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Time-dependent Effects of Dexamethasone on Glutamate Binding, Ornithine Decarboxylase Activity and Polyamine Levels in the Transected Spinal Cord

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Evidence exists that the spinal cord is a glucocorticoid-responsive tissue, and glucocorticoids have beneficial effects in cases of spinal cord injury. Using sham-operated rats, spinal cord transected (TRX) rats, and TRX animals receiving dexamethasone (DEX) 5 min or 24 h post-lesion, we have examined the following GC-sensitive parameters 6 h after DEX treatment: (1) binding of glutamate to NMDA-sensitive receptors; (2) the activity of ornithine decarboxylase (ODC); and (3) levels of polyamines. We found that glutamate binding in the dorsal horn (Laminae 1–2) and central canal were upregulated in TRX rats, whereas DEX had an additional stimulatory effect. 24 h post-lesion, glutamate binding was unmodified in TRX or TRX+DEX rats. ODC activity was increased 10-fold in rats killed on the day of transection but only 2-fold 24 h post-lesion. DEX reduced ODC activity on transection day but highly increased it when given 24 h after surgery. The content of the polyamines spermidine and spermine were unchanged after TRX or DEX treatment, in contrast to putrescine which increased in TRX rats and further increased in TRX+DEX rats when measured the day post-lesion. Thus, parallel increases in ODC and putrescine 1 day after the lesion, suggest that glucocorticoid effects on growth responses due to polyamines may develop at a late period. The changes of glutamate binding in the dorsal horn and central canal due to early glucocorticoid treatment, further suggest hormonal modulation of neurotransmission in sensitive areas of the deafferented spinal cord.

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INTRODUCTION

The spinal cord is a glucocorticoid-responsive tissue. It contains substantial amounts of receptors for adrenocortical steroids [1–4]. Of the two receptor types described in the central nervous system by Reul and De Kloet [5], it is the type II glucocorticoid receptor (GR) which is predominantly found in the ventral and dorsal horns and also in white matter [6, 7]. Additionally, the spinal cord shows sensitivity towards the glucocorticoid agonists methylprednisolone and dexamethasone (DEX). These hormones modulate the activities of the enzymes (Na+K)ATPase, glycerolphosphate dehydrogenase and ornithine decarboxylase (ODC) [8, 9]. The effects on (Na+K)ATPase may be genomically mediated, as DEX stimulated in the spinal cord not only the function but also the mRNA levels for the α_3- and β_1-subunits of the enzyme [10].

It has been shown that treatment with glucocorticoids has beneficial effects in cases of spinal cord trauma and favors regeneration. Megadose therapy with glucocorticoids results in fast recovery from spinal cord injury and in clinical improvement of patients with degenerative diseases [11, 12]. The mentioned effects on ODC and (Na+K)ATPase could mediate some of the trophic and regenerative properties of glucocorticoid hormones [13]. Another consequence of glucocorticoid therapy is the attenuation of lipid peroxidation due to injury or ischemia [14].

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Finally, glucocorticoid reduction of the inhibitory benzodiazepine receptor system in the dorsal horn, could favor glucocorticoid enhancement of excitatory synaptic neurotransmission [15].

Excitatory synaptic neurotransmission is mostly due, however, to glutamate [16]. Following spinal cord injury or ischemia, the excitatory amino acid transmitters glutamate and aspartate are released from presynaptic terminals [17]. Although accumulation of glutamate may induce Ca^{2+}-dependent cell damage after binding to *N*-methyl-D-aspartate (NMDA) receptors [18], it also protects against neuronal death by inducing neurotrophins and their receptors [19, 20]. NMDA receptor activation also stimulates the activity of ODC and polyamine synthesis; polyamines have been shown to regulate the NMDA receptor [21–23]. Literature data suggests that both parameters are under glucocorticoid regulatory control. Thus, glucocorticoids regulate ODC activity in the spinal cord [6,7] and binding of glutamate to its brain receptors [24,25]. Taking into account these data, we examined the effects of the GR agonist DEX on [^{3}H]glutamate binding to NMDA-sensitive receptors, the activity of ODC and the levels of polyamines (putrescine, spermine and spermidine) in spinal cord transected (TRX) rats. We speculated that activation of GR with DEX, could affect these parameters in a time-dependent manner. Data obtained from these experiments, therefore, would help us to understand the biochemical role of glucocorticoid hormones during spinal cord injury and regeneration [12, 13].

EXPERIMENTAL

Materials

L-[1-^{14}C]Ornithine (51.6 mCi/mol) and [^{3}H]glutamate (50 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Hyperfilm and NCS solubilizer were obtained from Amersham (U.K.). NMDA, unlabeled ornithine, putrescine, spermidine, spermine and pyridoxal phosphate were obtained from Sigma (St Louis, MO). All other chemicals used were of the highest purity available, and solvents were purchased from Merck (Darmstadt, Germany).

Animals

Male Sprague–Dawley rats (weighing 250–300 g) were used. Animals were anesthetized with ketamine (60 mg/kg b.w.) and a partial laminectomy was carried out at the level of vertebrae T_5–T_6. The spinal cord was transected with iridectomy scissors at the thoracic level (T_7–T_8) as previously described [7]. The wound was closed with sutures placed on the muscle and skin and the rats returned to their cages. Sham-operated rats had the skin, muscle and bone cut open but the spinal cord was left intact. TRX rats were divided into two groups: the first group received 5 mg/kg DEX s.c. 5 min after cordotomy, whereas the second group received DEX 24 h after surgery. Both groups were killed 6 h after hormone application. These conditions represented maximal response and optimal time for induction of ODC activity and occupation of GR in rats [9]. Animals were killed by decapitation, and the cervical enlargement (C_3–C_7) and the lumbar enlargement (T_9–L_3) were dissected [9]. Tissues were frozen on dry ice and kept at −70 C until used.

Glutamate binding

Coronal sections (16 μm) of the cervical and lumbar spinal cord were cut in a cryostat at −15°C. Sections were thaw mounted onto gelatine-coated slides and stored at −70°C until assayed. [^{3}H]Glutamate binding sites were determined using the method described by Halpain and McEwen [24]. Sections were preincubated at room temperature for two succesive 20 min periods in 50 mM Tris–acetate buffer pH 7.4, and dried under a cold air stream. Sections were then incubated at 4°C for 30 min in 50 mM Tris–acetate buffer containing 146 nM [^{3}H]glutamate, which is near the reported K_d for binding to the NMDA receptor [26,27]. Non-specific binding was determined by incubating adjacent sections in the presence of 1 mM NMDA. Under these conditions, specificity of labeling of the NMDA receptor was supported by the following data: (1) incubations were carried out in Tris–acetate buffer, which enhances [^{3}H]glutamate binding to the NMDA receptor subtype [26, 27]; (2) for non-specific binding we used NMDA, which displaces [^{3}H]glutamate from NMDA receptors but is not an agonist for AMPA sites [27]; (3) at least 80 % binding was displaced by 1 mM excess NMDA. The incubation was followed by four 30 s washes with cold buffer and a rapid rinse in ice-cold double distilled water. Sections were dried under a cold air stream and aposed against tritium sensitive film (Hyperfilm, Amersham) in the dark for 9 weeks. At the end of this period, films were developed using standard procedures [28]. Autoradiograms were analyzed using a computer-assisted densitometer, consisting of a CCD-X77 video camera, coupled to a Mackintosh computer with NIH-Image software (version 1.5, Dr W. Rasband, NIMH, Bethesda, MD, U.S.A.). Relative optical densities were converted to binding values using a standard curve derived from coexposed standards.

ODC activity

The method of Russell and Snyder [29] was followed as used in the spinal cord by Orti *et al.* [9]. Briefly, the spinal cord lumbar enlargement was homogenized in 50 mM KH_2PO_4 buffer, pH 7.2, 2 mM EDTA, 1 mM dithiothreitol and 30 mM NaN_3, and centrifuged at 12,000 ***g*** for 30 min at 4°C. The supernatant was incubated with 0.2 mM [^{14}C]ornithine

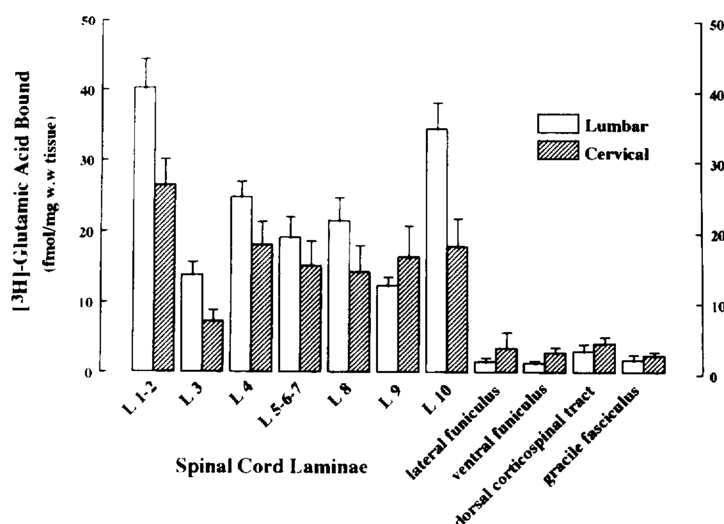

Fig. 1. Binding of [^{3}H]glutamate to NMDA-sensitive receptors in regions of the spinal cord. Results (mean ± SE, n=6 animals per group) are shown for the cervical enlargement (hatched columns) and the lumbar enlargement (open columns). Laminae (L) 1–10 correspond to gray matter. Ventral funiculus, lateral funiculus, dorsal corticospinal tract and gracile funiculus correspond to white matter. In the lumbar cord, binding in L1–2 was similar to L10 but higher than in the other Laminae (P<0.05 or less). Binding in all regions of the lumbar white matter was uniformly lower than in gray matter (P<0.05 or less). In the cervical cord, binding in L1–2 was similar to L10 and L4 but higher than in the other Laminae (P<0.05 or less). Binding in cervical white matter was lower than in all gray matter Laminae (P<0.05 or less) with the exception of Lamina 3 (p:NS). Statistical analysis by ANOVA and the Scheffe's F-test.

and 0.1 mM pyridoxal phosphate. After 60 min at 37°C, the reaction was stopped with 40% trichloroacetic acid, and the labeled CO_2 released was trapped in a well containing 0.2 ml NCS tissue solubilizer. ODC activity was expressed as pmol of [^{14}C]CO_2 released per h per mg protein.

Polyamine determination

The levels of putrescine, spermidine and spermine were measured [30] in perchloric acid extracts of spinal cords located below transection. The method of de las Heras *et al.* [31] was slightly modified as follows: after dansylation, polyamines were separated on silica-gel plates in a chloroform–triethylamine (9:1, v/v) system. The bands corresponding to dansyl derivatives of putrescine (R_f 0.62), spermidine (0.53) and spermine (0.45) were eluted with benzene–triethanolamine (95:5, v/v) and their concentration was measured fluorimetrically at 350 and 490 nm wavelength for excitation and emission, respectively. The intra-assay and inter-assay coefficients of variation were ±3–5% for both parameters, recovery was >90% and sensitivity was 0.125 nmol/sample [31]. Results were expressed as nmol/g of tissue.

Statistical analysis

One way ANOVA, followed by the Scheffe's F-test was used for statistical analysis [32].

RESULTS

Figure 1 shows the distribution of [^{3}H]glutamate binding sites measured by quantitative autoradiography in 11 regions of the spinal cord of normal, untreated rats. [^{3}H]Glutamate binding was highly concentrated in gray matter (Laminae 1–10), whereas in white matter areas, such as the ventral and lateral funiculus, dorsal corticospinal tract and gracile fasciculus, binding was low to negligible. In the lumbar cord, binding was significantly higher in the superficial areas of the dorsal horn (Laminae 1–2) and in Lamina 10, the area surrounding the central canal, than in the other Laminae (Fig. 1). In the cervical region, the highest binding was also found in Laminae 1–2, but without significant difference from binding in Laminae 10 and 4. Other areas of the cervical region showed moderate, but still significantly lower, binding values. Binding of [^{3}H]glutamate to white matter tracks was significantly lower than to any Laminae of the lumbar or cervical cord with the exception of Lamina 3 (Fig. 1).

Considering the regional distribution of binding sites, the effects of transection were evaluated in the dorsal horn (Laminae 1–2) and in the central canal (Lamina 10) of the lumbar region. Figure 2(A) shows, for the dorsal horn, the effects of DEX given 5 min or 24 h following spinal cord transection. In the 5 min group, deafferentiation *per se* significantly increased [^{3}H]glutamate binding (P<0.05 vs sham), while binding in DEX-treated rats was significantly higher than

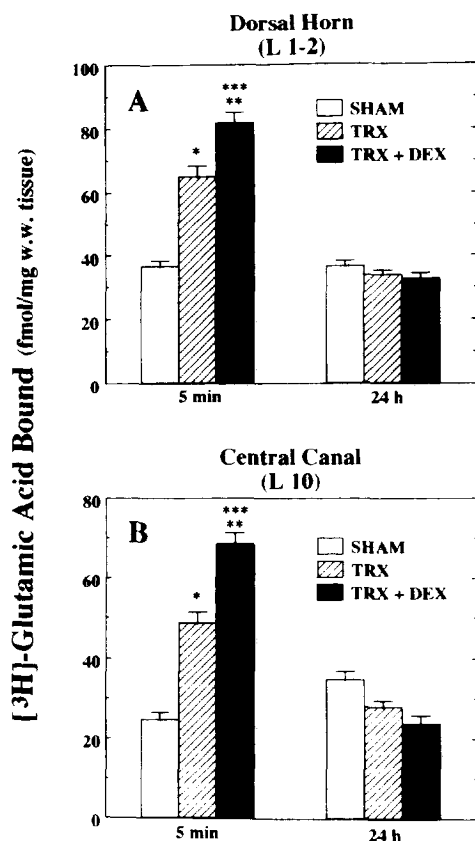

Fig. 2. Binding of [^{3}H]glutamate to NMDA-sensitive receptors in the dorsal horn and in the central canal (Lamina 10) of sham-operated (SHAM, open columns), transected (TRX, hatched columns) and TRX rats receiving DEX (DEX, closed columns). Results represent the mean ± SE of *n*=6 rats per group. (A) Binding in the lumbar dorsal horn of rats receiving vehicle or hormone administration 5 min or 24 h after SHAM or TRX, and killed 6 h afterwards. *TRX vs SHAM, *P*<0.05; **DEX vs SHAM, *P*<0.05; ***DEX vs TRX, *P*<0.05. (B) Binding in the lumbar Lamina 10 of the animals depicted in A. *TRX vs SHAM, *P*<0.05; **DEX vs SHAM, *P*<0.05; ***DEX vs TRX, *P*<0.05. Statistical analysis by ANOVA and Scheffe's *F*-test.

in sham-operated and TRX rats (*P*<0.05 vs sham or TRX). However, neither cordotomy nor DEX modified [^{3}H]glutamate binding in rats killed 24 h after surgery [Fig. 2(A)].

Receptor binding densities in the central canal (Lamina 10) are shown in Fig. 2(B). The response of this Lamina located in the lumbar region, however, wholly resembled that shown above for the dorsal horn. Here binding levels of rats injected with vehicle at 5 min were significantly increased by transection (*P*<0.05 vs sham), and DEX up-regulated binding over that of sham-operated and TRX rats (*P*<0.05 for both). In agreement with findings in Laminae 1–2, there were no significant changes in [^{3}H]glutamate binding in rats killed 24 h after cordotomy.

As the response of [^{3}H]glutamate binding to transection and glucocorticoid was time-dependent, results were compared with the induction of ODC activity, which is also affected by lesion and DEX treatment in a time-dependent manner [7, 9]. In these experiments, DEX was also given 5 min or 24 h after sham operation or spinal cord transection, with animals killed 6 h following vehicle or hormone application. Figure 3 (left side) shows that DEX treatment of sham-operated rats produced a 4-fold increase of ODC activity (*P*<0.05), in agreement with previous results [7]. Lesioning caused a 20-fold increase of ODC activity in the lumbar spinal cord from rats killed 6 h after surgery (*P*<0.0001 vs sham). In this experiment, ODC levels were also significantly higher in TRX rats receiving DEX than in the sham-operated group (*P*<0.001), but DEX treatment reduced by half the stimulated levels of TRX rats receiving vehicle only (*P*<0.02 vs TRX; Fig. 3, left side). In rats studied 24 h after surgery (Fig. 3, right side), DEX significantly stimulated ODC activity in sham-operated rats (*P*<0.01), similarly to animals killed on the day of surgery. Moreover, ODC activity was increased 5-fold in the lumbar cord 24 h after transection (sham vs TRX, *P*<0.02; Fig. 3, right side). At this time point, the TRX+DEX-treated group showed an almost 10-fold stimulation of ODC activity with respect to the sham-operated group, achieving levels significantly higher than TRX rats receiving vehicle (*P*<0.01). Thus, the increments of ODC activity produced by transection and DEX treatment 24 h after surgery, contrasted with the lack of effect of these maneuvers on [^{3}H]glutamate binding sites at this time period [see Fig.2(A and B)]. Rather, changes in ODC activity at 24 h resembled the profile of [^{3}H]glutamate binding sites detected on the day of surgery. In this period, transection increased amino acid receptor binding, and DEX increased the effect of transection on this parameter [see Fig. 2(A and B)].

The responses of putrescine, spermidine and spermine to transection and glucocorticoid treatment in the lumbar spinal cord located below the injury level are shown in Fig. 4 (A–C). As expected, the rank order of polyamine concentration in control tissue was spermidine (1236±72 nmol/g tissue)>spermine (160±21)>putrescine (117±15) [33]. Content of polyamines in TRX rats remained in the range of control rats when killing took place 6 h after surgery alone or accompanied by DEX administration [5 min group in Fig.4(A, B and C)]. However, in rats killed 24 h after transection, concentrations of putrescine increased above control levels [Fig. 4(A), *P*<0.05]. In 24 h TRX rats, DEX treatment given 6 h before killing further increased putrescine concentration above control [Fig. 4(A), *P*<0.05] and spinal cord injured rats [Fig. 4(A), *P*<0.01]. Experimental manipulations did not change the levels of spermidine or spermine [Fig. 4(B and C)]. Therefore, the synthesis of

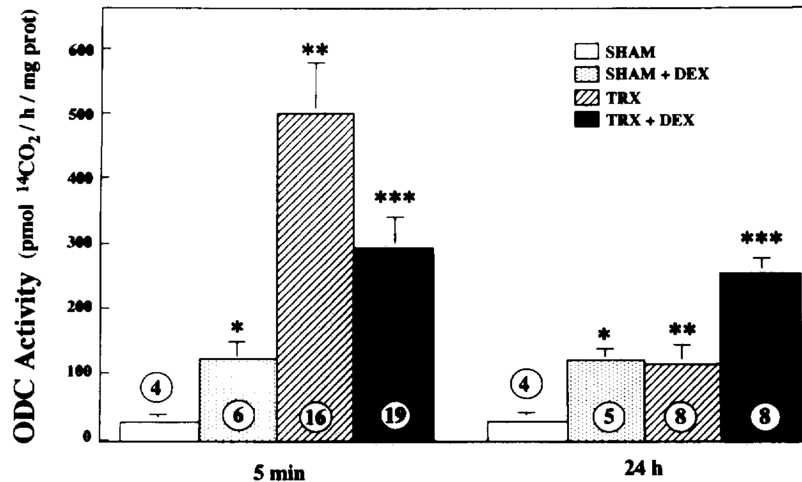

Fig. 3. Activity of ornithine decarboxylase (ODC) in the lumbar spinal cord of sham-operated rats (SHAM, open columns), sham receiving DEX (SHAM+DEX, dotted columns), transected (TRX, hatched columns) and TRX rats receiving DEX (TRX+DEX, closed columns). Results represent the mean ± SE of the number of animals shown inside the columns. Left-hand graph: ODC activity in lumbar cord of SHAM-operated and TRX rats receiving vehicle or DEX 5 min after surgery, and killed 6 h afterwards. *SHAM vs SHAM+DEX, $P<0.05$; **SHAM vs TRX, $P<0.0001$; ***TRX vs TRX+DEX, $P<0.02$. Right hand graph: ODC activity in lumbar cord of SHAM-operated and TRX rats receiving vehicle or DEX 24 h after surgery and killed 6 h afterwards. *SHAM vs SHAM+DEX, $P<0.01$; **SHAM vs TRX, $P<0.02$; ***TRX vs TRX+DEX, $P<0.01$. Statistical analysis by ANOVA and Scheffe's *F*-test.

putrescine, the product of ODC reaction, changed in parallel to ODC activity after transection and DEX treatment, provided the animals were killed 24 h following injury.

DISCUSSION

Analysis of the results of glutamate receptors, ODC activity and polyamine content in animals with transection of the spinal cord, suggests a complex, time-dependent interaction in the response of these parameters to trauma and glucocorticoid therapy. Our experiments in rats killed on the day of transection, showed enhanced binding of [^{3}H]glutamate to NMDA-sensitive receptors in Laminae 1–2 of the dorsal horn and Lamina 10 of the central canal. In the case of another brain region, the hippocampus, increased function of excitatory amino acid receptors and glucocorticoids act in synergy to damage neurons [34]. However, depending on the experimental condition, glucocorticoids can both activate [25] or reduce [24] [^{3}H]glutamate binding in hippocampus. Additionally, GR-mediated effects on glutamate binding in hippocampus may differ to that in the spinal cord, because GR of the spinal cord shows lower ligand affinity towards GC, than GR of hippocampus [35].

In TRX rats killed on the day of surgery, there was increased ODC activity in the lumbar spinal cord located below transection. In other systems, increases in ODC activity and overproduction of polyamines due to ischemia and tissue injury are linked to increased ligand binding to glutamate receptors [36] and to NMDA neurotoxicity [21, 23, 37, 38]. In the present work, administration of DEX to rats 5 min after transection, further increased glutamate binding, but in this case the injury effect on ODC activity was significantly attenuated by the glucocorticoid. Therefore, it is possible that DEX could afford early protection by preventing an excessive production of polyamines, which would further excite glutamate receptors [37]. Similarly, DEX prevented an early increase in ODC activity and polyamines in the cold-injured CNS which are implicated in blood–brain barrier breakdown [22].

In contrast, in animals killed the day after transection, binding to glutamate receptors remained normal in the dorsal horn and central canal. In these animals a small, albeit significant stimulation of ODC activity remained, which was further potentiated by DEX. An alternative explanation for the dissociation of DEX effects on ODC activity and glutamate binding after 24 h, is that the two measures are not linked, although more experiments are needed to define this issue. The effects on ODC activity are in full agreement with previous data in spinal cord injured animals [7]. The up-regulation of ODC activity after trauma could be due to loss of cell-to-cell contact after deafferentiation [39]. The high putrescine production due to glucocorticoid therapy would then exert trophic, regenerative properties [13, 40–42].

Therefore, it is possible that the early and late effects of glucocorticoids on ODC activity and putrescine generation may depend on the state of the NMDA-sensitive glutamate receptor. In the presence of increased binding of glutamate, the products of

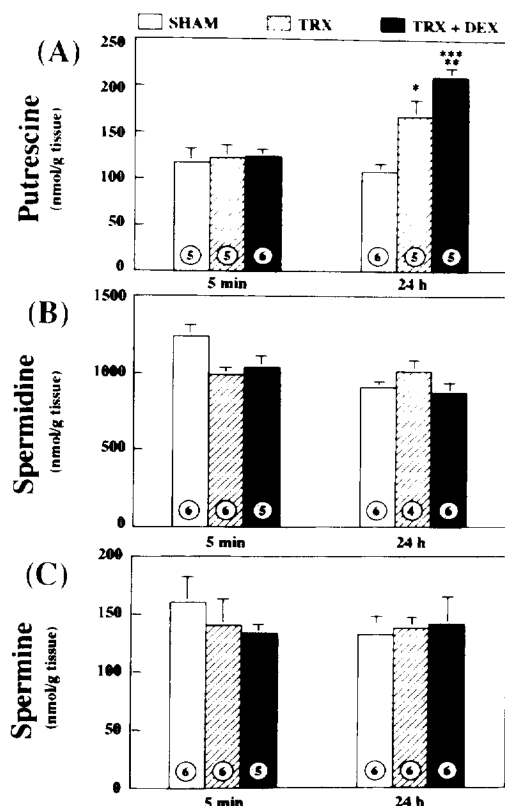

Fig. 4. Levels of polyamines in the lumbar spinal cord of sham-operated rats (SHAM, open columns), transected (TRX, hatched columns) and TRX rats receiving DEX (TRX+DEX, closed columns). Results represent the mean±SE of the number of animals shown inside the columns. (A) Concentrations of putrescine in the lumbar dorsal horn of rats receiving vehicle or hormone administration 5 min or 24 h after SHAM or TRX, and killed 6 h afterwards. *TRX vs SHAM, $P<0.05$; **DEX vs SHAM, $P<0.05$; ***DEX vs TRX, $P<0.05$. (B) and (C) Concentrations of spermidine and spermine in spinal cords from animals depicted in (A). There were no significant differences between sham vs TRX vs DEX-treated rats for these two polyamines.

ODC activity may be neurotoxic [21, 23, 36], an effect partly prevented by DEX. With normal binding of glutamate, the neurotrophic, regenerative effects of polyamines resulting from DEX stimulation may prevail. Literature reports support that increases of ODC following glucocorticoid administration leads to accelerated recovery after cell damage [7, 13, 39, 40]. After nerve transection, increment in the production of polyamines is linked to initiation of a regenerative response in neurons [42]. The fact that in our system only putrescine, but not spermidine or spermine, accumulated after DEX therapy, suggests putrescine metabolism to *n*-acetyl-derivatives [43]. Even putrescine levels may be underestimated, since it also gives origin to GABA [44].

To further substantiate our results, it would be necessary to disclose whether glucocorticoid effects on glutamate binding and ODC activity occur in the same cell population or different cells. ODC activity has been mapped in motoneurons, nerve cells processes and glial cells [45, 46]. ODC induction by glucocorticoids also occurs in neurons and glial cells [7, 39, 40]. Similarly, both neurons and glial cells express NMDA-sensitive glutamate receptors [16, 47]. The methods used in the present study did not allocate effects to specific cell types. However, [^{3}H]glutamate binding sites were preferentially found in the neuronal-rich gray matter horns, whereas white matter tracks were devoid of receptors. GR are found in neurons and glial cells of normal and TRX rats [48]. Therefore, to more precisely localize hormonal effects, it would be necessary to employ immunocytochemistry or *in situ* hybridization techniques to map ODC and glutamate receptor-producing cells. Steps in this direction will be taken in our laboratory.

The effects of glucocorticoids on NMDA-sensitive glutamate receptors of the spinal cord seem worthy of further consideration. Physiologically, NMDA receptors of the spinal cord have been implicated in polysynaptic responses of the dorsal horn neurons, and in monosynaptic and polysynaptic excitation of ventral horn motoneurons [49]. Pathologically, excessive NMDA receptor activation may be neurotoxic. However, it has been suggested that in the spinal cord, Ca^{2+} influx through NMDA-receptor channels induces mRNAs for early genes and brain-derived neurotrophic factor [20]. After brain insults, up-regulation of neurotrophins and their receptors by glutamate and Ca^{2+} influx protects against neuronal damage and stimulates sprouting and synaptic reorganization [19].

Based on these data, we propose that the early stimulatory effect of DEX on glutamate binding indicates hormonal modulation of neurotransmission in sensitive areas of the deafferented spinal cord. 24 h after trauma, glutamate binding was insensitive to DEX, but DEX highly stimulated ODC activity and putrescine production. In the brain, high putrescine levels after glucocorticoid therapy preceed increases in the rates of DNA, RNA and protein synthesis [33]. We suggest that the hormonal effects shown in the present work give biochemical support for glucocorticoids being the treatment of choice for acute phase human spinal cord injury, as demonstrated in the NASCIS II clinical study [12].

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