VALPROATE ANTINOCICEPTIVE AND ANTI-INFLAMATORY EFFECT IN FEMALE RODENTS

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Abstract:

Valproate, an indirect γ-aminobutiric acid agonist has been successfully used in various painful conditions. Despite its frequent use, limited pre-clinical and clinical data exist about its analgesic effect. We tested valproate p.o. in increasing doses (10, 25, 50, 100 mg/kg) in acute nociception models in mice (writhing test and formalin licking-paw test) and in acute primary thermal hyperalgesia induced by carrageenan in rats, comparing its effects to a non-treated control and morphine (5 mg/kg), amitriptyline (10 mg/kg) or indomethacin (10 mg/kg). Valproate showed a statiscally significant ($p<0.001$) dose-response effect in these models, both in male and female mice and in female rats. Antihyperalgesic effect of valproate in the plantar model was not reverted by reserpine pre-treatment. Interestingly, the maximum effect of valproate on this model occurred at a dose of 50mg/kg and the higher dose of 100mg/kg showed a minor effect. Additionally, we demonstrated that valproate has anti-inflammatory effect in the carrageenan-induced oedema model in male and female mice. Valproate antinociceptive and anti-inflammatory effects seem not to be gender-specific in animal models.

Introduction:

Valproic acid (valproate, di-n-propylacetic acid, VPA) is a short chain fatty acid that was used infrequently since its synthesis in 1882 as a "metabolically inert" solvent for organic compounds. In the 60s its effect as an anticonvulsant was discovered serendipitously in France. Since then it has been widely used for the treatment of seizures in adults and children (Cloyd, 2003). Valproate increases the brain concentration of γ-aminobutiric acid (GABA), indirectly acting as an agonist of this inhibitory transmitter. Unlike drugs that act as indirect GABA agonists by increasing GABA availability (eg, tiagabine and vigabatrin), valproate acts through more than one mechanism in providing its broad pharmacological activity: inhibition of

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degradation, increased synthesis and decreased turnover. The increase in glutamic acid decarboxylase (GAD) activity is rapid and temporally correlates with the acute anticonvulsant activity of valproate, though very high doses of valproate are associated with inhibition of GAD and reduce GABA concentrations. Although there are a number of other biochemical actions of valproate, their role in mediating its efficacy is not well established (Owens, 2003).

Anti-epileptic drugs (AED) are frequently used as analgesic in chronic but not acute pain conditions, even though a recent meta-analysis has demonstrated that there is little clinical evidence to support their widespread use (Wiffen, 2005). However, there is an extensive biological and preclinical rationale for their effect in painful diseases (Rogawski, 2004). Valproate is approved in the USA for migraine treatment (Rogawski, 2004) and has been shown to possess analgesic effect in acute migraine in adults and children (Freitag, 2003; Fragoso, 2003; Stillman, 2004; Reiter, 2005; Leniger, 2005). It has been used for more than 20 years for migraine patients and is frequently used by pain specialists for treating neuropathic pain of various etiologies (Freitag, 2003; Sindrup, 2003). Preclinical data and mechanism of action knowledge about its use as analgesic are scarce and inconclusive, even though its basic pharmacology indicates that it could be useful in neuropathic pain (Sindrup, 2003; Frenk, 1988; Loscher, 1985; Cutrer, 1996). Recently, some authors have issued moderate to strong recommendations of using valproate to treat diabetic neuropathy and migraine (Rogawski, 2004; Goodyear-Smith, 2009), whereas others have not cited it in their reviews (Veves, 2008).

In the last years, criticism surfaced about the exclusive use of male animal models when investigating painful syndromes. The prevalence of most common forms of pain is higher among women than men, women report greater pain after invasive procedures, and women display enhanced sensitivity to most forms of experimentally induced pain (with the exception

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of ischemic pain) (Fillingim, 2009). Gender can modify the response to painful stimuli in experimental animals. Once valproate is more often used for painful syndromes such as neuropathic pain and migraine we believe that using females animals as experimental models may lead to more appropriate results.

Because valproate is widely used but preclinical and clinical test data is largely lacking for its analgesic effect we sought to test it in widely used animal models of nociception. We tested it on the writhing test in male and female mice, on formalin test in female mice, and on a thermal hyperalgesia model, the Hargreaves plantar test in female rats (Hargreaves, 1988). In order to evaluate monoamine participation in the antinociceptive effect, we used a general catecholamine block by reserpine pre-treatment. We also wanted to rule out motor impairment or sedation with a rotarod test. Additionally, we evaluated the anti-inflamatory effect of valproate in female rats, comparing with male animals, using the carrageenan-induced paw oedema model.

Material and Methods:

Animals:

Inbred male and female Swiss mice, and female Wistar rats weighing a mean of 200 g in groups of ten animals each were used. Rats were housed in groups up to six per cage in a large colony room on a 12:12-h light/dark cycle (lights on 06:00 h), with food and water provided *ad libitum*. Each animal was used only once. Test sessions were performed between 08:00 and 18:00 h and animals habituated to the laboratory for a week prior to the tests. Food and water were withdrawn for 4 h before drug administrations. Estrous cycle was confirmed in each animal prior to the test performing. All experiments were conducted in accordance with the brazilian regulations of animal care and experimentation covered by CONCEA (National Council for Control of Animal Experimentation) documentations, and were approved by the local

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Institutional Animal Care and Use Committee.

Writhing test: The writhing test was performed acording as described earlier (Fontenele, 1997). Male and female Swiss mice (25-30g) were injected intraperitoneally with 0.6% acetic acid (10ml/kg) and the number of writhings was recorded over a period of 20 min. Animals were treated orally with valproate sodium in increasing doses of 10, 25, 100, and 250 mg/kg, or with morfine 5 mg/kg (positive control) 1h before acetic acid administration. Negative control animals were treated with the diluent of VPA.

Formalin test: Formalin-induced paw-licking test was determined essentially as described by Hunskaar and Hole (Fontenele, 1997). Female Swiss mice (25-30g) were injected by na intraplantar route in the right hindpaw with formalin (1%, 20 µl). The duration of paw licking was measured 0-5 min (first phase) and 20-25 min (second phase) after formalin administration. Animals were treated orally with valproate sodium in increasing doses of 10, 50, and 100 mg/kg 1 h before formalin administration, or with morfine 5 mg/kg (positive control) 30 min before formalin administration. Negative control animals were treated with the diluent of VPA.

Carrageenan-Induced Thermal Hyperalgesia. Plantar test was performed as previously described (Hargreaves, 1988). Briefly, each rat was placed in a Plexiglas cubicle with a glass floor through which an infrared photobeam was shown onto the plantar surface of the hind paws and the latency to withdrawal from the thermal stimulus was determined. The intensity of the infrared photobeam from the plantar reflex device (Plantar Test, Ugo Basile) was adjusted to produce a mean response latency in untreated rats (SHAM) of approximately 10-12 s (mean 11 s). The response latency was determined using a timer linked to the photodiode motion sensors in the plantar reflex device. Response latency was defined as the time from the onset of exposure to the infrared photobeam to the cessation of the photobeam when the photodiode motion sensors

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94 detected the withdrawal response of the paw of the rat. Response to the thermal stimulus was
95 reported as the withdrawal latency differences between the treated and untreated paws in
96 seconds. Groups of 10 rats were injected s.c. with λ-carrageenan (100 µl of a 1.5% solution) into
97 the plantar surface of the right hind paw at time zero followed immediately by an p.o. dose of
98 vehicle or a dose of drug, 120 min before plantar test. The drugs used were valproate sodium in
99 increasing doses of 10, 25, 50, and 100 mg/kg; amitriptyline 10 mg/kg and indomethacin 10
100 mg/kg. Two groups were pre-treated with reserpine (Sigma), 5.0 mg/kg, dissolved in 0.5 ml of
101 glacial acetic acid and 9.5 ml of saline and administered i.p. (0.2ml/animal), 6 h before plantar
102 test. Then, reserpine-treated animals were treated with valproate sodium in a single p.o. dose of
103 50 mg/kg, or vehicle. Doses were selected from usual dosing in published literature and from our
104 initial experiments with valproate (data not reported). Oral doses of drugs were given through a
105 metal rodent feeding tube in a volume of less than 1.0ml, after fasting as described.

106 **Carrageenan-induced paw oedema.** Valproate was administered orally (10, 50 and 100
107 mg/kg) to mice, 60 min before intraplantar injection of 0.1 mL 1% carrageenan solution in the
108 right hind paw. Indomethacin (2 mg/kg , p.o.) was used as a reference drug. Inflammatory
109 oedema was evaluated by the measurement of the hind paw swelling induced by the injection of
110 carrageenan using a plethysmometer (Ugo Basile, Italy). The hind paw was submerged to the
111 tibiotarsal joint into the liquid-filled cell of the instrument. The volume of the displacement,
112 which is equal to the paw volume, was then read on a digital display. The oedema (µL) was
113 defined as the difference between the paw volume before and 1, 2, 3, 4 and 24 h after the
114 carrageenan administration (Fontenele, 2009).

115 **Rotarod Test.** The effects of valproate on motor performance were evaluated using a Rotarod.
116 All animals were given 3 initial training trials of 120 s, approximately 10 min apart, to maintain

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posture on a Rotarod (model 7650; Ugo Basile, Comerio, Italy), 3 cm in diameter, and rotating at a constant 12 revolutions/ min. The day after the initial training trials, a 60 s test trial was conducted, 2 hours after administration of valproate sodium in a single p.o. dose of 50 mg/kg. The number of falls of each animal was recorded.

We used the number of animals per group necessary to obtain alpha = 0.05 (or less) with beta = 0.8 for an effect size between groups of at least 0.5 (calculation performed with R, package pwr). The results between all groups were compared using one-way analysis of variance, with Tukey's multiple comparison as a post hoc test if significant ($p < 0.05$). Anderson-Darling test for the composite hypothesis of normality was performed. Rotarod results were compared with Fischer's exact test of independence. The statistical packages used were GraphPad Prism 5.0 (La Jolla, CA – USA) and R 2.X (R Foundation for Statistical Computing, Vienna, Austria).

Results:

Valproate reduced dose-dependently the writhing number in both male and female Swiss mice. In female animals, all VPA doses reduced the writhing number in a statistically significant way comparing to negative control. The maximum dose of VPA showed an effect size comparable with that of morphine (Fig. 1). Valproate reduced dose-dependently the licking time both in the first and the second phases of formalin test in female Swiss mice. Again, the maximum dose of VPA showed an effect size comparable with that of morphine in the formalin test (Fig. 2).

While the heat-induced reaction time before carrageenan treatment varied between 10-12 seconds, the reaction time for controls treated with carrageenan alone was 6.0 +/- 1.6 s (mean +/- SD). Valproate showed an acute dose-response effect in the plantar test. Following valproate

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administration in carrageenan-treated animals, reaction times were 9.5 ± 3.0 (10 mg/kg), 11.4 ± 4.8 (25 mg/kg), 14.9 ± 4.8 (50 mg/kg) and 9.6 ± 3.3 (100 mg/kg). Valproate produced a significant prolongation of reactions times at the dose of 25 mg/kg and 50 mg/kg (Fig. 3). The maximum effect of valproate was elicited with the 50 mg/kg dose while the 100 mg/kg dose showed an effect with a magnitude comparable to the 10 mg/kg dose effect. We did not test any further dose, because we were concerned with possible acute toxicity complicating the results. Amitriptyline 10 mg/kg had a significant effect on plantar test, prolonging reaction time to 11.1 ± 4.7 seconds ($p < 0.05$ compared to control). In the same way indomethacin 10 mg/kg showed an effect on plantar test, prolonging reaction time to 12.7 ± 5.9 seconds ($p < 0.01$ compared to control). Pre-treatment with reserpine 5 mg/kg 6h before plantar test did not modify the effect of valproate (Fig. 4).

Valproate equally reduced dose-dependently the carrageenan-induced paw oedema both in male and in female rats. In rotarod test, all animals treated with valproate or carrageenan alone were able to complete the Rotarod test without falls.

Discussion and Conclusion:

Our results confirm that valproate has analgesic potential in the writhing test and in the formalin-induced licking paw test, and showed an anti-hyperalgesic effect in the plantar test model, seemingly as potent as a high dose of amitriptyline or indomethacin. This action seems not to be dependent on central nervous system effects, like sedation or motor skill impairment, once animals did not show any alteration in rotarod test. The analgesic effect of valproate was not reverted by pre-treatment with reserpine, which may indicate that it does not depend on monoamines. Since valproate induced marked effect in reserpine pre-treated animals, one can rule out the confounding factor of regulation of cutaneous vasoconstrictor tone and skin

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163 temperature by the sympathetic nervous system. Interestingly, there was a bimodal dose-
164 response relation of valproate in the plantar model. This was not demonstrated in the writhing
165 test or in the formalin-induced licking paw test. Additionally, valproate demonstrated anti-
166 inflamatory effect in the hindpaw oedema model.

167 Gender seemingly did not alter antinociception or anti-inflammatory effects of valproate,
168 as shown by writhing test and hindpaw oedema test. Despite interspecies differences in
169 pharmacokinetics and pharmacodynamics of valproate, it is rapidly absorbed by oral as well as
170 after different routes of administration and yields almost the same brain/plasma ratios in all
171 tested species (Loscher, 1999). Because of this characteristic of valproate, we preferred oral
172 dosing and not the more conventional parenteral route. Half-life of valproate in rats is 2-5h
173 (Loscher, 1999) and that prompted us to choose the amount of time between administration and
174 plantar test.

175 Analgesic effect of valproate has been observed for decades in clinical practice, and it is
176 widely used by pain specialists and others to treat different painful syndromes. Reports of its
177 clinical usage as analgesic in neuropathic pain are almost contemporaneous to its introduction as
178 anti-epileptic (Raftery, 1979; Carraz, 1967, Barnes, 1975). However, evidence supporting
179 analgesic effect by valproate is still scarce. Preclinical and clinical reports have shown since the
180 80's that valproate had possible efficacy in pain patients due to its effect on GAD and GABA
181 (Loscher, 1985; Hitchcock, 1982; Swerdlow, 1981). There has been no definitive progress in the
182 elucidation of the exact mechanisms of action of valproate in pain states, and it seems that
183 GABAergic theory is the only accepted paradigm, in the same fashion as with its anticonvulsant
184 action (Loscher, 1999; Balfour, 1994). Few randomized controlled clinical trials have been
185 performed testing valproate as analgesic, most of them for migraine (Jensen, 1994). A trial of

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valproate in central pain secondary to spinal trauma reported no analgesic effect (Drewes, 1994). A group has published results of a double blind, randomized trial showing that valproate was effective in diabetic neuropathy patients (Kochar, 2002). However, their results were criticized because of methodological issues (Sindrup, 2003). Recently, the analgesic effect of valproate has been questioned in the face of preclinical and clinical data (Otto, 2004; Munro, 2007).

Munro et al have found no effect of i.p. valproate (10-100mg/kg, single dose immediately before stimulus) in male rats, using formalin test (Munro, 2007). Nevertheless, Czuczwar et al reported effect of valproate in the same paradigm, but in female mice (Czuczwar, 2001). They reported an ID50 of 102.5 mg/kg (72.7-145.5). Moreover, they were able to show a significant impairment of motor coordination in mice using the chimney test, with an ID50 3-4 times that of formalin test. In a model of radiant heat-induced pain in mice, Aley & Kulkarni found no anti-nociception of acute treatment with valproate (Aley, 1989). By contrast, Abulaban *et al* reported anti-nociceptive effect of chronic orally administered valproate (administered in drinking water in incremental doses ranging from 679 to 1,456 mg/kg/day for up to 21 days) in mice using heat-induced pain (hotplate) (Abulaban, 1997). An associated group had found evidence of the antinociceptive and anti-inflammatory effect of valproate in male rodents (Ximenes, 2013). It seems that valproate analgesic effect may be dependent on the dose, route of administration, gender and animal model or the methodology used for testing it. We did not find a gender-specific difference in the models we tested, arguing if gender is indeed a factor to be considered in valproate anti-nociception.

Our results could indicate, additionally, that valproate effect can have an inverted u-shaped dose-response relationship, at least in the plantar test of thermal hyperalgesia. This knowledge could have implications for the interpretation of clinical trials in human, as well. We

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did not explore the possible mechanism of this bimodal effect, but as far as GABAergic mechanism of valproate is thought to be important for its effect, one could hypothesize a dose differential effect of valproate analogous to that on GAD. Other possibility could stem from the recently described effect of valproate in inhibiting hystone deacetylase related gene silencing, thus enhancing the expression of cell proteins (Hoffmann, 2008). The epigenetic effect of valproate can be observed in low as well as in higher doses and it may modify the expression of different sets of genes in variable cell types at different dose ranges.

We conclude that valproate, a drug clinically used for treatment of neuropathic pain and migraine almost since its initial use as anticonvulsant, showed a gender non-specific antinociceptive and anti-inflammatory effect in rodents and a bimodal analgesic effect in the plantar test thermal hyperalgesia model in female rats. It should be additionally tested in a greater number of pre-clinical models and in clinical trials with patients that suffer from chronic pain in order to assure its analgesic effectiveness and mechanism of action.

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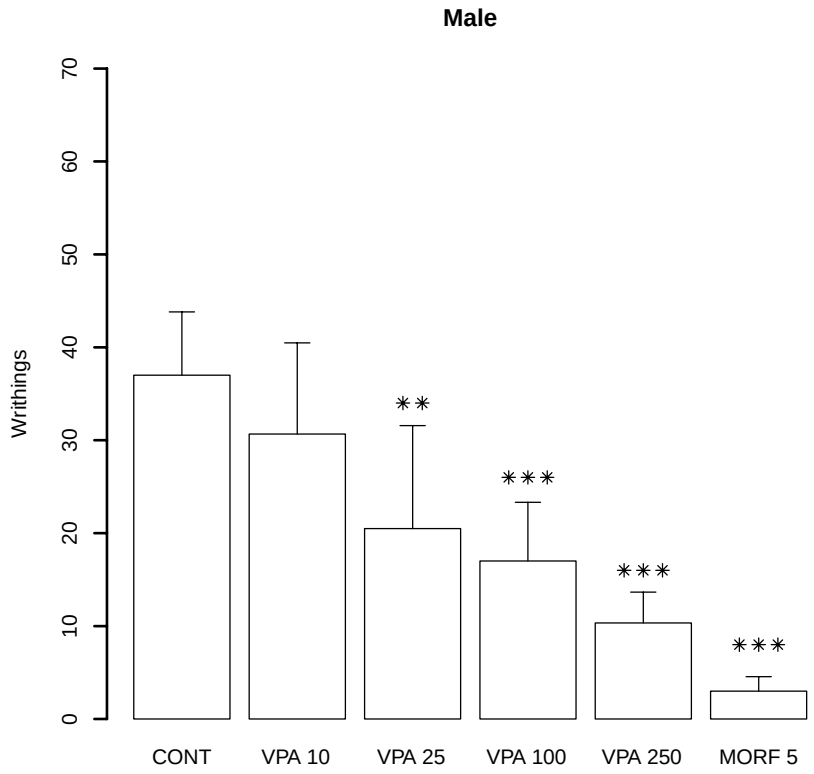

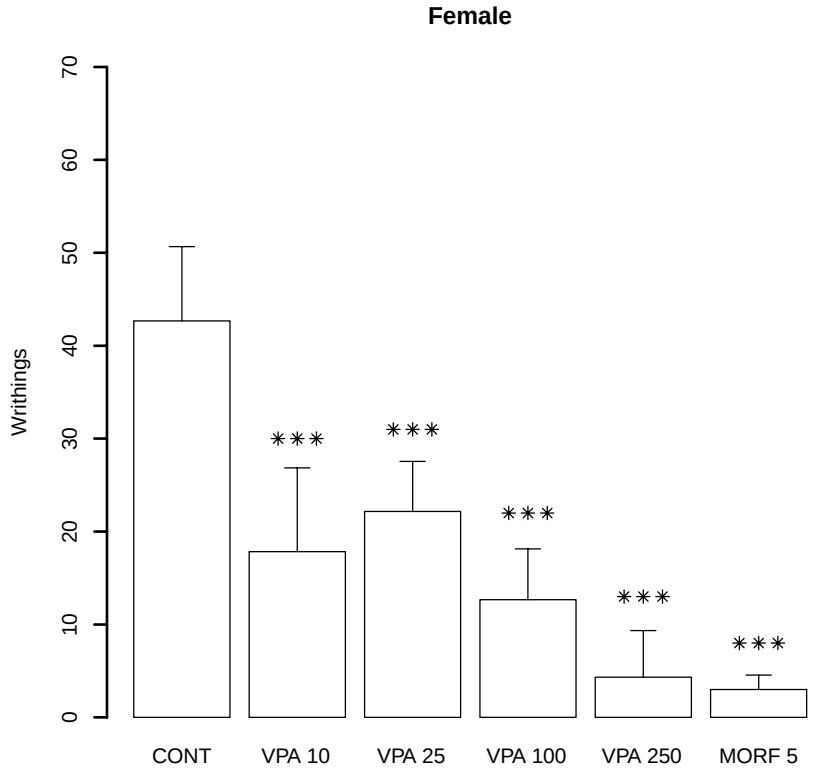

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Figure 1: Valproate reversal of acetic acid-induced nociception. The writhing test was performed as described in methods. Male and female Swiss mice (25-30g) were injected intraperitoneally with 0.6% acetic acid (10ml/kg) and the number of writhings was recorded over a period of 20 min. Animals were treated orally with valproate sodium in increasing doses of 10, 25, 100, and 250 mg/kg, or with morfine 5 mg/kg (positive control) 1h before acetic acid administration. Negative control animals were treated with the diluent of VPA. Number of writhings (mean ± SD) was plotted. Statistical significance is depicted when present (One-way ANOVA with Tukey as post-hoc test).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

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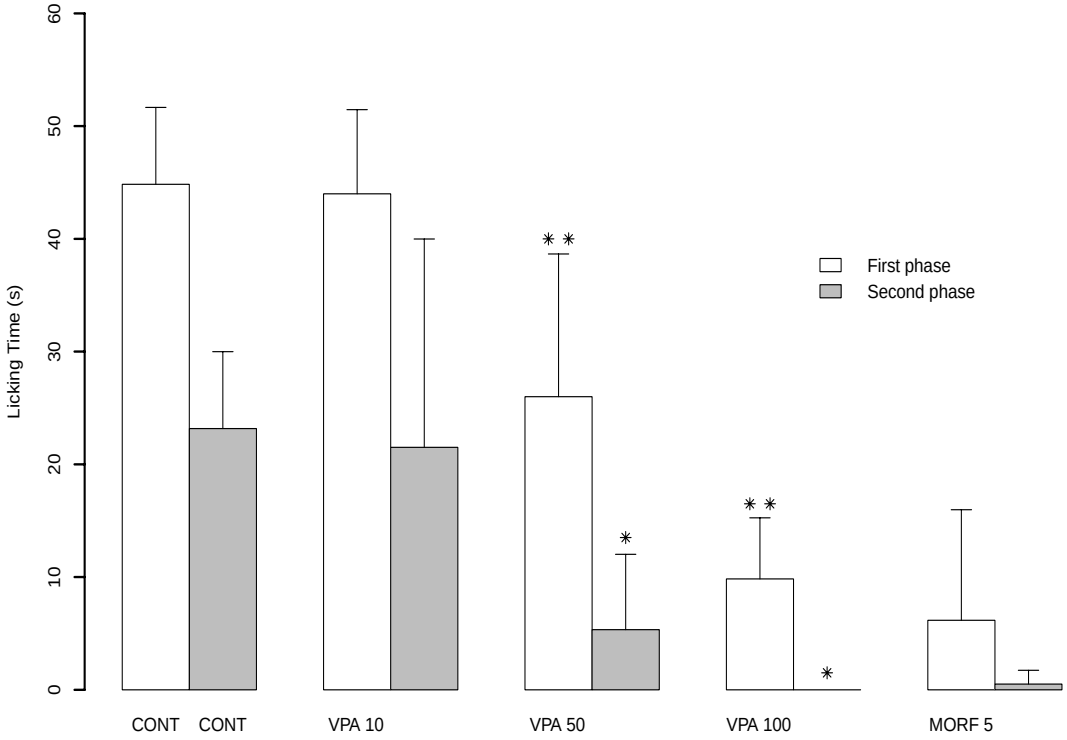

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Figure 2: Valproate reversal of formalin-induced nociception. Formalin-induced paw-licking test was determined as described in methods. Female Swiss mice (25-30g) were injected by na intraplantar route in the right hindpaw with formalin (1%, 20 µl). The duration of paw licking was measured 0-5 min (first phase) and 20-25 min (second phase) after formalin administration. Animals were treated orally with valproate sodium in increasing doses of 10, 50, and 100 mg/kg 1 h before formalin administration, or with morfine 5 mg/kg (positive control) 30 min before formalin administration. Negative control animals were treated with the diluent of VPA. Lickng time (mean ± SD) was plotted. Statistical significance is depicted when present (One-way ANOVA with Tukey as post-hoc test).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

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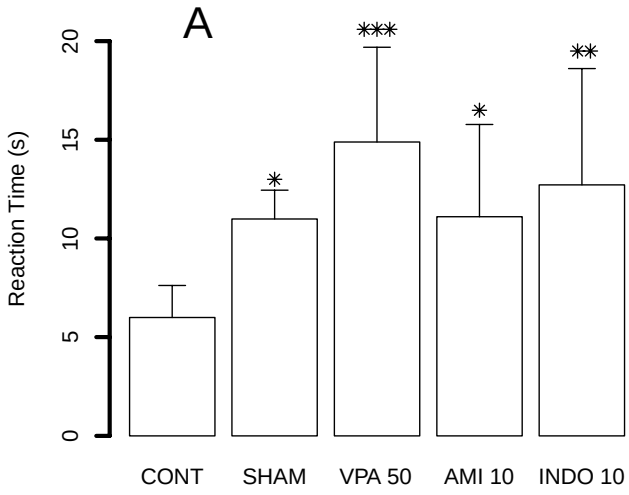

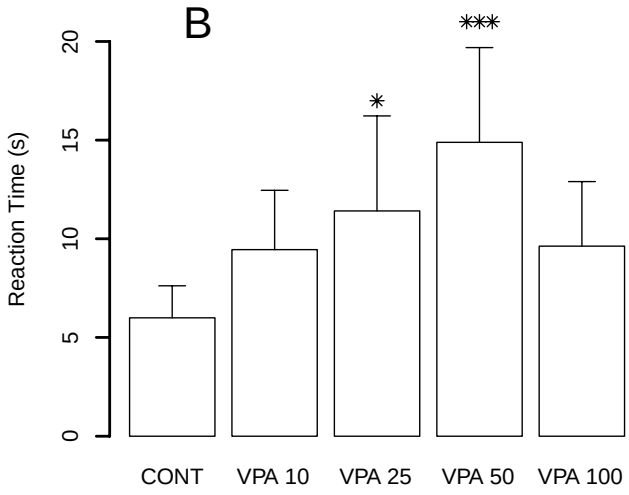

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Figure 3: Valproate reversal of carrageenan-induced acute thermal hyperalgesia. Plantar test was performed as described in methods. SHAM group (SHAM) had no pharmacological treatment. Control group had a 1.0 mg carrageenan plantar injection in right hindpaw 2 h before the test. Treatment groups (VPA 10, VPA 25, VPA 50, VPA 100, AMI 10, and INDO 10) were treated with valproate 10 to 100 mg/kg (B), amitriptyline 10 mg/kg or indomethacin 10 mg/kg (A) at the same time of carrageenan injection. Reaction time to thermal stimulus (mean ± SD) was plotted. Statistical significance is depicted when present (One-way ANOVA with Tukey as post-hoc test).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

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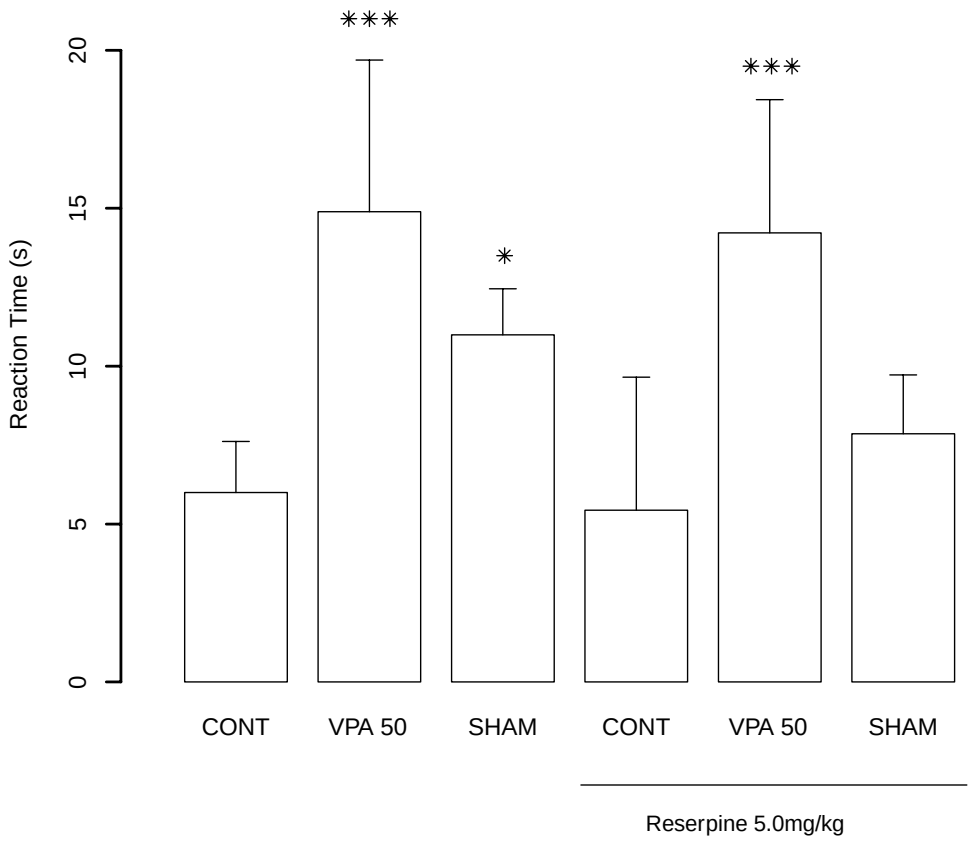

Figure 4: Reserpine did not modify the effect of valproate in the plantar test. Plantar test was performed as described in methods. Animals were pre-treated with reserpine (Sigma), 5.0 mg/kg, administered i.p. (0.2 ml/animal), 6 h before plantar test. Treatment groups (VPA 50)

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were treated with valproate 50 mg/kg at the same time of carrageenan injection. Reaction time to thermal stimulus (mean ± SD) was plotted. Statistical significance is depicted when present (One-way ANOVA with Tukey as post-hoc test).

* $p < 0.05$

*** $p < 0.001$

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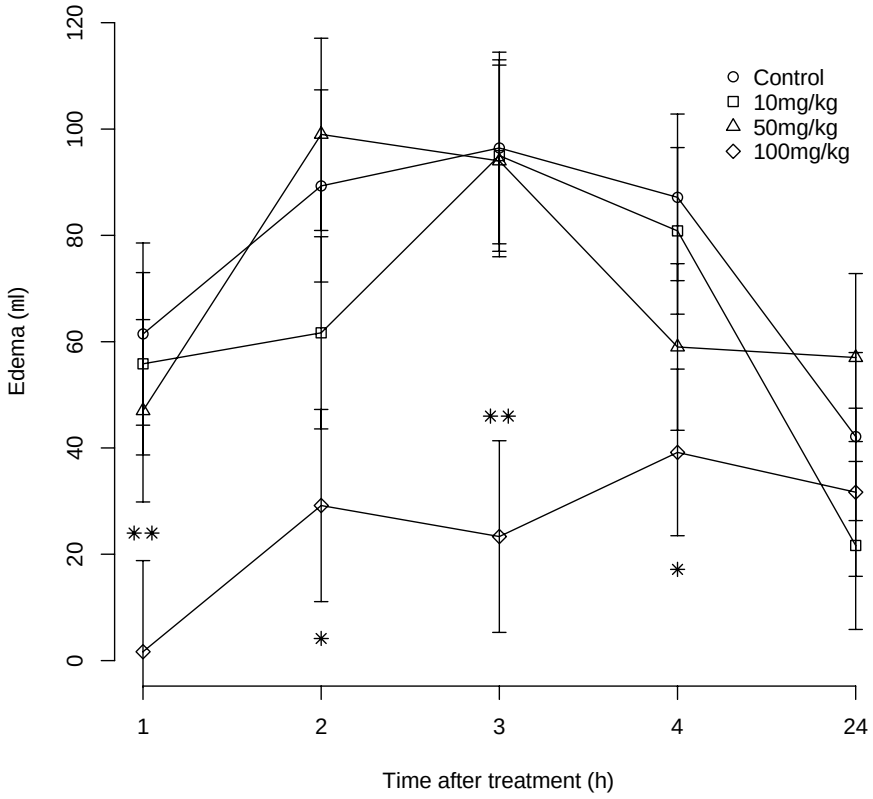
Male mice



Female mice

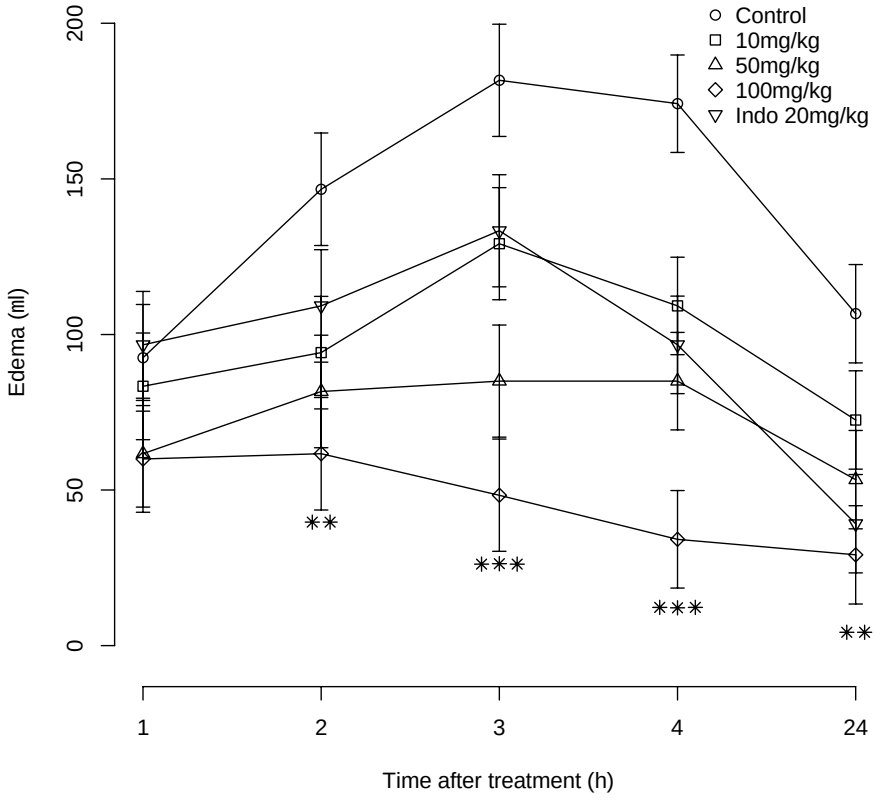


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Figure 5: Valproate reversal of carrageenan-induced paw oedema. Valproate was administered orally (10, 50 and 100 mg/kg) to mice, 60 min before intraplantar injection of 0.1 mL 1% carrageenan solution in the right hind paw. Indomethacin (2 mg/kg , p.o.) was used as a reference drug. Inflammatory oedema was evaluated by the measurement of the hind paw swelling induced by the injection of carrageenan using a plethysmometer (Ugo Basile, Italy). The hind paw was submerged to the tibiotarsal joint into the liquid-filled cell of the instrument. The volume of the displacement, which is equal to the paw volume, was then read on a digital display. The oedema (µL) was defined as the difference between the paw volume before and 1, 2, 3, 4 and 24 h after the carrageenan administration and plotted. Statistical significance is depicted when present (One-way ANOVA with Tukey as post-hoc test).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

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