INTRODUCTION

The main effect of hypothalamic-pituitary-adrenal (HPA) axis activation by a variety of stressful stimuli is stimulation of the release of glucocorticoid hormones from the adrenal cortex into the circulatory system. Excessive, repeated activation of the HPA axis due to prolonged stress may result in an enhanced corticotropin-releasing hormone (CRH) synthesis and release, downregulation of glucocorticoid receptors in the brain, enhancement of the corticosteroid response to acute stress and reduction of negative glucocorticoid feedback, which in turn may lead to numerous disorders (1, 2). Corticotropin-releasing hormone (CRH)-synthesizing neurons of the hypothalamic paraventricular nucleus (PVN) play a key role in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. It is well known that excitatory and inhibitory inputs that regulate the activity of these neurons may undergo stress-related modifications; however, the effect of repeated restraint stress on the function of glutamatergic and GABAergic synapses on PVN parvocellular neuroendocrine neurons has not been fully understood so far. Adolescent male Wistar rats were subjected to restraint lasting 10 min and repeated twice daily for 3 days. Brain slices were prepared 24 hours after the last restraint session and were studied ex vivo. Whole-cell patch-clamping was used to record spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs) from parvocellular neuroendocrine neurons of the PVN. Repeated restraint stress resulted in an increase in the mean frequency of sEPSCs and in a decrease in the rise time and the decay time constant of sEPSCs. There was no change in the mean amplitude of sEPSCs. The parameters characterizing sIPSCs also remained unaltered. In addition, the injected current vs. spiking rate ratio of parvocellular neurons was decreased. In conclusion, restraint stress, repeated for 3 days, selectively enhances excitatory synaptic inputs to parvocellular neurons of the PVN, these modifications being accompanied with a decrease in the intrinsic excitability of PVN neuroendocrine parvocellular neurons.

Key words: brain slices, corticosterone, excitatory postsynaptic currents, inhibitory postsynaptic currents, stress, hypothalamic paraventricular nucleus, glutamate, glutamine
parvocellular neurons, the present study was designed to examine the influence of restraint stress repeated for 3 days on spontaneous excitatory and inhibitory transmission in synapses located on those cells. Moreover, we tested the influence of such stress on their intrinsic excitability.

MATERIALS AND METHODS

Animals and stress protocol

All experimental procedures were approved by the Animal Care and Use Committee at the Institute of Pharmacology, and were carried out in accordance with the European Community guidelines and national law. Male Wistar rats, weighing approximately 100 g at the beginning of the experiment, were housed in groups of 4-5 animals in plastic cages (55×35×20 cm) on a controlled light/dark cycle (the light on: 7.00-19.00), with free access to standard food and tap water. The rats were restrained in metal tubes (diameter: 50 mm) for 10 minutes, 2 times a day for 3 days. Control group included naive animals remaining in home cages.

Slice preparation and whole-cell recording

The rats were anesthetized with halothane and decapitated using a guillotine 24 hours after the last stress session. Their brains were rapidly removed and immersed in an ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (130), KCl (5), CaCl$_2$ (2.5), MgSO$_4$ (1.3) K$_2$HPO$_4$ (1.25), NaHCO$_3$ (26) and D-glucose (10), bubbled with a mixture of 95% O$_2$ and 5% CO$_2$. Brain slices (thickness: 420 μm), containing part of the PVN, were cut a coronal plane using a vibrating microtome (VT1000, Leica Microsystems). The slices were incubated in aCSF at 30°C and were placed in the recording chamber after an at least 3-hour incubation. A single slice was placed in the recording chamber, submerged in and superfused at 2.5 ml/min with warm (32°C) modified aCSF in which [NaCl] was raised to 132 mM and [KCl] was lowered to 2 mM. Neurons were visualized (Fig. 1A) using a Zeiss Axioskop 2 upright microscope with the Nomarski optics, a 40× water immersion lens and an infrared camera (13). Small-bodied fusiform parvocellular neurons were distinguished from large-bodied magnocellular neurons (reviewed in: 14). Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical Instruments) using the Sutter Instrument P97 puller. The pipette solution contained (in mM): K-gluconate (130), NaCl (5), CaCl$_2$ (0.3), MgCl$_2$ (2), HEPES (10), Na$_2$-ATP (5), Na-GTP (0.4) and EGTA (1). Osmolarity and pH were adjusted to 280 mOsm and 7.2, respectively. Pipettes had an open tip resistance of approx. 6 MΩ. Signals were recorded using the Multiclamp 700B amplifier, filtered at 2 kHz and digitized at 20 kHz with the Digidata 1400A interface and Clampex 10 software (Molecular Devices).

![Fig. 1](image_url) Restraint stress reduces the excitability of PVN neurons. (A) A high-power microscopic image of a slice (under DIC optics) showing presumed neuroendocrine PVN parvocellular neuron (arrow). Scale bar: 10 µm. (B) A typical example of a control cell response (upper trace) and a response of a neuron originating from a stressed rat (middle trace) to a sequence of hyperpolarizing/depolarizing current pulses (lower trace), characteristic of PVN parvocellular neuroendocrine cells. (C) Example of an injected current vs. spiking rate relationship in a representative control cell (black symbols) and a cell from a stressed rat (white symbols). (D) The firing threshold (mean ± S.E.M.) and (E) the gain (a slope of injected current vs. spiking rate relationship; mean ± S.E.M.) of PVN parvocellular neuroendocrine cells prepared from control (black bars) and stressed (grey bars) animals. * - p<0.05.
Analysis of intrinsic excitability and spontaneous excitatory and inhibitory postsynaptic currents

Response characteristics of the recorded neurons were evaluated in current clamp mode. PVN neuroendocrine parvocellular neurons can be distinguished from non-neurosecretory parvocellular cells and from magnocellular neurons on the basis of the lack of expression of transient outward rectification and a low-threshold spike (15). To determine the relationship between the current and the firing rate, the neurons were first hyperpolarized from the resting membrane potential with intracellular injections of rectangular current pulses (-60 pA) and were then depolarized by current steps of increasing amplitude (an increment of 20 pA; Fig. 1B) (15). The number of spikes evoked by a respective current was determined and plotted (16). Gain (slope) and firing threshold (measured as a current extrapolated at a zero firing rate) parameters were determined from the straight lines fitted to the raw data (Fig. 1C) and averaged as described previously (17).

After electrophysiological characterization, the cells were voltage-clamped at -76 mV and spontaneous excitatory postsynaptic currents (sEPSCs) were recorded for 4 min. Then, the cells were voltage-clamped at 0 mV and spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded for 4 min. This approach allows recording without a need to change pipette solution (18). Recordings were analyzed off-line using the Mini Analysis software (Synaptosoft). Data were accepted for analysis when the access resistance ranged between 15 and 18 MΩ and was stable during recordings.

Statistical analysis

After checking for a normal distribution of data statistical analysis was carried out using Student's T-test (SigmaPlot 12, Systat Software).

RESULTS

The effect of stress on intrinsic excitability

In response to current pulses, all the cells subjected to the analysis showed a firing pattern characteristic of PVN neuroendocrine parvocellular neurons (Fig. 1B) (15) and no spontaneous spiking activity at the resting membrane potential. There were no statistically significant differences between neurons originating from stressed and control animals in either the resting membrane potential (58.40±0.34 vs. 58.72±0.33 mV, respectively, t=0.691, df=46, p=0.493) or the input resistance (58±21 vs. 546±14 MΩ, respectively, t=1.641, df=43, p=0.108). However, analysis of the relationship between the intensity of the injected current and the firing rate revealed that the intrinsic excitability of recorded neurons was reduced after stress. As shown in Figs. 1D and 1E, the mean threshold value of the injected current was similar in the experimental and the control groups, whereas the mean gain was significantly lower (p<0.05).

The effect of stress on spontaneous excitatory and inhibitory postsynaptic currents

In cells obtained from stressed rats, the mean frequency of sEPSCs (Figs. 2A1 and 2A2) was markedly higher than that in cells originating from control animals (Table 1). The stress did not affect the mean amplitude of sEPSCs (Table 1); however, the rise time and the decay time constant of the averaged sEPSCs (Fig. 2B) were lower than in the control group (Table 1).

In contrast to sEPSCs, no effect of restraint stress on parameters characterizing spontaneous IPSCs was found. The mean frequency and the mean amplitude, as well as the rise time and the decay time constant of sIPSCs (Fig. 3) did not significantly differ from the respective values in the control group (Table 2).

Table 1. The effects of restraint stress on the parameters characterizing spontaneous excitatory postsynaptic currents. Mean ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean frequency (Hz)</th>
<th>Mean amplitude (pA)</th>
<th>Rise time (ms)</th>
<th>Decay time constant (τ, ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>stressed</td>
<td>3.84±0.48*</td>
<td>15.53±0.58</td>
<td>1.01±0.04*</td>
<td>3.44±0.21*</td>
<td>37</td>
</tr>
<tr>
<td>control</td>
<td>2.44±0.31</td>
<td>16.20±0.89</td>
<td>1.27±0.08</td>
<td>4.37±0.37</td>
<td>25</td>
</tr>
</tbody>
</table>

* indicates a significant difference (p<0.05) between the experimental (stressed) and the control groups; n - the number of cells.
DISCUSSION

The results of the present study demonstrate that the repeated restraint of rats results in an enhancement of the excitatory input to PVN parvocellular neuroendocrine cells and in a decrease in the excitability of these neurons. Besides CRH, PVN parvocellular neurons may synthetize the thyrotropin releasing hormone, somatostatin, vasopressin, enkephalin and vasoactive-inhibitory peptide (3, 14). However, we have not determined the nature of the peptides synthesized and released by these cells.

Glutamatergic inputs to PVN parvocellular neuroendocrine neurons originate in the lateral, posterior, dorsomedial, and ventromedial hypothalamic nuclei as well as in other brain structures including the thalamus, amygdala, hippocampus and cerebral cortex (19). Also brainstem nuclei, including the periaqueductal gray, zona incerta, subparafascicular nucleus and lateral parabrachial nucleus, provide glutamatergic innervation of PVN cells (20). The parvocellular neuroendocrine PVN neurons receive GABAergic innervation from the neighboring hypothalamic nuclei and adjacent forebrain regions (3, 21, 22). Since we recorded spontaneous EPSCs and IPSCs, contribution of a specific projection to the observed effects remains to be established.

Some earlier reports demonstrated that acute restraint stress attenuated GABAergic inhibitory transmission in rat PVN. Restraint lasting 30 min was found to reduce the frequency of mIPSC (6). Similar effects were described after corticosterone administration; it was concluded that elevation of corticosterone level decreased the probability of GABA release from presynaptic terminals (6). Interestingly, the mechanism of the acute stress-related suppression of GABAergic input to PVN neurosecretory cells also involves a depolarizing shift in the reversal potential of GABA A-mediated IPSCs due to a postsynaptic mechanism (5). However, no changes in the parameters characterizing IPSCs were found in our study where brain slices were prepared 24 hours after the last restraint, which was consistent with the transient nature of those effects. Verkuyl et al. (8) reported suppression of the GABAergic input to PVN parvocellular neurons after 3-week unpredictable stress; however, chronic variable stress lasting 1 week was not shown to influence the number of GABAergic terminals in the parvocellular part of the PVN (10). Rats subjected to the stress regime identical to that used in the present study showed moderately increased plasma corticosterone, ACTH and interleukin-1β levels, measured 24 hours after the last restraint (11). It may be speculated that the stressing procedure used was too brief to exert any influence on inhibitory transmission in the PVN; on the other hand, it was sufficient to induce enhancement of the excitatory transmission.

Although corticosteroids, secreted to plasma as a result of the action of CRH (e.g. 23), were shown to directly suppress glutamatergic transmission in the PVN via a non-genomic mechanism (24), a direct influence of corticosterone on the activity recorded in our study was unlikely, as we incubated brain slices in the aCSF for at least 3 hours before the recording began. We observed an increase in the mean frequency of sEPSCs, but no change in their mean amplitude. This effect may potentially result from an increased spontaneous firing of cells synapsing on neurons from which the recording was performed.

Table 2. The effects of restraint stress on the parameters characterizing spontaneous inhibitory postsynaptic currents. Mean ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean frequency (Hz)</th>
<th>Mean amplitude (pA)</th>
<th>Rise time (ms)</th>
<th>Decay time constant (τ, ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>stressed</td>
<td>0.72±0.07</td>
<td>29.16±1.28</td>
<td>1.81±0.11</td>
<td>10.24±0.81</td>
<td>37</td>
</tr>
<tr>
<td>control</td>
<td>0.65±0.13</td>
<td>29.74±1.39</td>
<td>1.84±0.11</td>
<td>10.01±0.90</td>
<td>25</td>
</tr>
</tbody>
</table>

The differences between the experimental (stressed) and the control groups are not significant; n - the number of cells.
However, slices used for the study mainly contain distal axonal processes of neurons located in other brain structures (19, 20). Although it cannot be excluded that some sparse connections originating in the neighboring hypothalamic nuclei remained intact, we observed no spontaneous spiking activity of PVN neurons. The mean frequencies of sEPSCs and mEPSCs, recorded from the spinally projecting PVN neurons, did not significantly differ (25). Therefore we regard the majority of sEPSCs recorded in our study as a product of the spike-independent, spontaneous release of glutamate from presynaptic terminals, i.e. miniature EPSCs. The frequency of mEPSCs depends mainly on intracellular calcium released from ryano dine- and IP3-sensitive Ca2+ stores in presynaptic terminals (26, 27). Thus, an increase in the mean frequency of sEPSCs, described in the present study, is likely to rely on a presynaptic mechanism. It has been shown that immediate effects of a single episode of immobilization of a rat include modification of the short-term dynamics of glutamatergic input to PVN neurons, mediated by increased probability of release (7). It is conceivable that a similar mechanism may operate after repeated restraint. Variable stress lasting 1 week was also shown to result in an increase in the number of glutamatergic terminals in the parvocellular part of rat PVN (10). The observed decreases in the rise time and the decay time constant of sEPSCs are likely to result from a change in the kinetics of activation of postsynaptic glutamate receptors. In line with those findings, variable stress lasting 2 weeks induced selective reduction of the expression of mRNA for the GluN2B subunit (but not the GluN1 or the GluN2A ones), of the NMDA receptor (9); hence the altered GluN2B/GluN2A ratio is likely to change the time course of NMDA receptor-mediated currents. It is noteworthy that acute immobilization stress was found to increase the expression of mRNA for the GluN1 subunit in the PVN (28).

In addition to synaptic modifications, we observed a decrease in the gain of neurons originating from stressed animals. To the best of our knowledge, the influence of stress on the excitability of PVN neuroendocrine parvocellular neurons has not been investigated so far. Our results implicate that a 3-day repeated restraint modifies the excitatory/inhibitory balance of inputs to these cells, which is likely to tend to enhance their activity, as the action potential threshold remains unchanged. At the same time, the intrinsic excitability of neurons is reduced. These phenomena may be interpreted in terms of homeostatic plasticity mechanisms that act to stabilize neuronal and circuit activity (29). These mechanisms cooperate to adjust the excitability to compensate for enhanced excitatory synaptic drive. Thus, an excessive and potentially harmful spiking activity of PVN neurons is prevented by a decrease in the intrinsic excitability.

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Conflict of interests: None declared.

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