 100 nM corticosterone (right). Dentate granule cells from control animals have a significantly smaller capacitance than cells obtained from chronically stressed or mifepristone-treated animals; this difference was only found in cells that were incubated with 100 nM corticosterone 1-4 h prior to recording. Asterisk indicates significant difference between the groups ($P < 0.05$). (B) Membrane capacitance and input resistance per dentate granule cell showed a significant ($P < 0.001$) negative correlation.

In the whole-cell recording mode, capacitance is probably mostly determined by the cell surface near the pipette (i.e. the size of the soma and primary dendrite) (Anderson et al. 2001). Increased capacitance may thus point to an increased membrane surface close to the recording pipette which could result in a lower membrane resistance. In agreement, a strong and significant ($P < 0.001$) negative correlation was observed between capacitance and membrane resistance when analyzing all cells (Figure 2B). Statistical analysis further showed that mifepristone treatment and corticosterone incubation both had an overall effect on input resistance [$F(1,97) = 4.04$, $P < 0.05$ and $F(1,97) = 3.78$, $P = 0.05$ respectively]. Post-hoc tests showed that input resistance was significantly smaller in corticosterone-treated cells in the mifepristone-treated group, in accordance with the enhanced capacitance in this group. However, the reduction in
resistance in the corticosterone-treated slices from chronically stressed rats did not reach significance \( P = 0.28 \).

**Calcium current amplitude**

In dentate granule cells, in general small low voltage-activated calcium currents are found (Akaishi et al. 2004; Gorter et al. 2002; Karst and Joels 2001). Also in the present study, the current / voltage relationships of the total calcium current showed small currents when voltages from -70 to -40 mV were applied. No clear differences in the voltage dependency of total calcium currents were observed between the groups. Voltage of half-maximal inactivation, indicating the steady-state inactivation properties mostly of the low-threshold calcium current (Karst et al. 1997b) was not significantly affected by chronic stress \([F(1,51) = 0.002, P = 0.96]\), mifepristone treatment \([F(1,51) = 0.19, P = 0.67]\) nor corticosterone incubation \([F(1,51) = 0.97, P = 0.33]\) (Table 2). Recovery of inactivation, as tested with the double pulse protocol shown in Figure 1E, showed near-maximal recovery with an interpulse interval of 250 ms or more in all groups. It was found that the recovery of inactivation, expressed as the tau of the inactivation curve, showed no statistically significant differences due to chronic stress \([F(1,47) = 1.06, P = 0.31]\), mifepristone treatment \([F(1,47) = 0.13, P = 0.72]\) or corticosterone incubation \([F(1,47) = 1.65, P = 0.21]\) (Table 2).

In each experimental group, the largest average calcium current amplitude was found with a voltage of -20 mV (example in Figure 3). We here illustrate the main findings for these representative currents evoked by a voltage step to -20 mV. For statistical analysis, the recorded current over the entire range of -80 to 0 mV was the dependent (within subjects) variable, while chronic stress, mifepristone treatment, and *in vitro* corticosterone incubation represented the independent (between-subjects) variables.

![Figure 3: Examples of (averaged) current/voltage relationship of total calcium currents evoked in dentate granule cells. The curves here summarize the data from slices of control animals incubated with 100 nM corticosterone or vehicle solution. Very small currents are evoked with potentials of -80 to -50 mV. The largest total calcium current amplitude was found with a pulse of -20 mV (this was the same in all groups). For this reason, current amplitude evoked by a voltage step to -20 mV was depicted in Figure 4.](image-url)
For total calcium currents, evoked after a prepulse of -130 mV, no overall effects of mifepristone treatment \( [F(1,75) = 0.286, P = 0.59] \) nor \textit{in vitro} corticosterone incubation \( [F(1,75) = 1.67, P = 0.20] \) were found (data not shown). Although not significant, trends to an overall effect of chronic stress \( [F(1,75) = 3.34, P = 0.07] \) and an interaction effect of the three factors \( [\text{stress} \times \text{mifepristone} \times \text{corticosterone}; F(1,75) = 2.94, P = 0.09] \) were found. Because no significant overall effects were observed, we did not apply post-hoc multiple comparison of the means. We conclude that neither chronic stress, mifepristone treatment, nor corticosterone incubation significantly affected total calcium currents in dentate granule cells.

When stepping to -20 mV directly from holding potential (-65 mV), only part of the calcium current is activated (i.e. the component that is not liable to steady state inactivation). Because L-type currents mostly show calcium-dependent rather than steady state inactivation (Budde et al. 2002), this protocol favors the generation of high-voltage activated (HVA) L-type currents. No overall effects of chronic stress \( [F(1,81) = 1.33, P = 0.25] \), mifepristone treatment \( [F(1,81) = 0.01, P = 0.92] \), or \textit{in vitro} corticosterone incubation \( [F(1,81) = 0.05, P = 0.81] \) were observed. However, a significant interaction effect of the three independent factors on high-voltage activated currents was found \( [\text{stress} \times \text{mifepristone} \times \text{corticosterone}; F(1,81) = 4.23, P < 0.05] \). Post-hoc multiple comparisons of the means showed that under basal conditions (i.e. when slices were incubated with vehicle solution) HVA currents were not affected by chronic stress and/or mifepristone treatment (Figure 4, left side). However, when slices were incubated with a high dose of corticosterone 1-4 hours before recording, HVA currents were significantly increased in slices from chronically stressed rats when compared to control handled animals (Figure 4, right side; \( P < 0.05 \)). Slices from chronically stressed animals cotreated with mifepristone yielded current amplitudes that were comparable to the handled control group. Incubation of slices from control handled animals with 100 nM corticosterone tended to decrease the HVA calcium current when compared to incubation with vehicle solution, but this was not significant \( (P = 0.17) \).

![Figure 4: High-voltage activated (HVA) calcium current amplitude evoked by a voltage step from holding potential (-65 mV) to -20 mV in cells incubated with vehicle solution (left) or 100 nM corticosterone (right). Asterisk indicates significant difference between the groups (\( P < 0.05 \)).](image-url)
As mentioned above, significant differences in membrane capacitance were found between the groups (Figure 2A). In the whole-cell recording mode, capacitance is probably mostly determined by the cell surface near the pipette (i.e. the size of the soma and primary dendrite) (Anderson et al. 2001). Altered currents might thus be explained by changes in the size of the cell surface close to the patch pipette rather than increased calcium channel density. Indeed, current density (defined as current amplitude divided by membrane capacitance) of the high voltage-activated current was not significantly affected by any of the treatments (Figure 5), suggesting that at least part of the changes in current amplitude could be attributable to changes in cell size.

**Discussion**

In the present study, we examined the effects of 21 days of unpredictable stress on whole-cell calcium currents in rat dentate granule neurones. We also studied the effects of 4 days treatment with the GR antagonist mifepristone in chronically stressed as well as control animals. These effects were studied under basal corticosterone levels (vehicle incubation) as well as 1-4 h after *in vitro* incubation with 100 nM corticosterone.

**Neuroendocrine parameters**

Animals subjected to the chronic stress paradigm showed signs of long-term stress, as is evident from neuroendocrine parameters measured 1 day after a 21-day period of unpredictable stress. The chronic unpredictable stress paradigm resulted in decreased body weight gain and increased adrenal weight, as was reported before (Cullinan and Wolfe 2000; Herman et al. 1995; Joels et al. 2004). Similar to an earlier study from our group using the same stress protocol, no change in thymus weight was found (Joels et al. 2004). Basal plasma corticosterone concentration was not significantly elevated one day after the last stress exposure. This parameter has been found to show considerable variation between animals, and only yields significant results with very large group sizes.
Treatment with the GR antagonist mifepristone did not reverse the changes in body or adrenal weight induced by chronic stress. This may indicate that any changes in neuroendocrine parameters take place early in the course of the chronic stress protocol and therefore are not prevented or reversed by treatment with a GR antagonist during the last 4 days of the 21-day paradigm. A certain degree of adaptation to repetitive stress is indeed known to occur over the course of several weeks (Magarinos and McEwen 1995; Spencer and McEwen 1990; Watanabe et al. 1992), even with an unpredictable stress protocol similar to the one used in the present study (Chappell et al. 1986). Mifepristone treatment resulted in a significant increase in adrenal weight in chronically stressed animals. This is in line with a study in which increased adrenal weight was found when mifepristone was administered i.c.v. for a period of 10 days (van Haarst et al. 1996).

Effects on calcium currents

Chronic stress was earlier found to affect among other things neurogenesis (Gould and Tanapat 1999; Heine et al. 2004), LTP (Alfarez et al. 2003), and excitability of granule cells (Karst and Joels 2003) in the dentate gyrus. Furthermore, mRNA expression of neuropeptide Y (Sergeyev et al. 2005) as well as relative expression of AMPA, GABA, and calcium channel subunits (Qin et al. 2004) is altered after chronic stress. Importantly for the present study, mRNA expression of α1C relative to other calcium channel subunits in dentate granule cells was found to be affected by chronic stress, but only when hippocampal slices were incubated in vitro with 100 nM corticosterone (Qin et al. 2004), indicating an interdependence of these two factors. In the present study, we investigated whether these changes in mRNA expression are reflected in functional changes in calcium currents. This indeed seems to be the case, particularly for the HVA currents. Chronic stress was found to result in larger current amplitudes compared to the control group only when cells were recorded 1-4 h after corticosterone treatment, but not in vehicle treated slices, which were prepared from animals with very low circulating corticosterone levels. Given the space clamp limitations associated with whole-cell recording, some care with the interpretation of changes in calcium current amplitude is required. Although this confounding factor may be relatively minor in granule cells, which were reported to be electrotonically quite compact when compared to, for example, CA1 neurons (Carnevale et al. 1997), we cannot exclude that space clamp problems occurred in our experiments. Still, the present data strongly suggest that chronic stress affects the way granule cells respond to an acute high amount of corticosterone. We therefore confirm and substantiate our earlier conclusion that dentate granule cells are exposed to a higher calcium load after GR activation when they have a history of chronic stress as opposed to handling.

Current density (defined as total current divided by capacitance) did not differ between the groups, indicating that at least part of the changes in calcium current might be explained by changes in size of the cell surface close to the pipette (i.e. the soma and
Chronic stress the primary dendrite) (Anderson et al. 2001), rather than in the properties or density of the channels; the latter could be verified by recording calcium currents in excised patches. Morphological changes after chronic stress have been reported repeatedly in the CA3 area, where atrophy of dendrites was found (Galea et al. 1997; Magarinos and McEwen 1995; Magarinos et al. 1996; Magarinos et al. 1997; Watanabe et al. 1992). In the dentate gyrus, smaller and simpler dendritic trees have been found after adrenalectomy, when corticosterone levels are extremely low (Wossink et al. 2001). Considering the present findings, it is possible that the dendritic tree of dentate granule cells is also affected by chronic stress in combination with a high dose of corticosterone. However, we have to take into account that capacitance is not a very precise reflection of cell size when recording from cells in slices. It mostly reflects the size of the soma/primary dendrites and could also depend on the lipid structure of the membrane. Detailed morphological studies of dentate granule cells after chronic stress and corticosterone treatment could provide more insight in this matter.

Finally, we also studied the effect of 4 days treatment with the GR antagonist mifepristone on calcium currents, both in control and chronically stressed animals. Under basal conditions (i.e. when slices were incubated with vehicle solution), no effect of mifepristone on calcium currents was found, neither in the control group nor in animals exposed to chronic stress. In mifepristone-treated animals, the significant combined effect of chronic stress and corticosterone treatment was no longer seen. However, it should be noted that mifepristone did not reverse or prevent effects of chronic stress and corticosterone treatment in the dentate gyrus to the extent that significant differences with the corresponding milk-treated groups were observed. This differs from the strong effects of mifepristone on parameters that were tested in the CA1 area from the same animals (Karst and Joels 2007; Krugers et al. 2006a). The latter excludes that mifepristone was ineffective in general. Precisely how mifepristone exerted its effects on calcium currents is hard to judge using the present paradigm. Thus, mifepristone may have blocked GR-mediated events directly in the hippocampus. However, we can not exclude the possibility that the compound might also have interfered with corticosteroid actions in other brain regions projecting to the hippocampus, or even in peripheral organs which indirectly affect brain function. It is even possible that mifepristone counteracted the effects of a high levels of corticosterone by means of its neuroprotective (Behl et al. 1997; Ghoumari et al. 2003; McCullers et al. 2002) or antioxidative (Parthasarathy et al. 1994) properties.

In conclusion, the present data shows that chronic stress in combination with acute corticosterone application not only results in enhanced α1C mRNA expression (Qin et al. 2004) but that most likely these changes are translated to a functional effect. This potentially leads to a higher calcium load in dentate granule cells after exposure to an acute stress when the animal has a prior history of chronic stress. This may lead to enhanced vulnerability to cell death of cells that have relatively large HVA currents to start with (i.e. ‘older’ granule cells) (Karst et al. 1997b). Moreover, this condition may
endanger cells that are simultaneously challenged by strong depolarizations such as may occur in association with epilepsy.