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Original experimental

Effects of the excitatory amino acid transporter subtype 2 (EAAT-2) inducer ceftriaxone on different pain modalities in rat

Laila Eljaja[a], Ole J. Bjerrum[a], Per Hartvig Honoré[a], Bjarke Abrahamsen[b,*]

[a] *Department of Pharmacology and Pharmacotherapy, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark*
[b] *Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark*

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ABSTRACT

Glutamate is the major excitatory amino acid in the mammalian CNS and is involved in transmission of pain together with processes for cognition, memory and learning. In order to terminate glutamatergic neurotransmission and avoid excitotoxic damage, a balanced glutamate homeostasis is of critical importance. The level of glutamate in the synaptic cleft is regulated through the action of five subtypes of excitatory amino acid transporters (EAAT1-5). Ceftriaxone, a β-lactam, induces EAAT-2 and has proven effect for the treatment of neuropathic pain. This pilot study investigated the effects of ceftriaxone upon acute and inflammatory pain and additionally, the analgesic effect of ceftriaxone after introduction of neuropathic pain.

Methods: Rats were tested before, during and after treatment of ceftriaxone for changes in response to both mechanical and thermal stimuli, using calibrated von Frey filaments and Hargreaves instrument, respectively. Inflammatory responses were investigated by assessing the response to intra-plantar injections of formalin; lastly, neuropathic pain was introduced using the spinal nerve ligation (SNL) model after which changes in both mechanical and thermal responses were again investigated.

Results: A significant increase in mechanical withdrawal threshold was observed following acute pain inducement in ceftriaxone treated rats. A marked increase in thermal withdrawal latency was also observed. In response to intra plantar administered formalin, ceftriaxone delayed the intensity of nocifensive behaviours. Applying the SNL model of neuropathic pain on naive rats created significant mechanical allodynia, but only a negligibly different response to thermal stimulation. After treatment with ceftriaxone the treated rats developed a hypoalgesic response to thermal stimulation, whilst the response to mechanical pain was insignificant.

Conclusion: In conclusion, ceftriaxone clearly interfered in the transmission of noxious signalling and proved in this study to have an effect upon acute *thermal and mechanical* pain thresholds as well as pathologic pain conditions. The present results are a piece in the large puzzle where administration route, dosage and pain models must be thoroughly investigated before a study can be planned for a proof of concept in different clinical pain states.

Implications: The current study demonstrates that ceftriaxone has a mitigating effect upon many pain modalities including acute and inflammatory, and that these modalities should be included in future studies characterising the anti-nociceptive effect of beta-lactams such as ceftriaxone. The fact that β-lactams also has antibiotic properties implies that similar chemical structures could be identified with the positive effect upon expression levels of EAAT2, but lacking the antibiotic side effect.

1. Introduction

The primary excitatory amino acid neurotransmitter in the central nervous system is glutamate [1,37]. Glutamate is involved in complex physiological processes such as learning, memory and pain [2,34,35]. By activating pre- and postsynaptic receptors, glutamate takes part in the transmission of nociceptive information

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* Corresponding author. Tel.: +45 39 17 96 54; fax: +45 35 33 60 20.
E-mail address: ba@farma.ku.dk (B. Abrahamsen).

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[2,36]. Sodium dependent high-affinity glutamate transporters regulate the extracellular glutamate homeostasis. A total of five subtypes have been cloned; GLAST (EAAT-1), GLT-1 (EAAT-2), EAAC-1 (EAAT-3), EAAT-4 and EAAT-5 [3–6]. GLAST, GLT-1 and EAAC1 (EAAT1-3) are expressed throughout the CNS, EAAT4 is exclusively expressed in Purkinje cells in the cerebellum [5] and finally EAAT5 distribution is confined to the retina [3]. At the cellular level GLAST/GLT-1 are expressed in glial tissue whilst EAAC1/EAAT4 are expressed in neuronal tissue [7]. For the regulation of the glutamate homeostasis, GLT-1 is of major importance, being responsible for the vast majority of the total glutamate clearance [8] which is already proven through the lethal knock out of GLT-1 [9].

Changes in EAAT function contribute to the development of chronic pain and other types of neurological disorders such as epilepsy, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [10–12]. During neuropathic pain expression levels of glutamate transporters are significantly changed, contributing to the increased activation of post-synaptic glutamate receptors [13,14]. Considering nociceptive signalling, modulation of EAATs may present a unique alternative for a treatment of pain also in the clinic [11,15,16].

Recently, a screening of 1040 FDA approved drugs resulted in identification of a number of antibiotics, all with a β-lactam structure in common, able to induce an up-regulation of GLT-1 expression through the nuclear factor-κβ signalling pathway [1,17]. The induced increased expression of GLT-1 by ceftriaxone led to several studies on the changes in nociceptive response [2,18,19]. Using different routes of administration, β-lactams have been reported to attenuate nociceptive responses both after induction of neuropathic pain and of visceral pain ([2,18]; Ramos et al.). A number of hypotheses were investigated in the present pilot study in order to understand the effects of ceftriaxone with respect to acute responses after mechanical or thermal stimulation, intraplantar formalin and finally after introduction of neuropathic pain using the SNL neuropathic pain model, as this model had not yet been used in studies on ceftriaxone induced analgesia.

2. Materials and methods

2.1. Animals

Outbred, male Sprague–Dawley rats (SPRD) (Taconic, Denmark), 6 weeks old at the time of arrival, were used in the experiments. Rats were housed in groups of four per cage. All rats were maintained at standard 12 h light/dark cycle with free access to food and water. Experiments were performed during the light period of the day. All experimental procedures were approved by The Animal Experiments Inspectorate, The Danish Ministry of Justice (license number: 2007/561-1284 C7).

2.2. Ceftriaxone

Rats were divided into two groups, one receiving 200 mg/kg ceftriaxone (Ceftriaxone hydrochloride salt, ACSD generics) intraperitoneally daily and the other received an equivalent volume of saline (0.9% NaCl). Treatment was continued for 9 days in total. For practical reasons, blinding was not possible due to development of diarrhea from the rats receiving ceftriaxone.

2.3. Behavioural models

All behavioural procedures were performed by the same investigator, in the same laboratory, and at the same time of the day with animals allowed to acclimatize before start.

2.4. Hargreaves instrument

For the Hargreaves test (Plantar Test Analgesia Meter Model 400, IITC Life Science, Woodland Hills, CA, USA), rats were placed in individual Perspex chambers (20 cm × 10 cm × 12 cm) for about 20 min before testing, performed as described [20]. A total of three independent baseline measures were obtained before initiating treatment. Using the guide light a focused light beam was pointed towards the plantar surface of the left paw and the latency in paw withdrawal monitored. A cut-off latency of 20 s was used. The measurements were repeated three times with an interval of 3–5 min.

2.5. von Frey filaments

To test for mechanical allodynia calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) were used [19]. Three independent baseline measurements were obtained before initiating treatment. Rats were placed in elevated individual Perspex chambers (20 cm × 20 cm × 12 cm) with a metal mesh floor and left to acclimatize until settled before starting. von Frey filaments were applied using the Chaplan 'up and down paradigm' [21]. A 50% threshold was calculated using the Dixon method [22].

2.6. Randall Selitto apparatus

The Randall Selitto paw pressure meter (IITC Life Science, Woodland Hills, CA, USA) was used to quantify mechanical hyperalgesia. The instrument consists of a digitally controlled paw pressure meter and a sling suit. The rat was placed in the sling and left to acclimatize for about 2 min before mechanical hyperalgesia was assessed from uniformly increasing pressure applied to either tail or paw.

The tail was placed between the arms of the pressure applicator. Responses to pressure applied to the tail were measured twice with at least 3 min in between each test terminating when any of the defined endpoints was reached. For pressure applied to the tail the endpoint was withdrawal [23]. The same area of the tail was tested at each occasion. For measures on the paw, the paw was gently supported and placed between the arms of the pressure applicator with the tip pointing towards the dorsal surface. Pressure was applied as described until the selected endpoint defined as paw withdrawal was observed.

2.7. Formalin test

Responses to intraplantar administered formalin (Nomeco A/S, DK) were performed as described [24]. In brief, 50 μl 5% (v/v) formalin was injected subcutaneously into the plantar surface of the left hind paw. The rat was immediately placed in a clear Perspex chamber (20 cm × 20 cm × 12 cm). Paw licking or biting, flinching or lifting was monitored for 60 min and recorded for every 5 min. Phase 1 was defined as the nocifensive behaviour from 0 to 10 min with phase 2 lasting from 10 to 60 min.

2.8. Spinal nerve ligation (SNL)

To introduce neuropathic pain a tight ligation was introduced around L5 of the segmental spinal nerve as described by [25]. The rat was anesthetized and prepared for the surgical procedure by performing an incision and blunt dissection of connective tissue and muscle to expose L5 between processus transversi of L5 and L6. Following identification of L5 a tight ligation (Ethicon, 4-0, Johnson and Johnson International, Belgium) was introduced and muscle and skin was carefully closed using sutures (Ethicon, 4-0, absorbable and non-absorbable). Post-operative treatment included free access to food and water and assessing symptoms

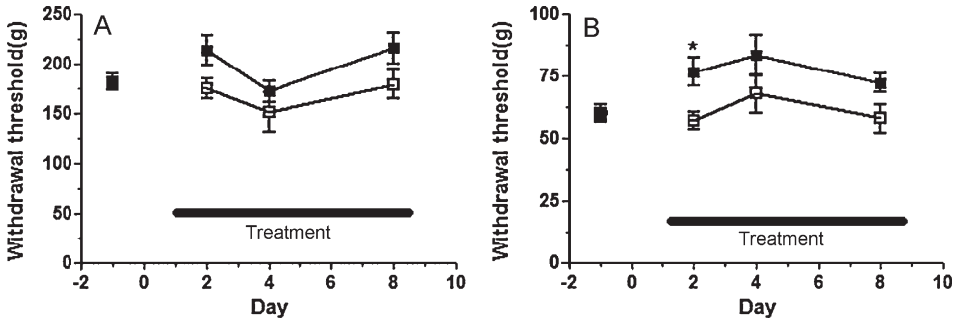

Fig. 1. Effect of ceftriaxone on acute noxious mechanical thresholds in naïve rats: (●) ceftriaxone treated; (♦) control. Significant differences were observed for responses applying the Randall Selitto paw pressure meter to (A) tail ($p = 0.026$) and (B) paw ($p = 0.05$). Ceftriaxone group $n = 7$, control group $n = 5$. The threshold value shown day 1 (−1) corresponds to the mean of three baseline measurements in each rat, performed daily for 7 days.

of neuropathic pain such as an elevation of the ipsilateral paw. No behavioural procedures were performed until day 4 after surgery. All SNL surgery was performed on the same day.

2.9. Statistics

Statistical analysis was performed using Graphpad Prism 4. A parametric unpaired *t*-test with two-tailed *p*-values and a confidence interval of 95% was employed. The analysis of variances used a two-way ANOVA as repeated measures test, where each row of data represents a different time point. The ANOVA was followed by the Bonferroni post-test. Statistical significance was considered at $p \leq 0.05$. An unpaired Student's *t*-test was used when comparing sets of data from specific days or time points. For acute pain thresholds, a mean difference in percent between ceftriaxone treated and controls ± the standard error of the mean was calculated by accumulating the differences from the days of experiments dividing by the number of days.

3. Results

3.1. Ceftriaxone increased noxious threshold in naïve rats

Mechanical threshold. Rats were treated with intraperitoneal ceftriaxone and responses to noxious mechanical stimulation using the Randal Selitto apparatus were measured on both paw and tail (Fig. 1). A significant increase in thresholds were demonstrated when comparing ceftriaxone treated rats ($n = 7$) with control rats ($n = 5$) with paw and tail responses which increased by 27 ± 3.6% and 19 ± 2.5%, respectively, based on 3 days of experiments ($p < 0.05$).

Thermal threshold. Responses to thermal stimulation were assessed using the Hargreaves instrument (Fig. 2). A significant, 26 ± 3.2% increase in thermal threshold was monitored for the ceftriaxone treated rats ($n = 7$) when compared with control rats ($n = 5$) based on 4 days of experiments ($p < 0.05$).

3.2. Effect of cetriaxone on inflammatory response

Changes in inflammatory responses, induced by intraplantar injection of formalin were assessed by quantifying defined inflammatory pain behaviours for 60 min (Fig. 3). Intraplantar formalin resulted in a characteristic biphasic response with the first phase due to direct stimulation of the nerve terminals and the second phase considered as inflammatory [26]. No significant difference for phase 1 was observed when comparing behavioural responses for ceftriaxone treated rats ($n = 7$) with control rats ($n = 5$). During the second phase a decrease in inflammatory pain defined behaviour was seen for the ceftriaxone treated rats starting after 40 min and lasting until the end of the study at 60 min. A significant difference at time point 40–45 min ($p = 0.041$) was recorded.

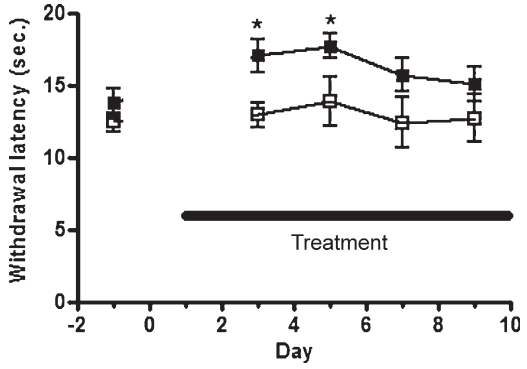

Fig. 2. Effect of ceftriaxone on acute noxious thermal thresholds in naïve rats: (●) ceftriaxone treated; (♦) control. Using the Hargreaves instrument a significantly increased noxious threshold ($p = 0.038$) was found between the ceftriaxone treated and control. Significant difference for individual days was found at day 3 ($p = 0.023$) and 5 ($p = 0.049$). Ceftriaxone group $n = 7$, control group $n = 5$. The threshold value shown day 1 (−1) corresponds to the mean of three baseline measurements performed daily for 6 days in each rat.

3.3. Effects of ceftriaxone on SNL induced neuropathic pain

Neuropathic pain was induced using the SNL model and the effect of ceftriaxone on mechanical allodynia and thermal hyperalgesia were investigated. The development of mechanical allodynia

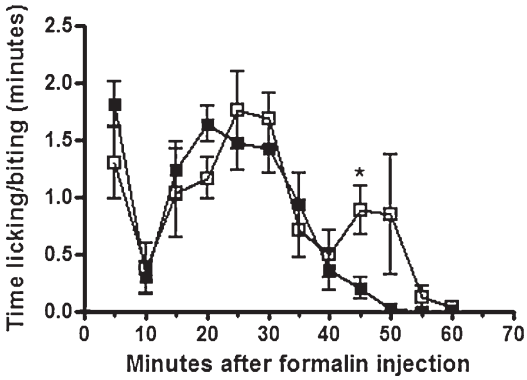

Fig. 3. Effect of ceftriaxone on inflammatory responses: (●) ceftriaxone treated; (♦) control. Inflammatory responses were monitored for 60 min after intraplantar injections of 5% formalin. A classical bi-phasic response developed with a delayed analgesic effect seen for the ceftriaxone treated rats ($n = 7$) by the end of the experiment when compared to controls ($n = 5$). A significant difference was observed at 40–45 min post-injection ($p = 0.041$).

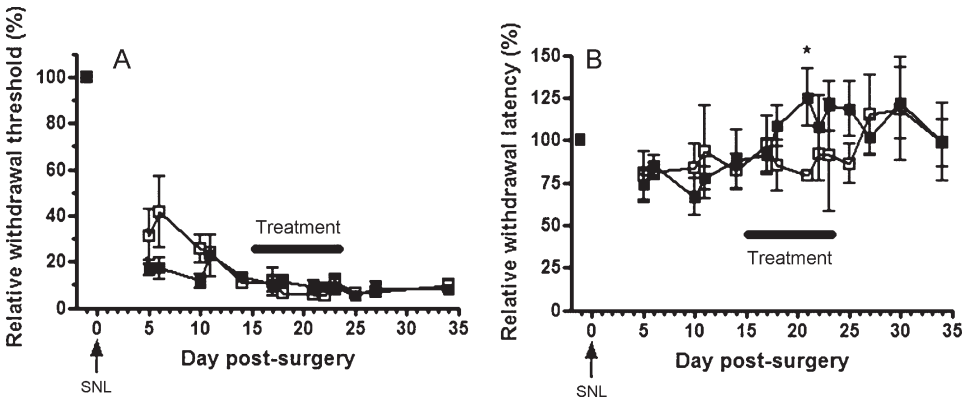

Fig. 4. Effect of ceftriaxone on responses after induction of neuropathic pain: (●) ceftriaxone treated; (♦) control. Mechanical allodynia (A) was significant after nerve damage, but no effect of ceftriaxone in treated rats ($n = 6$) was found when compared with controls ($n = 4$). A thermal hyperalgesia (B) trend developed after introducing nerve damage with ceftriaxone giving rise to a hypoalgesic profile when compared with control. The measurement at day 21 was significantly different between groups ($p = 0.036$). The value for day −1 was the mean of two baseline measurements for each rat, performed daily for 3 days.

was assessed using calibrated von Frey filaments with rats showing a fast decrease in pain threshold. Ceftriaxone treatment did not have any effect on mechanical allodynia, as interpreted from absence of any significant differences in effect between control rats ($n = 4$) and treated rats ($n = 6$) at any time point (Fig. 4A).

No significant development of thermal hyperalgesia was observed due to induction of neuropathic pain although a trend was observed (Fig. 4B). The effect of ceftriaxone was demonstrated by a trend of hypoalgesic responses seen as an elevation of the threshold starting 3 days after initiating treatment and ending 4 days after termination of treatment. Measurement at day 21 was significantly different between ceftriaxone and control group ($p = 0.036$). For both ceftriaxone treated and control treated rats a return to baseline was observed at day 27 post-surgery and until the end of study. The period of anti-nociceptive effect from ceftriaxone lasted for at least 7 days.

4. Discussion

The β-lactam ceftriaxone enhances the expression of the glial glutamate transport-1 (GLT-1) [1] resulting in attenuated neuropathic pain induced using the chronic constrictive injury model in rats ([2]; Ramos et al.). This extended study on ceftriaxone analgesic effects also showed a clear impact on acute pain thresholds, but no effects were seen on immediate effect and a limited effect upon late inflammatory pain. Ceftriaxone also had a clear impact on thermal hyperalgesia during, and continuing through to after, termination of treatment as assessed in neuropathic pain by the SNL model, which has not been used previously with respect to ceftriaxone induced analgesia.

The effects of ceftriaxone were investigated over a period of 6 days demonstrating a significant decrease in acute pain measures. Ramos et al. [19] have recently investigated acute thresholds of ceftriaxone with no difference in response to controls and opposing the present results (Ramos et al.). A number of differences between the two studies may explain the contradictory results. Ramos et al. [19] used von Frey filaments in the investigation of mechanicals thresholds whereas the current study used the Randal Selitto apparatus. Application of von Frey filaments gave rise to non-noxious stimuli [27] compared with the noxious stimuli induced using the Randall Selitto apparatus [32]. Acute thermal thresholds were measured in both the current study and in that of Ramos et al. [19] using the Hargreaves instrument. Administration of ceftriaxone differed between the two studies with intrathecal administration [19] compared to intraperitoneal administration in the present study. These differences of administration route and unknown dose equivalence may have major impact on the response seen in the two studies. In this study the effect was already obvious 1 day after initiating treatment and lasted throughout the period of treatment. Ramos et al. [19] administered ceftriaxone for 5 days before investigating acute thresholds 24 h after the last injection. Elevation of GLT-1 mRNA was demonstrated lasting for at least 24 h after end of the administration (Ramos et al.). An effect of ceftriaxone was hypothesized with glutamate being the main excitatory transmitter mediating acute noxious signalling [28,29] supporting the present results.

Effect of ceftriaxone upon acute thresholds following induction of inflammatory pain was investigated, monitoring the nociceptive behaviour after intraplantar formalin injections. Receptors for glutamate are significantly *up*regulated following inflammation thus implicating glutamate evidently in the transmission of noxious signalling [30]. The results suggest that ceftriaxone and an induced elevation of GLT-1 expression have an effect in the second phase of the formalin test, but not in the first. The strong impact of the formalin-induced inflammatory pain responses blunted the monitoring of peak response. Other inflammatory pain models must be investigated over a large dosage range of ceftriaxone to clarify its effect upon responses to inflammation.

Two independent groups have reported a significant effect of ceftriaxone attenuating neuropathic pain modalities using the chronic constriction injury model [2,19]. In the current study the spinal nerve ligation model was employed instead and the results indicated only a limited effect of ceftriaxone upon induced neuropathic pain modalities. Mechanical allodynia developed rapidly after induction of nerve damage and persisted even up to 9 days of ceftriaxone treatment, with no impact of ceftriaxone upon the persistence of mechanical allodynia. Operated rats did not develop thermal hyperalgesia. Ceftriaxone treated rats, however, developed a trend demonstrating an increased latency in response to the focused light from the Hargreaves instrument as compared with measures obtained before start of treatment. This suggests that ceftriaxone may have an analgesic effect using the SNL model. Rat studies using the chronic constrictive injury (CCI) model for ceftriaxone effect upon neuropathic pain have demonstrated conflicting results. In one, the highest impact of ceftriaxone was found on development and persistence of thermal hyperalgesia [2] whereas the other found that ceftriaxone had the greatest impact in attenuating the persistence of mechanical allodynia [19]. Generally, neuropathic pain models show similar results to mechanical and thermal stimuli [31], although the CCI displayed higher pain intensities in rats [33].

It is noteworthy that the ceftriaxone attenuation of the neuropathic pain response continued for at least 2 days after termination

of treatment, probably due to long survival of the induced GLT-1 protein. This may imply that ceftriaxone might be administered as infrequently as every 2 or more days. Little or no development of tolerance to ceftriaxone might be another possible advantage of the suggested intermittent dosing. These possibilities must, however, be proven.

It has, undoubtedly, been shown that elevation of GLT-1 expression has a role in pain signalling [19]. The result of the present study demonstrated that ceftriaxone had a clear impact on acute mechanical and thermal thresholds and some influence in inflammatory pain as a probable result of the induced promotion of GLT-1. The conflicting results found in the two neuropathic pain models in rats might pose demands for further investigations on the optimal conditions for administration of ceftriaxone and exploitation of its tentative up-regulation of GLT-1 to treat chronic pain.

In conclusion, ceftriaxone has demonstrated the potential for treatment in both acute and chronic pain, probably due to increased uptake of glutamate from the synaptic cleft. Studies investigating the effect of ceftriaxone on pathological pain conditions have emerged over the last few years and the present *pilot* study add experiences from both acute pain levels and after inflammation. To complete the picture, many pieces of investigation are still needed before proof of concept in human would be possible. These include methods of administration, optimization of dose regimen as well as exploration of other β-lactams without any antibacterial effect.

Conflict of interest statement

The authors declared no conflict of interest.

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