The effect of 6-hydroxydopamine infused into
the third cerebral ventricle on
the plasma cortisol concentration in sheep
subjected to repeated and prolonged stress stimuli

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Abstract. It has previously been observed that sheep
subjected to repeated and prolonged stress stimuli
showed biphasic cortisol responses. On the first and
second day of stimulation an elevation was observed,
while on the subsequent days and on the day after the
stimulation a marked suppression of plasma cortisol level
and a disappearance of its circadian rhythm was noted. It
was hypothesized that these changes in the secretion of
the hormone were caused by the alteration of catechol-
aminergic systems in the CNS. To verify this suggestion
chemical lesions of the catecholergic systems of the
diencephalon were carried out by the infusion of
6-hydroxydopamine (6-OHDA) into the third cerebral
ventricle and animals treated in this fashion were sub-
jected to repeated and prolonged electrical mild foot-
shocking (applied during 3 days). The pretreated ani-
mals lost the circadian rhythm in cortisol secretion on the
days before as well as during and after the electrical
stimulation. The animals pretreated with 6-OHDA
showed a significant rise of the plasma cortisol level
during stimulation. This rise, as the highest daily con-
centration, occurring within about 1 h after the beginning of
footshocking, was significantly accelerated in time with
respect to the physiological acrophase, occurring in the
early morning hours at the end of prestimulatory days.
On the other hand, the pretreated animals did not show
the decrease of plasma cortisol levels on the day after the
stimulation, observed in normal non-pretreated ones. It
is suggested that the absence of the suppression of
plasma cortisol concentration in the animals pretreated
with 6-OHDA on the day after the stimulation may be
due to the blockade of the ventral noradrenergic bundle
innervating the medial basal hypothalamus, while dis-
appearance of the circadian rhythm of cortisol secretion
was due to the disturbance in the function of retinohypo-
thalamic projection.

Previous studies from this laboratory on cortisol
secretion in sheep subjected to repeated and pro-
longed stress stimuli have shown changes in the
circadian rhythm and in plasma cortisol concentra-
tions during the first and subsequent days of elec-
tric stimulation and after the stimulation (Przekop
et al. 1985). On the third or even on the second day
of stimulation the circadian rhythm disappeared.
The mean mesor values of plasma cortisol concen-
tration showed a biphasic release of the hormone;
during the first day(s) of stimulation the mean
mesor values rose by about 50%, while during the
subsequent days of stimulation, and after the stimu-
lation, there was a decrease of about 50%. A
decrease of plasma cortisol concentration was par-
cularly characteristic of pregnant animals. On the
basis of these data, the suggestion was put forward
that these changes in cortisol secretion were caused
by the action of the catecholaminergic system of
the CNS, because it is known that the retinohypo-
thalamic pathway, encompassing noradrenergic
neurons (Moore 1979) is involved in hormonal
rhythms, that the tuberoinfundibular catechol-
aminergic system is involved in the control of
hypothalamic neurohormone release (Moore et al.
1978), and the mesolimbic and mesocortical dopa-
minergic system in the circuitry of stress emotiona-
lity. The last mentioned, autonomic system in rats treated with α-methylparatyrosine and subjected to electric footshocks, showed a 60% decrease in DA levels (Thierry et al. 1978). To verify this suggestion, chemical lesions of the catecholergic system of the hypothalamus were carried out by infusion of 6-OHDA into the third cerebral ventricle and, after such pretreatment, the animals were subjected to repeated and prolonged mild electrical footshocking applied for 3 days. The plasma cortisol concentration in these animals was studied as an index of the organism’s responsiveness to stress.

Materials and Methods

Animals

Seventeen Polish Merino ewes 2.5–3.5 years old in the anoestrous phase served as subjects. They were kept singly in 2 × 2 m compartments under natural light conditions, fed hay and concentrates and water ad libitum. Ambient temperature (February) was maintained at 14 ± 2°C. Each animal bore an indwelling catheter in the jugular vein for drawing blood samples.

Experimental procedures

The animals were assigned randomly to three groups. Group I as the control group was subjected to electrical stimulation without pretreatment with 6-OHDA. Group II was treated with vehicle of 6-OHDA (see below), while group III was pretreated with 6-OHDA and then subjected to electrical stimulation. To determine the physiological variations of plasma cortisol level blood samples were taken at 2 h intervals during 3 days from animals of group III before infusion of 6-OHDA. After this preliminary blood sample collection, stainless steel cannulae of 0.5 mm diameter were implanted into the third cerebral ventricle of all animals of groups II and III, using a stereotaxic technique. Four to 5 days after surgical operation the animals of group III were infused into the third cerebral ventricle with 200 µg of 6-OHDA (2,4,5-trihydroxyphenylethylamine hydrochloride, Sigma) dissolved in 300 µl of vehicle (5% glucose containing 1% ascorbic acid) at a rate of 2.5 µl/min. The animals of group II were infused in the same way with vehicle only. During 3 to 5 days after the infusion of 6-OHDA the ewes showed a rise of body temperature of about 1.5°C, decreased locomotor activity and diminished appetite.

These symptoms disappeared within a few subsequent days and 10 days after the infusion of 6-OHDA the animals showed complete recovery and were suitable for further stages of the experiment. These transient disturbance following 6-OHDA infusion were similar to those obtained in rats after lesions of the latero-ventral hypothalamus or infusion of 6-OHDA in the third cerebral ventricle. Ungerstedt (1971) ascribed these disturbances to the injury of some dopaminergic nigrostriatal projections. Twelve days after the infusion of 6-OHDA or its vehicle blood samples were collected from all the animals over 2 days for determination of plasma cortisol concentration. Since the plasma cortisol values of ewes pretreated with the vehicle and those of the untreated ones were similar and a similar circadian rhythm was observed in plasma hormone levels in both groups the vehicle-treated ewes were regarded as normal and were not used for further experimental procedures, i.e. for electrical stimulation. The ewes of group III pretreated with 6-OHDA, showing marked differences in the circadian rhythm of hormone secretion as compared to that before pretreatment, were stimulated over 3 days. During stimulation and one day thereafter the plasma cortisol concentration was estimated. Each experimental day began and ended at 09.00 h (day after day).

Blood samples were taken from the jugular vein at 2 h intervals. Collection of blood samples started at 09.00 h on the day preceding stimulation and continued until the end of the day following stimulation.

Plasma prolactin (Prl) concentration was also determined as an indirect index of the destructive action of 6-OHDA on the catecholergic system. This indirect method was based on the multiple experiments carried out on rats and especially on the original works of Thoenen & Tanzer (1968) and Thoenen et al. (1973) on rats and cats in which they documented that 6-OHDA induced degeneration and destruction of dopaminergic as well as of noradrenergic terminals. Reader & Gautier (1984) showed that infusion of this substance into the third ventricle reduced the hypothalamic concentration of dopamine by 63%, and that of noradrenaline by 75%. Moreover, in our experiments on the secretion of Prl in sheep after infusion of 6-OHDA into the third ventricle we found a significant rise of plasma Prl concentration and histological degenerations of axones in the medial basal hypothalamus in the third week after this treatment (Domański et al. 1980). The concentration of cortisol in blood plasma was determined using the RIA method applied in our laboratory (Stupnicki 1979), while that of the Prl method was described by Kosowicz (1979).

Stressing procedure

The state of stress in animals was induced by applying similar electrical footshocks of an enduring and repetitive character during 3 days from 09.00–18.00 h as in the animals described in our previous paper (Przekop et al. 1985).
Statistics
The data were processed using 'Mera' 400 computer and analyzed with the cosinor test (Halberg et al. 1965) to test the circadian rhythmicity in the cortisol secretion. Some principles of the method and interpretation of this analysis were described in our previous paper (Przekop et al. 1985).

Detailed comparison of mesors was made with the Bartlett's method testing the homogeneity of the reaction (values of plasma cortisol concentrations) then with Duncan's multiple range test after one way analysis of variance.

Results

Behavioural response to footshock
At the onset of footshocking the animals were agitated or sometimes attempted to escape. Later on this behaviour subsided and changed to mild restlessness synchronous with the stimulation periods. The animals interrupted eating, drinking and rumination for the time the shocks were delivered, but resumed these activities during no-shock periods. Daily food intake was not affected; body weight did not change during the period of stimulation. Observation of the animals during 2 months after stressing showed no disturbances in the general state of health.

Plasma cortisol levels in non-pretreated and stimulated animals (group I)
The results summarized in Table 1 show a biphasic response of the animals of this group to prolonged stimulation. During the first and second day of stimulation the mean 24 h plasma cortisol concen-

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Experimental procedure</th>
<th>Mean 24h plasma cortisol conc. (mesor) ng/ml</th>
<th>Amplitude (A) ng/ml</th>
<th>Acrophase (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mesor</td>
<td>SE</td>
<td>A</td>
</tr>
<tr>
<td>I Non-pretreated with 6-OHDA (5)*</td>
<td>before stimulation</td>
<td>8.85 ± 0.58</td>
<td>41.97</td>
<td>5.23 - 79.38</td>
</tr>
<tr>
<td></td>
<td>stimulation (1st day)</td>
<td>15.97b ± 0.59</td>
<td>29.07</td>
<td>9.91 - 56.11</td>
</tr>
<tr>
<td></td>
<td>stimulation (2nd day)</td>
<td>12.90b ± 0.74</td>
<td>31.89</td>
<td>2.93 - 71.17</td>
</tr>
<tr>
<td></td>
<td>stimulation (3rd day)</td>
<td>10.67c ± 1.43</td>
<td>73.99</td>
<td>36.20 - 111.79</td>
</tr>
<tr>
<td></td>
<td>day after stimulation</td>
<td>4.67b ± 0.46</td>
<td>49.55</td>
<td>7.66 - 123.17</td>
</tr>
<tr>
<td>II Pretreated with vehicle (6)</td>
<td>collection of blood samples during 2 days</td>
<td>10.16 ± 1.80</td>
<td>7.22</td>
<td>2.18 - 11.58</td>
</tr>
<tr>
<td>III Pretreated with 6-OHDA and stimulated (6)</td>
<td>before pretreatment with 6-OHDA (collection of blood samples 3 days)</td>
<td>8.96 ± 1.77</td>
<td>5.66</td>
<td>1.28 - 8.46</td>
</tr>
<tr>
<td></td>
<td>after pretreatment with 6-OHDA (collection of blood samples 2 days)</td>
<td>9.24 ± 1.50</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>stimulation (1st day)</td>
<td>13.83b ± 2.22</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>stimulation (2nd day)</td>
<td>12.67a ± 2.13</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>stimulation (3rd day)</td>
<td>11.39c ± 3.11</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>day after stimulation</td>
<td>8.34c ± 2.19</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The figures in parentheses indicate the number of animals.
CI: confidence interval for the amplitude. CA: confidence interval for the acrophase.
a, b: Significantly different from the values found on the day before stimulation in non-pretreated and pretreated animals (groups I (1) and III (2). P < 0.05 and P < 0.01, respectively.
c: NS from the values found on the days before stimulation in animals before pretreatment with 6-OHDA and after pretreatment with 6-OHDA (group III).
Table 2.
Pattern of plasma cortisol acrophases in controls and animals pretreated with 6-OHDA before, during and after the stimulation.

<table>
<thead>
<tr>
<th>Group of animals and experimental procedure</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
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<tr>
<td>I non pretreated</td>
<td></td>
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<tr>
<td>1. before stimulation</td>
<td></td>
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<td>2. stimulation, 1st day</td>
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<td>3. stimulation, 2nd day</td>
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<td>4. stimulation, 3rd day</td>
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<tr>
<td>5. after stimulation</td>
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<tr>
<td>II pretreated with vehicle</td>
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<tr>
<td>III pretreated with 6-OHDA</td>
<td></td>
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<tr>
<td>1. before pretreatment</td>
<td></td>
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<tr>
<td>2. after pretreatment</td>
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<tr>
<td>3. stimulation, 1st day</td>
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<tr>
<td>4. stimulation, 2nd day</td>
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<tr>
<td>5. stimulation, 3rd day</td>
<td></td>
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<tr>
<td>6. after stimulation</td>
<td></td>
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</tbody>
</table>

~ time of stimulation
\[\square\] acrophase
\[\square\] confidence interval of acrophase
95% CA
\[\square\] highest daily concentration and lack of the rhythm

Cortisol (mesor) was significantly elevated, on the third day of stimulation remained non-significantly higher, while it on the day after the stimulation fell to approximately 50% of the prestimulation value. Analysis of variance of mean 24 h plasma cortisol concentration revealed significant differences between pre-shock, first and second footshock and post-shock days (see Table 1).

The plasma cortisol levels on the day preceding stimulation, during stimulation and on the day after the stimulation showed circadian rhythmicity as verified by the cosinor test. However, the acrophase of the rhythm, occurring on the day preceding stimulation in the early morning hours with a peak at 06.21, was significantly accelerated on the days of stimulation, but returned again to the early morning hours on the days after the stimulation (Tables 1 and 2).

**Plasma cortisol levels in ewes treated with the vehicle (group II)**
The mean 24 h plasma cortisol concentration in 6 animals treated with the vehicle (5% glucose containing 1% ascorbic acid) amounted to 10.16 ± 1.80 ng/ml and was similar to the value of group I before stimulation. It also showed a timing of the circadian rhythm similar to that in non-pretreated animals on the prestimulatory days (Tables 1 and 2). Therefore, the animals of this group, as was mentioned above, were not subjected to electrical stimulation.

**Plasma cortisol levels in ewes pretreated with 6-OHDA and subjected to prolonged electrical stimulation (group III)**
The mean 24 h plasma cortisol concentration of 6 animals during 3 days (preliminary collection of blood samples before infusion of 6-OHDA) amounted to 8.96 ± 1.77 ng/ml. Variations in the hormone concentration showed circadian rhythmicity with the peak at 06.50 and acrophase (CA) with 95% confidence interval between 03.24–10.42 h (Tables 1 and 2).

After infusion of 6-OHDA the same animals did not show the circadian rhythm in plasma cortisol concentration but their mesor values were of the
same magnitude (9.24 ± 1.50 ng/ml) as before infusion. The pretreated animals also did not show circadian rhythmicity on the days of electrical stimulation, but their plasma hormone concentrations (mesor values) rose significantly on the first and second and non-significantly on the third day as compared to the values before stimulation (Table 1). The highest daily concentration of plasma cortisol in these animals occurred about 1 h after the beginning of footshocking and as in the 6-OHDA non-pretreated and stimulated animals, was significantly accelerated in time with respect to the physiological acrophase, occurring in the early morning hours. This acceleration is clearly seen in Table 2. The animals of this group also did not exhibit a circadian rhythm of hormone secretion on the day after the stimulation. Their plasma cortisol concentration on this day was not suppressed as in the untreated controls, but returned to the level before stimulation (Table 1). Plotting of the daily mean values and cosinor diagrams of plasma cortisol concentrations designing the confidence ellipses in individual groups were omitted, for all these data are summarized in Tables 1 and 2. It is also noteworthy that the mean 24 h plasma Prl concentration after pretreatment with 6-OHDA rose about 3-fold over that before treatment (from 118.20 to 338.90 ng/ml).

Discussion

The 3-fold rise of the plasma Prl concentration after infusion of 6-OHDA into the third cerebral ventricle provides evidence of injury to the catecholaminergic system of the hypothalamus. Although the rise of Prl is an indirect index of this process, it may nevertheless, be considered sufficiently reliable. The results presented in this study clearly show that the reaction of the animals pretreated with 6-OHDA and subjected to prolonged and repeated stimuli differed markedly from that of the untreated ones. Three features of this difference should be emphasized: 1) the disappearance of the circadian rhythm in plasma cortisol levels, 2) the rise in plasma cortisol concentration during footshocking and 3) the absence of its suppression after prolonged stimulation.

The disappearance of the circadian rhythm in plasma cortisol concentration after infusion of 6-OHDA into the third cerebral ventricle and lack of this phenomenon in animals during and after stimulation imply that the catecholaminergic system of the brain is involved in the regulation of the circadian rhythm in the secretion of this hormone. It is claimed that the retinohypothalamic projection to the suprachiasmatic and from there to the arcuate nuclei, entraining and regulating the rhythm of cortisol secretion, also encompasses the noradrenergic neurons (for references see Moore 1979). The presence of the noradrenergic system in this pathway explains the action of 6-OHDA abolishing the phenomenon of the rhythm. It is also noteworthy that in pretreated animals the highest daily concentrations of plasma cortisol during stimulation, like the acrophase in the untreated ones, were significantly accelerated as compared to the physiological acrophase (Table 2). However, on the day after stimulation the highest concentrations occurred in the morning hours (at the time of physiological acrophase). This acceleration of the hormone concentrations and synchronization with the footshocking indicate that the surge of cortisol, normally triggered at acrophase by the ‘biological clock’, can be induced by stress conditions and considered as mobilization and active adjustment possibilities of the organism.

The daily trends, and values, of the rise of plasma cortisol concentration during stimulation were similar in untreated and 6-OHDA pretreated animals. This rise seemed to be consistent with the finding of Di Renzo et al. (1979) that the stress response to low intensity stimuli (incision of the skin), as measured by corticosterone secretion, was reduced in rats pretreated with 6-OHDA. If, however, the stress was rather a severe one, e.g. sham surgery of bilateral adrenalectomy, the magnitude of the response was greater and similar in both the vehicle alone and 6-OHDA injected animals. It is noteworthy that the stress response in sheep, as measured by cortisol secretion, to electrical footshock, despite its low intensity, was rather high. A similar response was observed in rats also by Cuello et al. (1974); injection of 6-OHDA into the third ventricle produced a transient increase in ACTH secretion.

The data presented, consistent with those cited above, obtained in rats, show an increased propensity to secrete ACTH and cortisol after 6-OHDA treatment. This phenomenon may be interpreted on the basis of the recent hypothesis of Smythe et al. (1983) that ACTH responses to stress are mediated by increased hypothalamic noradrenerg-
gic (NA) neuronal activity. Since current evidence favours presynaptic autoinhibitory control of NA neuronal activity by NA itself, acting at α-adrenoceptors (Starke 1981; Chesselet 1984), then the removal of a functional pool of presynaptic NA through the action of an agent such as 6-OHDA would lead to increased NA neuronal activity (provided storage pools of neuronal NA were not grossly affected). There is a precedent for this in the actions of α-methyl-p-tyrosine which may reduce NA concentrations but increase neuronal firing (Engberg et al. 1981) and neuronal activity and ACTH release (Smythe & Bradshaw 1983).

Hence, the data of the present paper are consistent with the cited hypothesis of Smythe et al. (1983) it is proposed that 6-OHDA treatment primarily interferes with a functional pool of NA that normally auto-inhibits NA neuronal release. This results in reduced control of NA neuronal activity, particularly in response to stress; the resultant tonic increase in NA neuronal activity is presumably also responsible for the failure of cortisol (via ACTH) to be suppressed following stimulation as seen in normal sheep.

It is important to stress that central NA neuronal activity is not to be confused with NA concentrations as has been done in the past; indeed, they are inversely related. In view of all the doubts and ambiguities discussed above more detailed investigation is needed to obtain a more satisfactory explanation of the role of the catecholaminergic system in the function of the hypothalamo-hypophysial-adrenal axis in prolonged stress.

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References


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