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Research report

Effect of rat spinal cord injury (hemisection) on the *ex vivo* uptake and release of [^{3}H]noradrenaline from a slice preparation

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ABSTRACT

We measured the *ex vivo* uptake and release of [^{3}H]noradrenaline ([^{3}H]NA) from perfused rat spinal cord slice preparations at 1, 3 and 14 days after unilateral hemisection-induced spinal cord injury (SCI) compared with control slice preparations.

After surgical hemisection under anaesthesia, the rats showed characteristic signs of hemiplegia, with no movement of the ipsilateral hindlimb. After 3 days, the electron microscopy images showed overall degeneration of neuronal organelles and the myelin sheath, but the synapses seemed to be intact. In *ex vivo* experiments, the spinal cord injury did not influence uptake but increased [^{3}H]NA release at rest and in response to axonal stimulation. The effect of a selective noradrenaline reuptake inhibitor, nisoxetine, was studied to identify the mechanisms underlying the increase in NA release. Nisoxetine potentiated stimulation-evoked [^{3}H]NA release from the non-injured tissue, but it gradually lost its effectiveness after injury, depending on the time (1 and 3 days) elapsed after hemisection, indicating that the noradrenaline transporter binding sites of the terminals become impaired after decentralisation.

1. Introduction

Spinal cord trauma is an injury with devastating consequences, including high societal costs in developed countries and high mortality rates in developing countries (Lee et al., 2014; Jazayeri et al., 2015). Current therapies do not provide major improvements in neuronal and motor function (Yilmaz and Kaptanoglu, 2015); thus, spinal cord injury results in a permanently disabling condition. According to epidemiological data, an estimated 180,000 new injuries occur per year worldwide, but this rate is increasing, reflecting the increase in motor vehicle use (Cripps et al., 2011; Lee et al., 2014; Jazayeri et al., 2015).

In addition to the primary mechanical damage, increasing evidence has shown many secondary factors that contribute to spinal cord damage (Wrathall et al., 1992; Park et al., 2004; Liu and Xu, 2012), such as ischaemia, inflammation and excitotoxicity resulting from the excessive release of various neurotransmitters (Liu et al., 1990; Simpson et al., 1990; Uchihashi et al., 1998; Nakai et al., 1999; Sumiya et al., 2001; Padro and Sanders, 2014; Yilmaz and Kaptanoglu, 2015).

Inflammation and overactivation of the sympathetic nervous system have been increasingly accepted as key contributors to the pathophysiology of ischemic damage of stroke and spinal cord injury (Akpınar et al., 2016; Wei et al., 2016; Zhang et al., 2016a, 2016b; Zuo et al., 2016). In addition, it is also known that this condition may lead to immunosuppression (Meisel et al., 2005; Zhang et al., 2016a, 2016b), so the treatment of stroke remains a great global challenge (Prass et al., 2003; Kumar et al., 2010).

The excessive release of glutamate has neurotoxic effects (Faden and Simon, 1988; Wrathall et al., 1992; Lipton and Rosenberg, 1994; Brassai et al., 2015) and leads to the release of noradrenaline from the spinal cord (Klarica et al., 1996). As shown in previous *in vitro* neurochemical studies, ischaemic conditions induce the excessive release of noradrenaline from the spinal cord (Uchihashi et al., 1998; Nakai et al., 1999; Sumiya et al., 2001), which may be at least partially responsible for the neurotoxicity resulting from the toxic aldehyde metabolites of this compound, 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) (Burke et al., 2004) and for immune suppression (Elenkov et al., 1995; Woiciechowsky et al., 1998; Haskó et al., 1998; Elenkov et al., 2000;

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Vizi and Elenkov, 2002; Szelényi and Vizi, 2007; Zhang et al., 2013). Therefore, we examined whether injured spinal cord preparations exhibit changes in noradrenaline release from decentralised nerve terminals, i.e., the side where motor function was completely lost. These findings will improve our current understanding of the processes of secondary spinal cord injury, which is essential for the development of new therapeutic strategies.

2. Materials and methods

2.1. Experimental animals

Female Wistar rats weighing 210–340 g (average weight: 256 g, average age: 137 days) were housed in cages and maintained on 12-h light-dark cycles. Food and water were provided *ad libitum*. All animal procedures were performed in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals, 6th Edition, 2010. The minimum number of animals was used for these experiments, and care was taken to reduce any suffering.

2.1.1. Uptake experiments

Sixteen animals were randomly divided into 4 groups: control, control + nisoxetine, 3 days after SCI, and 3 days after SCI + nisoxetine.

2.1.2. Fractional release experiments

Forty-nine animals were divided into the following groups: control, 1 day after SCI, 3 days after SCI, and 14 days after SCI. In the first three groups, half of the measurements were performed in the presence of a noradrenaline reuptake inhibitor, as described below.

Electron microscopy was performed on samples from untreated and operated animals 3 days after SCI.

2.2. Surgical procedure

The spinal cord was hemisected at the 4th lumbar segment (L4) using the procedure described below (Nosrat et al., 2001). The rats were anaesthetised intramuscularly with a mixture of ketamine (80 mg/kg, Calypsol, Richter Gedeon, Budapest, Hungary) and xylazine (8 mg/kg, CP-Xylazine, CP-Pharma, Burgdorf, Germany). The first lumbar vertebra was exposed by shaving and cutting the skin above this region to free it from the adhering muscles. A laminectomy was performed to provide access to the spinal cord, which was cut on the right side with a crescent-shaped blade (BB512, B. Braun, Melsungen, Germany) under sterile conditions. The muscles and skin were sutured in layers.

2.3. Tissue preparation

Spinal cord segments L5-S1 located caudally and ipsilaterally to the hemisection were prepared at 1, 3 or 14 days following surgery. The caudal half of the vertebral column was removed and excised from the attached muscles. Subsequently, the lumbar and upper sacral spinal cord segments were removed and placed into ice-cold Krebs solution (in mmol/l: NaCl, 113; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 2.5; glucose, 11.5; ascorbic acid, 0.3; and Na_2EDTA, 0.03) aerated with 95% O_2 and 5% CO_2. The spinal cord was freed from the adhering meninges/arachnoid membranes and nerves. The right side (ipsilateral to the hemisection) of spinal cord segments L5 to S1 was isolated and cut into 400 μm slices using a McIllwain tissue chopper. The average weight of the slices was 13.1 ± 2.9 mg, n = 65.

2.4. Tissue loading with [^{3}H]NA

Spinal cord tissue slices were preincubated in aerated (95% O_2 and 5% CO_2) Krebs solution for 20 min at 37 °C and subsequently incubated in a similar solution containing 5 μCi/ml radiolabelled [^{3}H]NA (10.8 Ci/mmol) (PerkinElmer, Waltham, MA, USA) for 45 min (Umeda et al., 1997). The slices were subsequently washed 3 times with Krebs solution and placed in superfusion chambers (Vizi et al., 1985). The chambers were perfused with aerated Krebs solution at 37 °C at a rate of 0.5 ml/min. A preperfusion period of 60 min was applied to remove all of the [^{3}H]NA isotope that was not taken up by the tissues.

In half of the uptake experiments, 1 μM nisoxetine (Tocris Bioscience, Bristol, UK) was added to the preincubation and incubation solutions and maintained throughout the experiments.

2.5. Measurement and calculation of noradrenaline uptake

At the end of the perfusion experiment, the tissue slices were weighed and homogenised in 0.5 ml of 10% trichloroacetic acid. Then, 0.1 ml of the homogenised tissue was mixed with 2 ml of liquid scintillation cocktail (Ultima Gold, PerkinElmer, Waltham, MA, USA). The radioactivity of these samples was determined using a Packard 1900 Tricarb liquid scintillation spectrometer (Packard, Canberra, Australia). The radioactivity was expressed as the number of disintegrations/sec/gram of spinal cord tissue (Bq/g).

2.6. Stimulated noradrenaline release

Following preperfusion, the chamber effluents were collected in 19 consecutive fractions, each of which was 200 s in length. Electrical field stimuli (40 V, 3 Hz, and 1 msec impulse duration) were applied with platinum electrodes for 80 s at the beginning of the 3rd (S_1) and 13th (S_2) fractions.

In half of the fractional release experiments, 1 μM nisoxetine was added to the perfusion solution starting at the 8th fraction and was maintained throughout the experiments.

2.7. Measurement and calculation of fractional noradrenaline release

After the fractions were collected, 0.5 ml of each perfusate was mixed with 2 ml of liquid scintillation cocktail. Radioactivity was determined as described above. The fractional release value represents the percentage of the total [^{3}H]NA content present at the beginning of the fraction collection period that was released from the tissue in a specific fraction. The transmitter release observed in the 4 fractions following electrical stimulation (fractions 3–6 and 13–16) in addition to resting release was considered the release resulting from stimulation (FRS_1 and FRS_2). The release in the two subsequent consecutive fractions (7–8 and 17–18) was considered resting release.

2.8. Electron microscopy

The transmission electron microscopy images were obtained from healthy (control) and hemisected (3 days after SCI) spinal cord tissue ipsilateral to the injury at segment L5, lamina VIII, where most of the locus coeruleus axons terminate (Clark and Proudfit, 1991). The surgical procedure and tissue preparation were performed as described above. The spinal cord slices were fixed in 4% paraformaldehyde, post-fixed in 1% OsO_4 (Taab, Aldermaston, Berkshire, UK) for 20 min, dehydrated in a graded ethanol series and embedded in Taab 812 (Taab). During dehydration, the sections were treated with 1% uranyl acetate in 50% ethanol for 20 min. Ultrathin sections were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems, Vienna, Austria) and analysed using a Hitachi 7100 transmission electron microscope (Hitachi, Tokyo, Japan) equipped with a Veleta side-mounted TEM CCD camera (Olympus, Tokyo, Japan). Contrast and brightness of electron micrographs were edited using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

Table 1
Effects of spinal cord hemisection and nisoxetine on [^{3}H]noradrenaline uptake in spinal cord slices. 1 μM nisoxetine was added to the slices during preincubation and maintained throughout the experiments. n = 16.

	Uptake (kBq/g)		
	Control	3 days after SCI	p
No drug	149 ± 32	158 ± 10	not significant
Nisoxetine, 1 μM	37 ± 9	61 ± 16	< 0.01
p	< 0.01	< 0.01	

2.9. Statistical analysis

Radioactivity values corresponding to [^{3}H]NA uptake and release were statistically analysed using two-way ANOVA with RopStat statistical software (RopStat, Budapest, Hungary). The data are presented as means ± SD. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Uptake and release in the control experiments

Following tissue loading, the average uptake of radioactivity in the spinal cord slices from the control group was 149 ± 32 kBq/g (Table 1, n = 4). Over a 200 s resting period, the average release from the slices was 0.67 ± 0.13% of the total radioactivity content of the tissue (FRR_1). In response to electrical stimulation (S_1) (3 Hz, 240 shocks), the release was 2.60 ± 0.45% of the total radioactivity present in the slices in the 3rd fraction. Subsequently, the rate slowly decreased to its resting value (Fig. 1a). In response to the second electrical stimulation (S_2), the increase was less pronounced (FRS_2 = 1.94 ± 0.57%) and the FRS_2/FRS_1 ratio was 0.73 ± 0.21 (n = 7).

3.2. Effects of surgical hemisection

At up to 3 days after surgical hemisection, [^{3}H]NA uptake did not change compared to control experiments (158 ± 10 kBq/g, p > 0.05) (Table 1, n = 4). However, surgical hemisection resulted in an overall increase in the amount of [^{3}H]NA released at rest. FRR_1 was 0.81 ± 0.09% 3 days after and 1.01 ± 0.46% 14 days after hemisection; these values were significantly higher (p < 0.01) than the values measured in control slices. These findings are shown in Fig. 1, and the release observed 3 days after SCI was compared with the release observed in the control group. After electrical stimulation, the maximum release (3rd fraction) increased from 2.60 ± 0.45% to 3.12 ± 0.53%, and the FRS_1 increased from 2.73 ± 0.68% to 3.21 ± 0.74% (Fig. 2); both of these changes were statistically

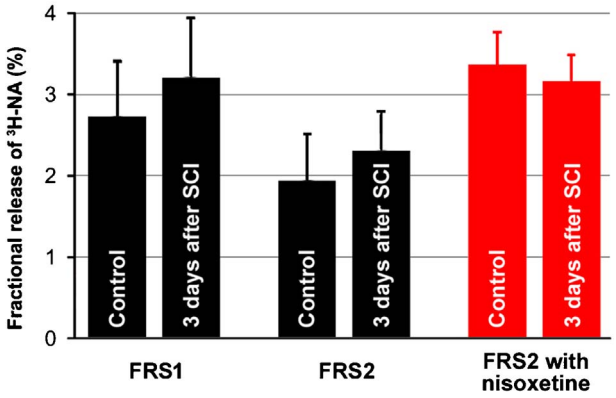

Fig. 2. FRS_1 and FRS_2 values, which represent the release following electrical stimulation (S_1 and S_2) in addition to the resting release. n = 28. Note, that after SCI by hemisection nisoxetine (1 μM) failed to increase the release of [^{3}H]NA in response to field stimulation. [^{3}H] release values expressed in kBq/g: FRS_1 (control 4.07 ± 1.01, after SCI 4.85 ± 1.12), FRS_2 (control 2.72 ± 0.80, after SCI 3.18 ± 0.66), FRS_2 with nisoxetine (control 4.43 ± 0.33, after SCI 4.35 ± 0.44).

significant (p < 0.05). These numbers correspond to a 20% increase in resting release and an additional 18% increase in stimulated [^{3}H]NA release following hemisection. A significant change in the FRS_2/FRS_1 ratio was not observed after hemisection. The ratio was 0.63 at 1 day after SCI and 0.70 at 3 days after SCI (n = 7 in each group).

Fourteen days after hemisection, large variations were observed in the fractional release values, and only 3 out of the 7 experiments resulted in a regular curve characteristic of stimulated transmitter release. Thus, we did not perform experiments with nisoxetine in this group.

3.3. Effect of nisoxetine

Noradrenaline uptake was potently inhibited by the application of the noradrenaline reuptake inhibitor nisoxetine at a concentration of 1 μM during the preincubation and loading period, resulting in an average radioactivity of 37 ± 9 kBq/g (control + nisoxetine) in the tissues, a 75% decrease in uptake compared with the control group. Three days after hemisection, nisoxetine decreased noradrenaline uptake to a significantly (p < 0.01) lesser extent (61 ± 16 kBq/g) (Table 1, n = 4 in each group).

In half of the release experiments, nisoxetine was added to the superfusion solution starting at the 8th fraction to inhibit [^{3}H]NA reuptake. Thus, the release evoked by the second stimulation (S_2) was significantly higher and more [^{3}H]NA remained in the collected superfusion solution (Fig. 3b), resulting in a significant (p < 0.01) increase in noradrenaline release (FRS_2/FRS_1 = 1.26 ± 0.20, n = 7).

An examination of the effect of nisoxetine on slices prepared from the injured spinal cord revealed a significant increase in the FRS_2/FRS_1

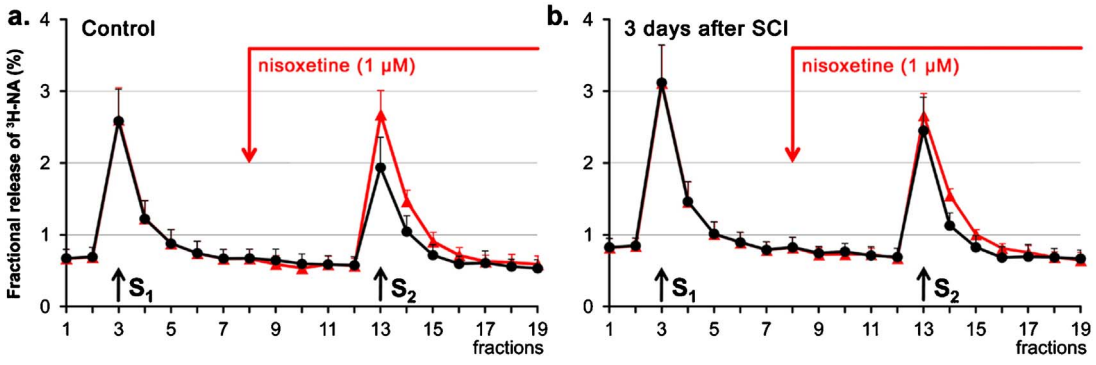

Fig. 1. Fractional release of [^{3}H]noradrenaline from spinal cord slices in response to electrical field stimulation at the beginning of the 3rd and 13th fractions. 1 μM nisoxetine was added to the superfusion solution starting at the 8th fraction. Non-injured tissue (a), 3 days after SCI by hemisection (b). n = 28.

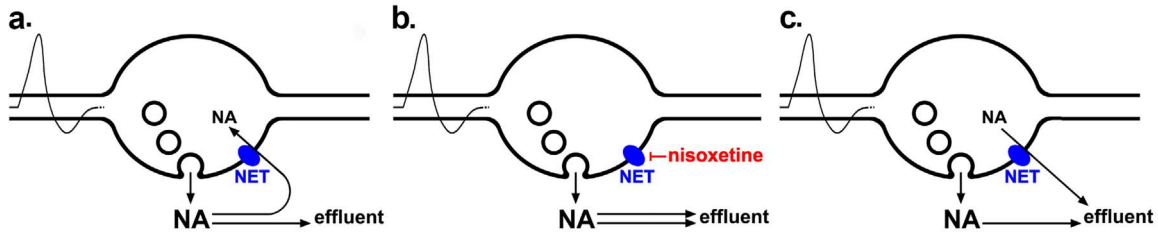

Fig. 3. Following an action potential or external electrical stimulation, [^{3}H]noradrenaline is released from vesicles into the synaptic cleft. Some of this compound is taken up again by noradrenaline transporters (NET), whereas the remaining [^{3}H]noradrenaline is degraded by enzymes or washed out into the superfusion solution and collected as effluent, as in the present study (a). When reuptake is inhibited with nisoxetine, a larger part of the released transmitter is washed out, resulting in a higher value for the measured noradrenaline release (b). A similar increase occurs when the transporter function is reversed in response to spinal cord injury (c).

ratio ($p < 0.01$) in each group: 1.05 ± 0.21 at 1 day after SCI and 1.07 ± 0.11 at 3 days after SCI. In the presence of nisoxetine, FRS_2 was increased by 74% in the control group; at 1 and 3 days after SCI, this value decreased to 61% and 37%, respectively (Fig. 2).

3.4. Electron microscopy images

Images of the healthy and injured spinal cord tissue were obtained 3 days after SCI to evaluate how the morphology of the tissue correlates with the findings of the release experiments.

At 3 days after SCI, the L5 spinal cord segment, which is located approximately 2 mm caudal to the injury, showed overall degeneration of the neuronal organelles and myelin sheath. However, many intact synapses were observed (Fig. 4).

4. Discussion

Noradrenergic innervation is a major neurotransmitter system in the CNS (including the spinal cord) that is involved in many behavioural processes. Three different ponto-spinal noradrenergic pathways originating from A5, A6 (locus coeruleus), and A7 cell groups innervate the ipsilateral spinal cord (Clark and Proudfit, 1991; Bruinstroop et al., 2012). NA release in the spinal cord has been shown to be involved in the control of somatosensory transmission (Bruinstroop et al., 2012).

This study is the first to reveal the modulation of NA release from the decentralised nerve terminals of noradrenergic nerves by unilateral hemisection of the spinal cord in adult rats. The experiments in the present study were performed to determine whether an artificially induced spinal cord injury had similar effects on noradrenaline uptake and release as the simulation of ischaemic conditions in healthy tissues *in vitro* (Uchihashi et al., 1998; Nakai et al., 1999; Sumiya et al., 2001; Sircuta et al., 2016) and *in vivo* (Globus et al., 1989). In contrast to the above-mentioned studies, normal Krebs perfusion solution was used in the present study, but the spinal cord tissue was isolated caudally and in close proximity (2–5 mm) to an artificial injury (Schoultz and DeLuca, 1974). After surgical hemisection, the rats showed typical signs of hemiplegia, with no movement of the right hindlimb. After 3 days, the macroscopic appearance of the spinal cord resembled healthy tissue. Electron microscopy images revealed overall degeneration of the neuronal organelles and myelin sheath, but many synapses remained intact, despite the surrounding inflammation and their detachment from the cell body. Both resting and stimulation-evoked NA release were observed. Thus, NA release was observed from the terminals several days after and caudal to hemisection. However, both resting and stimulated [^{3}H]NA release increased significantly after injury, indicating that the reuptake of the transmitter was impaired.

Surprisingly, the [^{3}H]NA uptake values observed 3 days after SCI were similar to the values observed in the control group, consistent with the findings of Globus et al. (1989) and Hofmeijer and Putten (2012), who showed that the changes in synaptic function observed during ischaemia are reversible following the restoration of blood flow in the brain.

Based on the results of the present study, hemisection prevented nisoxetine, a potent inhibitor of noradrenaline reuptake in the spinal cord, from binding to the noradrenergic terminals and increasing stimulation-evoked NA release. Hemisection induced significant changes in the characteristics of inhibition. Although FRS_2 was 74% higher in the nisoxetine-treated group than in the control group (without nisoxetine), the effect of nisoxetine was decreased to 61% and 37% at 1 and 3 days after SCI, respectively, indicating that the noradrenaline reuptake inhibitor gradually lost its effectiveness after hemisection. A similar trend was observed in the uptake experiments.

This finding likely reflects the observation that some of the noradrenaline transporters (NET) act as reverse carriers after injury (Fig. 3c) (Vizi et al., 1982), suggesting that there is a different magnitude but similar underlying mechanism of increased NA-release during ischaemia and decentralisation. As proposed in the study by Uchihashi et al. (1998) [^{3}H]NA release from the spinal cord during ischaemia is not subject to presynaptic modulation by α_{2A}-adrenoceptors, indicating that release was not associated with exocytosis but

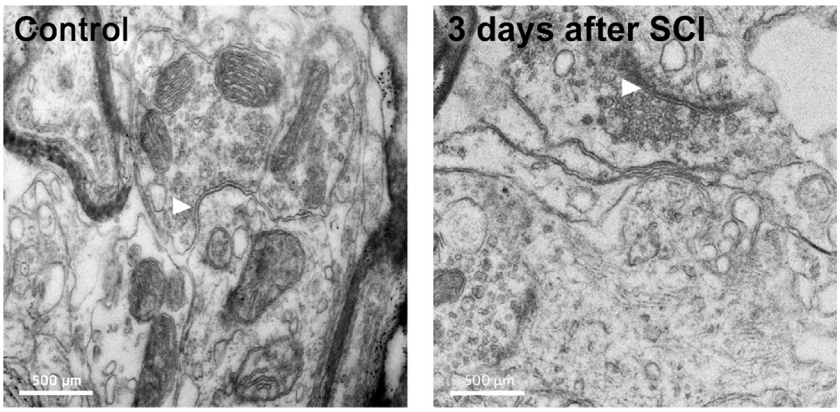

Fig. 4. Electron microscopy image of the spinal cord (segment L5, lamina VIII, ipsilateral to the injury). Synapses with intact morphology (arrow) were observed, even at 3 days after SCI.

rather the reverse operation of the carrier (Uchihashi et al., 1998; Sumiya et al., 2001).

The finding that NET loses its capacity to take up NA is consistent with the observations of Szot et al. (2012), who showed that the unilateral destruction of the locus coeruleus by 6-hydroxydopamine reduces the NET binding sites for presynaptic α_1- and α_2 receptors, including the sites on the contralateral side. Notably, the latter observation requires further explanation. Based on our experiments, hemisection at the lumbar level exclusively affects the terminal part of noradrenergic neurons.

Glutamate release is enhanced and uptake processes are concomitantly impaired in response to spinal cord injury (Demediuk et al., 1989; Inquimbert et al., 2012) or ischaemia of the spinal cord (Simpson et al., 1990) and the transmitter exhibits local neurotoxic effects (Wrathall et al., 1992) by inducing calcium-mediated neuronal death. The activation of NMDA and AMPA receptors induces noradrenaline release (Klarica et al., 1996; Sundström et al., 1998), and conversely, noradrenaline potentiates NMDA-mediated effects on the spinal cord (Wohlberg et al., 1987). Plausibly, noradrenaline and glutamate mutually reinforce the excessive release and injurious excitotoxic effects of the other. This phenomenon may play a role in the inflammation and subsequent loss of function following spinal cord injuries.

Based on the results of the present study, spinal cord hemisection increases the extracellular levels of NA in the affected spinal cord tissue, which may be cytotoxic due to the presence of its metabolites (DOPAL and DOPEGAL) (Burke et al., 2004). However, this increase is less pronounced than the ischaemic injury-induced increase observed in other studies (Uchihashi et al., 1998; Nakai et al., 1999; Sumiya et al., 2001).

5. Conclusions

The results of the present study suggest the following:

- A 20% increase in resting release and an additional 18% increase in stimulated noradrenaline release from the perfused spinal cord are observed 3 days after spinal cord hemisection. Although this change is statistically significant, it is well below the magnitude measured during ischaemia.
- Nisoxetine inhibits noradrenaline reuptake in healthy tissue, but after hemisection, this compound gradually loses its effectiveness at inhibiting NA reuptake, presumably reflecting a gradual loss of the binding capacity for the noradrenaline transporter, which acts as a reverse carrier.

We conclude that spinal cord injury leads to an increase in the extraneuronal concentration of NA, primarily reflecting an impairment in NA uptake.

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