The Effect of Fluorocitrate at the Spinal Level in a Rat Monoarthritic Pain Model: Possible Modulatory Role of D-serine

Mariana Quiroz-Munoz¹, Alejandro Hernandez², Teresa Pelissier³ and Claudio Laurido²

1 Introduction

Chronic pain is a clinical problem difficult to solve due to the incomplete knowledge we have about the adaptive changes that occurs in the neural substrates of the nociceptive system and glial cells in response to episodes of persistent pain. N-methyl Aspartate (NMDA) receptor antagonists inhibit the hyperexcitability of nociceptive neurons in the spinal cord induced by stimulation of C fibers (Davies & Lodge, 1987; Dickenson & Sullivan, 1987). This receptor is important in the establishment of chronic pain; furthermore, we know other factors that may modulate this pain, such as glial cells (Milligan & Watkins, 2009). Fluorocitrate (FC) is a specific inhibitor of glial metabolism. The application of 0.001 µM of FC administered intrastratally in the rat causes a 95% reduction of glutamine formation from acetate, a substrate that permeates astrocyte cells selectively (Hassel et al., 1992). Glutamine is synthesized by astrocytes and constitutes an important precursor for the synthesis of glutamate and GABA, both in vitro and in vivo. FC also blocks the Krebs cycle enzyme aconitase in the astrocytes, dramatically decreasing the production of ATP (Serres et al., 2008) "shutting down" them metabolically and thus

¹ Laboratory of Renal Physiology, Faculty of Biological Sciences, Catholic University of Chile, Chile
² Department of Biology, Department of Biology, Faculty of Chemistry and Biology, University of Santiago of Chile, Chile
³ ICBM, University of Chile, Chile
preventing the synthesis, among others, of proinflammatory cytokines (Guo et al., 2007). Furthermore, FC (also minocycline, and SB203580) effectively inhibited IL-1b–induced reactive astrocytosis, microgliosis, and thermal hyperalgesia (Sung et al., 2012). In this context, the exogenous application of D-serine should reconstitute their levels and concomitant to this, the nociceptive neuronal function.

## 2 Material and Methods

Experiments were carried out in 60 adults Sprague-Dawley (250–300 g) rats, normal and monoarthritic. The experiments presented in this work were performed according to the Ethics Committee of the University of Santiago of Chile and the Ethical Guidelines for Research on Experimental Pain in Conscious Animals (Zimmermann, 1983). In order to minimize the unnecessary suffering of animals, we utilized a maximum of six animals in each experiment. They were kept in a chamber with controlled temperature (25 °C ± 2) and light/dark cycles of 12/12 hours, starting at 8:00 AM, with food and water ad libitum.

### 2.1 Experimental Groups

Three groups of animals were prepared: rats injected with saline (saline means Artificial Cerebrospinal Fluid. It consists of two separate solutions. The first solution was composed of 7.597 g of NaCl, 0.231 g of KCl, 0.203 g of MgCl₂, 2.058 g of NaHCO₃, 0.69 g of NaH₂PO₄, and 500 ml of H₂O. The second solution was prepared with 5.376 g of glucose, 0.175 g of CaCl₂, and 500 ml of H₂O. Then the two solutions are mixed together) (Nakamura et al., 1987) or FC, 0.002 µM at time zero and then tested as follows:

a) Normal rats injected with saline at time zero, 15, 30, 45, 60, 120, 240 and 360 minutes. \((n = 6 \text{ rats})\)  
   1. Monoarthritic rats injected with saline at time zero, 15, 30, 45, 60, 120, 240 and 360 minutes. \((n = 6 \text{ rats})\)  
   2. Normal rats injected with FC at time zero, 15, 30, 45, 60, 120, 240 and 360 minutes. \((n = 6 \text{ rats})\)  
   3. Monoarthritic rats injected with FC at time zero, 15, 30, 45, 60, 120, 240 and 360 minutes. \((n = 6 \text{ rats})\)

b) Rats injected with saline or FC, 0.002 µM i.t. at time zero and then tested as follows:  
   1. Normal rats injected with saline and tested at time zero, 15, 30, 45, 60, 90 and 120 minutes. \((n = 6 \text{ rats})\)  
   2. Normal rats injected with FC at time zero and submitted to spinal wind-up at times zero, 15, 30, 45 and 60 minutes. At time 60 minutes D-serine (300 µM) was injected and tested at times 60, 90 and 120 minutes. \((n = 6 \text{ rats})\)  
   3. Normal rats injected with FC at time zero and tested at time zero, 15, 30, 45,
60, 90 and 120 minutes. \((n = 6 \text{ rats})\)

c) Rats injected with saline or FC, 0.002 \(\mu\text{M}\) i.t. at time zero and then tested as follows:

1. Monoarthritic rats injected with saline and tested at time zero, 15, 30, 45, 60, 90 and 120 minutes. \((n = 6 \text{ rats})\)

2. Monoarthritic rats injected with FC at time zero and submitted to spinal wind-up at times zero, 15, 30, 45 and 60 minutes. At time 60 minutes D-serine (300 \(\mu\text{M}\)) was injected and tested at times 60, 90 and 120 minutes. \((n = 6 \text{ rats})\)

3. Monoarthritic rats injected with FC at time zero and tested at time zero, 15, 30, 45, 60, 90 and 120 minutes. \((n = 6 \text{ rats})\)

Intrathecal administration of drugs was performed by percutaneous injections, according to the method described by Mestre et al. (Mestre et al., 1994). Briefly, the i.t. injection consist of administering either saline, FC or D-serine into the subarachnoid space between lumbar vertebrae L5 and L6, using a Hamilton syringe with a needle 26G \(\times\) 1/2'' in a maximum volume of 10 \(\mu\text{L}\). To assure the needle has penetrated into the subarachnoid space, a slight movement in the tail of the rat occurs as a result of the needle mechanical stimulation penetrating the meninges of the spinal cord.

Chemicals: D-serine was obtained from Sigma-Aldrich and dissolved in saline. Fluorocitrate (Sigma-Aldrich) was prepared from barium fluorocitrate. Freund’s complete adjuvant (Difco Lab.), experimental arthritis inductor. Briefly: 300 \(\mu\text{g}\) of Mycobacterium butyricum suspended in 0.6 ml of paraffin, 0.4 ml NaCl, 0.9% and 0.1 ml Tween 80, injected in one intra articular bolus of 50 \(\mu\text{L}\).

2.2 Experimental Model of Chronic Pain

Monoarthritis: This model of pathological pain was described by Butler et al., (Bendele, 2001). Briefly, rats weighing 120–150 g approximately are inoculated with 50 \(\mu\text{L}\) of complete Freund’s adjuvant containing 300 \(\mu\text{g}\) of Mycobacterium butyricum in the right ankle joint. This injection causes a localized arthritis syndrome, proving to be stable between four and six weeks post inoculation, establishing a persistent pain in the presence of neurogenic hyperalgesia. This arthritic model has been used in preclinical testing of numerous anti arthritic agents (Laurido et al., 2003). It is maintained for a period exceeding two months.

2.3 Electrophysiological Determinations

Spinal wind-up: The C fibers reflex was assessed in the normal and monoarthritic paw using rats anesthetized with urethane (1g/Kg i.p.). Initially, C fibers were stimulated by electrical shocks applied to the fourth and fifth toes of the hind paw, (territory innervated by the sural nerve), by means of two stainless steel electrodes. An intensity of 6–10 mA, duration of 2 ms and a frequency of 0.1 Hz is enough to evoke an electromyo-
graphic (EMG) activity. This EMG is recorded from the ipsilateral biceps femoris (Laurido et al., 2003) by means of two stainless steel electrodes placed inside the muscle. This stimulation is maintained for around 20 minutes to allow stabilization of the response. Then, the threshold of stimulation is sought by diminishing the amplitude of stimulation until one half of the EMG elicits an electrical response and the other half don’t. The values obtained for the C-fiber activation was shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>FC, 0.002 µM</th>
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<tbody>
<tr>
<td>Normal rats</td>
<td>6.0 ± 0.8</td>
<td>9.1 ± 1.1†</td>
</tr>
<tr>
<td>Monoarthritic rats</td>
<td>3.2 ± 0.3*</td>
<td>8.2 ± 0.9†</td>
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Table 1: The table shows the stimulant current required for the threshold activation of the C-fiber evoked responses in normal and monoarthritic rats treated with FC or saline.

Values are means ± standard error of the mean of stimulating current required (in mA) in the normal and FC-treated rats for the C-fiber activation. Two-way analysis of variance (ANOVA) identified the FC treatment and the monoarthritic condition as significant factors influencing the stimulating current required for threshold activation of the C-reflex. No FC treatment × monoarthritic condition interaction was observed. Significant differences ($P < 0.01$) between FC-treated and saline-treated groups are denoted by †, while significant differences between monoarthritic and normal groups ($P < 0.01$) are indicated by an asterisk (according to the Bonferroni post hoc test). $n = 6$ animals in each group.

Then, stimulation at 0.1 Hz with intensity twice the threshold was maintained throughout the experiment as seen in Figure 1. In order to obtain the electromyographic response, the cumulative integral with a duration of 300 ms, (which corresponds to a time interval of 150–450 ms after the stimulus), was obtained for each stimulus. The maximum value of this integral corresponds to the electromyographic response (Figure 1, lower panel). It can be observed that the integral values are stable (between 0.025–0.034 V.s). These responses were fed into a computer equipped with a digital to analog converter (Powerlab/4S), and processed with the Chart 5.0 software. To evoke synaptic potentiation or wind-up, twelve stimuli at 1.0 Hz were applied.

Figure 2 shows a representative record of stimulation showing the resultant EMG (upper panel). The wind-up is observed as a synaptic potentiation manifested for the progressive increase in the number of muscle action potentials produced by the stimulation. This enhancement is verified up to the stimulus number 9, and later a decrease is observed. This decrease was associated with activation of descending inhibitory mechanisms from supraspinal structures. The lower part shows the cumulative integral with a duration of 300 ms, which corresponds to a time of 150–450 ms after the stimulus. This initial test was the control. After injecting FC or D-serine, the maximum values of each stimulus (only those that show an increasing trend, usually between the seventh to
Figure 1: Representative electromyogram and cumulative integral of the C-reflex response.

Figure 2: Representative electromyogram and cumulative integral of the spinal wind-up response.
eighth stimuli) were used and the slope was expressed as a percentage with respect to the normal animals (Laurido et al., 2001). The results were expressed as mean ± SEM.  

The upper part shows a representative electromyogram with stimulation at 0.1 Hz. In order to obtain only the C-fiber activity, a time window of 300 ms is set. This window starts at 150 ms after the stimulus. This allows us to collect all the C-fiber activity discarding other responses such as Aδ fiber activity. The lower part shows the cumulative integral of the C-reflex responses. The maximum values of the sampling interval correspond to the C-reflex response for each time. This figure is valid for normal and monoarthritic rats. The only difference is the activation threshold for evoking the C-fiber evoked responses as shown in Table 1.

Electromyogram (upper part) and their associate integral (lower part). The sampling frequency is increased to 1 Hz in order to induce the C-fiber potentiation. Each sampling interval corresponds to a time window of 300 ms of duration, initiated 150 ms after the stimulus. This sample collects all the C-fiber activity discarding other responses such as Aδ fiber activity. The lower part shows the cumulative integral of the C-reflex responses. The C-fiber potentiation can be seen during the first four time intervals, where the integral increases the Vs value (from around 0.03 to 0.06 V.s). The slopes of the maximum of these four values correspond to the Wind-up response. This figure is valid for normal and monoarthritic rats. The only difference is the activation threshold for evoking the wind-up as shown in Table 1.

3 Results

Figure 3 shows the effect of FC applied to normal and monoarthritic rats at time zero. It can be observed that the antinociceptive effect of FC is maintained on time, reaching a plateau for both rats at around 120 minutes and sustained over the time until at least 360 minutes of testing. No longer times were attempted since that test is invasive in the sense that electrodes are placed into the muscle tissue and inside the toes, and might produce local inflammatory problems in the interface tissue electrode, altering the results.

Effect of saline on normal (S NR) and monoarthritic (S MR) rats: FC 0.002 µM was i.t. applied at time zero to both normal (FC NR) and monoarthritic (FC MR) rats. It can be seen that the antinociceptive effect spans at even 360 minutes. No longer times were tested (see text). n = 6, number of rats for each series of experiments.

Effect of saline, D-serine and FC on the spinal cord wind-up on normal rats: Figure 4 shows the effect of FC and D-serine in the wind-up cord of normal rats. At time zero was injected saline i.t. to the rats. The wind-up in rats with saline (black column, Saline Normal Rat (NR)) did not produce any significant change or was slightly algesic from zero to 120 minutes. A second series of experiments were done. FC was applied at time zero and the development of antinociception was followed at times 15, 30, 45, 60, 90 and 120 minutes (Green, FC Normal Rat (NR)). It can be observed that there is an increment in the antinociception over time, reaching a maximum of around 60 minutes,
Figure 3: Time course of the effect of FC alone on normal and monoarthritic rats.

Figure 4: Effect of saline, FC and D-serine in the wind-up cord of normal rats.
and then maintained up to 120 minutes. A third series of experiments were done. (Red, FC plus DS (60) NR). FC was applied at time zero observing a development of antinociception very similar to the one of FC alone. At time 60 minutes, D-serine (300 µM i.t., black arrow) was injected and the wind-up measured at times 90 and 120 minutes. It can be observed that there is a decrease in the antinociception (around 65%, at 90 minutes and 34% at 120 minutes) statistically significant ($P < 0.05$) for both times when compared with the time 60 minutes.

**Effect of saline (black column, Saline Normal Rat (NR)):** FC was applied at time zero and the development of antinociception was followed at times 15, 30, 45, 60, 90 and 120 minutes; In the green bar, FC Normal Rat (NR), FC was applied at time zero observing a development of antinociception very similar to the one of FC alone. At time 60 minutes, D-serine (300 µM i.t., black arrow) was injected and the wind-up measured at times 90 and 120 minutes. (Red column, FC plus DS (60) Normal Rat (NR)). $n = 6$, number of rats for each series of experiments. $* = p < 0.05$ according to one way ANOVA.

**Effect of saline, D-serine and FC on the spinal cord wind-up on monoarthritic rats:** Essentially, the same results as the normal ones appear in the monoarthritic rats. Figure 5 shows the effect of the application of saline at time zero (Black column, Saline Monoarthritic Rat). It can be observed that the effect of saline is more pronounced that the same treatment for normal rats. A second series of experiments were done. FC was applied at time zero and the development of antinociception was followed at times 15, 30, 45, 60, 90 and 120 minutes (Green, FC Monoarthritic Rat (MR)). It can be observed that there is an increment in the antinociception over time, which is maintained up to 120 minutes. A third series of experiments were done. (Red, FC plus DS (60) MR). FC was applied at time zero observing a development of antinociception very similar to the one of FC alone. At time 60 minutes, D-serine (300 µM i.t. black arrow) was injected and the wind-up measured at times 90 and 120 minutes. It can be observed that there is a decrease in the antinociception, around 50 %, at 90 minutes and 80% at 120 minutes) statistically significant ($P < 0.05$) for both times, when compared with the time 60 minutes.

**Effect of saline (black column, Saline Monoarthritic Rat (MR)):** FC was applied at time zero and the development of antinociception was followed at times 15, 30, 45, 60, 90 and 120 minutes (Green, FC Monoarthritic Rat (MR)); FC was applied at time zero observing a development of antinociception very similar to the one of FC alone. At time 60 minutes, D-serine (300 µM i.t., black arrow) was injected and the wind-up measured at times 90 and 120 minutes. (Red, FC plus DS (60) Monoarthritic Rat (MR)). $n = 6$, number of rats for each series of experiments. $* = p < 0.05$ according to one way ANOVA.

4 Discussion

The results obtained in this work showed that the analgesic effect of FC i.t. injected into normal rats or those presenting an experimental model of arthritis, can be possibly modulated by the i.t administration of D-serine. These experiments were done using the spinal wind-up as a test for nociception. Electrical stimulation of the leg is a very suita-
assay to quantify the effect of drugs, since it constitutes a demanding test requiring the action of very efficient antinociceptive drugs to achieve a notable or statistically significant effect. This test triggers nociceptive fibers that can be isolated because of his speed, allowing us to have mainly a C-fiber reflex response. The C-fiber response to electrical stimulation provides objective parameters of nociception. This is advantageous compared to behavioral approaches in which there is a subjective assessment of pain-like responses.

There is abundant evidence that glial cells are crucial to produce hyperalgesia/allodynia, which eventually generates the central sensitization phenomena, establishing the pain as a chronic condition and usually resistant to a number of anti-inflammatory drugs and analgesics used as first-line treatment for acute pain (Scholz & Clifford, 2007). Previous reports indicate that the nociceptive response in several animal models of chronic pain is attenuated due to inactivation of the glia or by preventing the action of pro-inflammatory cytokines (Watkins & Maier, 2003). For example, the chronic administration of intrathecal propentofylline reduces mechanical allodynia in a neuropathic pain model using von Frey filaments to assess the nociceptive response to tactile stimuli (Obata et al., 2010). FC has been used to reduce formalin-induced hyperalgesia and mechanical allodynia induced neuropathy produced by sciatic nerve inflammation (Watkins & Maier, 2002). Electro acupuncture has been used also as an antagonizing agent in thermal hyperalgesia and mechanical allodynia induced in the paw by adjuvant induced arthritis. The use of FC has helped to produce a synergistic effect enhancing the analgesia induced by electro acupuncture (Sun et al., 2006). The antinociceptive

Figure 5: Effect of saline, FC and D-serine in the wind-up cord of monoarthritic rats
action of FC can be attributed to the metabolic and functional inhibition of the astrocytes located in the spinal cord. Once the astrocytes are activated under physiopathological conditions such as inflammation, neuropathies or oncological pain, these cells are capable of release into the extracellular media, pro-inflammatory cytokines and other neuromodulators such as ATP. These substances could affect the second order neurons favoring the glutamate pathway mediated by the NMDA receptors. One of these substances is D-serine. D-serine is an endogenous ligand for the glycine site of the NMDA receptor (Mothet et al., 2000) and represents an important new source of modulation of glial brain cells. FC also inhibits the up-regulation of NOS expression, activity and production in the spinal cord, induced by the formalin test in rats (Sun et al., 2009). D-serine in the brain of the rat cerebral cortex is enriched in the rostral hippocampus, anterior olfactory nucleus, striatum and amygdala. In addition, D-serine has been found in the vertebrate retina (Stevens et al., 2003). On the role of D-serine in pain, D-serine has a pronociceptive effect when applied intrathecally. D-serine produces a facilitation of the rat tail flick, and is blocked by co-administration of 7-chlorokynurenic acid (Kolhekar et al., 1994). D-serine levels can be modified by inhibiting the racemization of L-serine by the serine racemase inhibitors, L-Serine-O-Sulfate (LSOS), and L-Erythro-3-Hydroxyaspartate. Both compounds injected intrathecally decreased the wind-up in normal and monoarthritic rats. Interestingly, the antinociceptive effect was abolished when 300 µg/10 µL was injected intrathecally (Laurido et al., 2012). On the other hand, in dynamic mechanical allodynia (DMA) (from rat chronic infraorbital nerve constriction), the astrocytes play a role in the synthesis of D-serine, since the administration of LSOS decreased pain behavior in allodynic rats. Administration of FC alleviated DMA, indicating a role of astrocytes since this compound block astrocyte metabolism, (Dieb & Hafidi, 2013) situation similar to the results found in this work for normal and monoarthritic rats.

5 Conclusions

These results may have applications in the knowledge of the mechanisms involved in the maintenance of chronic pain, in conditions were chronic pain is poorly controlled by drugs currently in use. In conclusion, the prevention of spinal glial activation by FC may not only alleviate the early symptoms of a disease, but also prevent, for example, the development of opioid dependence, increasing the possibility of maintaining prolonged pain relief.

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References


