

Peripheral Amitriptyline Suppresses Formalin-Induced Fos Expression in the Rat Spinal Cord

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We examined the effects of systemically, spinally, and peripherally administered amitriptyline on formalin-induced Fos immunoreactivity in the lumbar spinal cord. Formalin (2.5%), injected subcutaneously into the rat hindpaw, increased Fos immunoreactivity in laminae I–II, III–IV, and V–VI of the dorsal L5 spinal cord. Amitriptyline, administered both systemically and spinally before formalin, increased flinching and concurrently decreased biting/licking behaviors, but neither route of administration produced any statistically significant change in Fos immunoreactivity. Amitriptyline

coadministered with the formalin reduced both flinching and biting/licking behaviors, and significantly reduced Fos immunoreactivity, particularly in laminae I–II. These immunohistochemical changes reflect the net behavioral effects observed after the different routes of drug administration. The profile of amitriptyline action after peripheral administration may be of clinical importance because of the potential use of antidepressants as topical analgesics.

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The formalin test is a widely used model of continuing pain involving peripheral inflammation and central sensitization (1,2). Subcutaneous (SC) injection of formalin into the rat hindpaw produces a biphasic pain response that consists of an early, acute phase and a late, tonic phase that is manifested behaviorally as flinching (a brisk raising and shaking) and biting/licking of the affected paw, and these behaviors are robust and readily quantifiable (1,3). The Fos phosphoprotein is a transcription factor that arises from the *fos* protooncogene, an immediate-early gene that is upregulated in spinal dorsal horn neurons in response to noxious stimulation, and its expression reflects neural pathways activated by such stimuli (4). Upregulation of Fos immunoreactivity (Fos-IR) occurs in the superficial layers (laminae I–II) and neck (laminae V–VI) of the dorsal horn, and the pattern of expression is consistent with the central terminations of nociceptive afferents from the hindpaw (5). Fos is upregulated in the L5 region of the spinal cord in response to peripheral formalin injection (5), as well as in other models of noxious stimulation, including topical application of mustard oil (6), noxious heat and

pressure (7), carrageenin injection (8), and peripheral nerve injury (9).

Amitriptyline, a tricyclic antidepressant, is often used in the treatment of various forms of chronic and neuropathic pain (10,11). When examined in the formalin model, amitriptyline produces paradoxical behavioral effects when administered systemically and spinally; it increases flinching behaviors but decreases biting/licking behaviors (12). In contrast, when amitriptyline is administered peripherally with formalin, both behaviors are reduced (13). The latter observation raises the possibility that topical formulations of amitriptyline might be a useful strategy for producing analgesia (13,14).

In this study, we have compared the effects of systemic, spinal, and peripheral administration of amitriptyline on the increase in Fos-IR in the rat lumbar spinal cord after injection of 2.5% formalin into the hindpaw to determine to what extent the various effects of amitriptyline on nociceptive behaviors are reflected in changes in Fos expression.

Methods

Male Sprague-Dawley rats (125–175g) were housed in pairs under a 12:12-h light/dark cycle with water and rat chow available *ad libitum*. The protocol was approved by the University Committee on Laboratory

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Animals. On the day of testing, rats were placed individually in Plexiglas testing boxes and acclimated for 15–20 min. Rats received saline, or amitriptyline dissolved in saline, systemically (15 mg/kg intraperitoneally [IP] 20 min before formalin), spinally (60 μ g intrathecally [IT] 10 min before formalin), or peripherally (300 nmol SC, coinjected with formalin). Systemic injections were administered in a volume of 5 mL/kg. For spinal injections, rats were briefly anesthetized with halothane (1.5%–2%) and injected by lumbar puncture between L4 and L6 in a volume of 20 μ L. A characteristic tail flick signified penetration of the spinal space and was deemed evidence of a successful injection. Formalin 2.5% or the formalin/amitriptyline combination was administered by SC injection into the dorsal surface of the rat hindpaw in a volume of 50 μ L. After injections, animals were returned to testing boxes and flinching and biting/licking behaviors recorded for 60 min. Animals were tested two at a time in 2-min bins for 60 min. Phase 2 behaviors recorded 16–60 min after formalin are reported. Behaviors were compared by using Student's *t*-tests.

Marked Fos expression occurs 1–2 h after injection of formalin (5). At 90 min after the formalin injection, animals were killed with sodium pentobarbital (65 mg/kg IP) and transcardially perfused by using 350 mL of cold rinse (sodium nitrate in 0.5 M phosphate-buffered saline [PBS]), followed by 350 mL of cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer). The spinal cord was excised and post-fixed at 4°C for 48 h, then placed in 20% sucrose in phosphate buffer for 12 h. Tissues were sectioned on a freezing microtome at 40 μ m and placed in PBS. Sections were washed in 0.9% hydrogen peroxide for 30 min followed by two washes (10 min each) of 0.2% Triton-X in PBS and one wash of PBS. Sections were then incubated in sheep polyclonal antibody to *fos* oncoprotein (Genosys Biotechnologies, Woodlands, TX) in 2% rabbit serum in 1% Triton-X in PBS for 12 h at room temperature. After three washes in PBS (10 min each), sections were incubated in biotinylated rabbit antisheep immunoglobulin G (1:500; Vector, Burlingame, CA) in 2% rabbit serum for 90 min. Sections were then washed three times in PBS (10 min each) and incubated for 90 min in avidin-biotin horseradish peroxidase complex (Vectastain ABC-Elite; Vector, Burlington, ON). A final wash was followed by a 2-min incubation in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) before addition of 0.9% hydrogen peroxide to begin the visualization reaction. The reaction was terminated with three washes in PBS; the sections were stored at 4°C and then mounted on gelatin-subbed slides and air-dried for 24 h. Slides were dehydrated in an ascending alcohol series and defatted in xylene. They were coverslipped with Entellan[®] coverslipping medium

(Merck, Darmstadt, Germany) and dried in a fume-hood for 48 h.

Coverslipped sections were qualitatively assessed with standard microscopy, and representative sections were captured for quantitative analysis by using Adobe Photoshop (version 5; San Jose, CA). The dorsal horn region of each section was divided into laminae I–II, III–IV, and V–VI. Three sections per animal were selected for the L5 level, and counts were performed on each laminar region by using NIH Image software (version 1.61). Counts for drug-treated animals were compared with respective saline controls by using Student's *t*-tests.

Results

Formalin (2.5%) injected into the dorsal surface of the rat hindpaw produces characteristic flinching and biting/licking behaviors 16–60 min after injection (Phase 2). The systemic administration of amitriptyline, at a dose of 15 mg/kg, produced an increase in flinching behaviors (Fig. 1A) and a concurrent decrease in biting/licking behaviors (Fig. 1B). Similarly, a 60- μ g dose of amitriptyline administered spinally increased flinching (Fig. 1A) and decreased biting/licking behaviors (Fig. 1B). In contrast, 300 nmol of amitriptyline coadministered with formalin decreased both flinching (Fig. 1A) and biting/licking behaviors (Fig. 1B).

Injection of formalin into the dorsal surface of the hindpaw in saline-pretreated rats resulted in marked expression of Fos-IR in the ipsilateral dorsal horn of the spinal cord, particularly at the L5 level (Fig. 2, A and C). Figure 2E represents coadministration of saline with formalin and thus represents rats that received formalin alone. These panels indicate that expression of Fos-IR was concentrated in the dorsomedial aspect of the dorsal horn, primarily in laminae I–II and with some staining in laminae III–IV and laminae V–VI on the side ipsilateral to the formalin injection. There was no expression of Fos-IR in the contralateral spinal cord or in the ventral spinal cord (data not shown).

Neither 15 mg/kg amitriptyline administered systemically (Figs. 2B and 3A) nor 60 μ g amitriptyline administered spinally (Figs. 2D and 3B) produced any statistically significant change in the amount of Fos-IR expressed in the L5 dorsal horn compared with animals pretreated with saline. In contrast, 300 nmol of amitriptyline administered peripherally with formalin resulted in a marked and significant reduction in the amount of Fos-IR in laminae I–II compared with rats who received formalin alone (Figs. 2F and 3C). In the latter case, there was also a significant reduction in Fos-IR in laminae V–VI in amitriptyline-treated animals, but this reduction was slight compared with that observed in laminae I–II. No effect was observed in laminae III–VI.

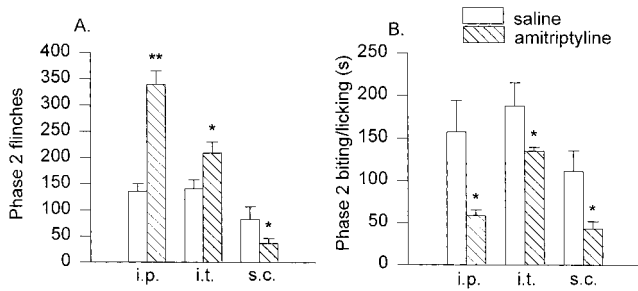


Figure 1. Effect of amitriptyline administered intraperitoneally (i.p.), intrathecally by lumbar puncture (i.t.), or peripherally by coinjection with formalin (subcutaneously [s.c.]) on (A) flinching and (B) biting/licking behaviors in Phase 2 (16–60 min) after injection of 2.5% formalin. The systemic injection was administered 20 min before formalin, the spinal injection was administered 10 min before formalin, and the local administration was coinjected with formalin. Data are presented as mean \pm SEM ($n = 6$ per group). * $P < 0.05$, ** $P < 0.01$ compared with corresponding saline-treated groups.

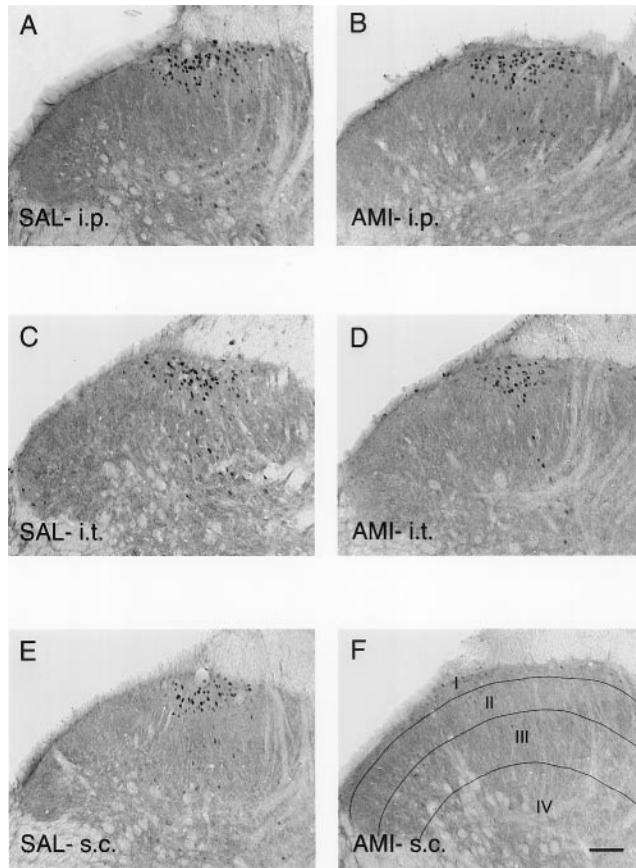


Figure 2. Representative sections showing Fos immunoreactivity induced by 2.5% formalin in the dorsal quadrant of the L5 spinal cord after amitriptyline administered by different routes. Saline (SAL) or amitriptyline (AMI) was injected intraperitoneally (i.p.) (A, B), intrathecally (i.t.) by lumbar puncture (C, D), or subcutaneously (s.c.) into the dorsal hindpaw (i.e., coinjected) (E, F). Bar = 50 μ m.

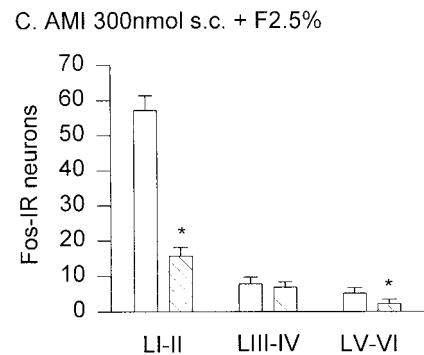
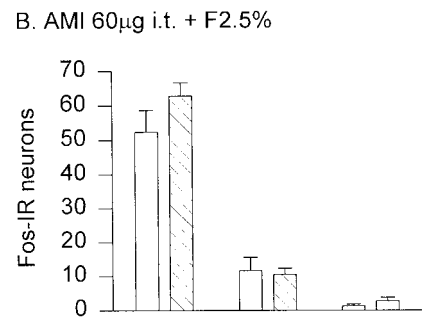
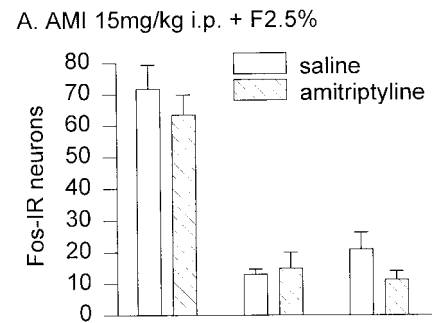


Figure 3. Fos immunoreactivity (Fos-IR) induced by 2.5% formalin (F) in the L5 region of the dorsal horn of the spinal cord after amitriptyline (AMI) treatment. (A) Saline or 15 mg/kg amitriptyline administered intraperitoneally (i.p.) 20 min before formalin, (B) saline or 60 μ g of amitriptyline administered intrathecally (i.t.) by lumbar puncture 10 min before formalin. In (C), formalin was delivered alone or coadministered with 300 nmol of amitriptyline subcutaneously (s.c.) into the paw. The dorsal horn was divided into laminae I–II, III–IV, and V–VI for quantitation of Fos-IR. Data are presented as mean \pm SEM ($n = 4$ per group, three sections counted per animal). * $P < 0.05$ compared with the saline-pretreated group (A, B) or formalin alone (C).

Discussion

This study was designed primarily to compare the effects of amitriptyline given by three different routes of administration (IP, IT, or SC) on the expression of Fos-IR in the spinal cord induced by injection of 2.5% formalin into the hindpaw. Behavioral effects of amitriptyline given by these three routes had previously been reported (12,13), and those effects were essentially reproduced here. Thus, both IP and IT amitriptyline increased flinching and decreased biting/licking behaviors, whereas

amitriptyline coadministered SC with the formalin suppressed both behaviors. Fos-IR after formalin was primarily observed in the ipsilateral dorsal horn of the spinal cord, as noted previously (5), and this was most prominent in the superficial layers of the dorsal horn of the spinal cord, which corresponds to the main area of termination of nociceptive primary afferent neurons (15). Neither IP nor IT amitriptyline altered the expression of Fos-IR in the dorsal horn of the spinal cord. In contrast, peripherally administered amitriptyline substantially decreased Fos-IR, particularly in laminae I-II. The immunohistochemical changes and presumed pattern of neural activation seem to directly reflect the behavioral profile observed with each route of administration. Thus, the outcome after systemic and spinal administration indicates a lack of net effect, as might be anticipated from a mix of augmented and suppressed behaviors. Peripherally-administered amitriptyline was the only route of administration found to decrease both behaviors and the expression of Fos-IR. This finding indicates that multiple behaviors contribute to neural activity and expression of Fos-IR and that suppression of a single behavior may be insufficient to alter such expression. It also supports the recommendation that multiple behaviors be determined in assessing the analgesic profile of drugs after the administration of formalin (16).

In a clinical context, amitriptyline is currently given exclusively orally (10,11). Although spinal application exhibits analgesia in animal studies (12,17,18), concern has been expressed regarding potential toxic effects of spinally-administered amitriptyline (19). Analgesic properties after local peripheral administration of amitriptyline have been reported relatively recently in pre-clinical studies (13,20), and these observations have led to a consideration of the potential for administering amitriptyline as a topical analgesic in a clinical context (13,14,20). It is interesting to note that the topical application of another tricyclic antidepressant, doxepin, was recently reported to produce analgesia in humans with neuropathic pain (21,22). This study indicates that in conditions in which there is a clear peripheral afferent drive involved, the topical route might be very useful because of larger local drug concentrations, a more complete suppression of nociceptive behaviors and circuitry activated by nociceptive stimulation, and, potentially, fewer adverse effects, which can limit the effectiveness of oral preparations.

Amitriptyline is a complex drug that exhibits multiple pharmacological actions, and a number of these are believed to contribute to its analgesic activity after systemic and spinal administration (14,18,23). These include block of biogenic amine reuptake, block of *N*-methyl-*D*-aspartate receptors, interactions with opioid systems, inhibition of ion channels, and block of adenosine uptake. A number of these mechanisms may also contribute to peripheral analgesia, albeit with a different balance of contributions (14). The lack

of similarity of outcome with systemic and peripheral administration in this study, both behaviorally and immunohistochemically, argues against a prominent peripheral component of action when amitriptyline is administered systemically. Such an action is also unlikely to be caused by dose considerations, because local tissue levels after systemic administration are unlikely to attain the levels that occur after local peripheral administration.

Fos is regarded as a marker of neuronal activation after noxious stimulation (4-7) and acts to regulate the transcription of a number of genes, such as proenkephalin and prodynorphin, whose protein derivatives can produce analgesia in inflammatory pain states (4). A number of systemically-administered drugs, such as morphine (5), α_2 -adrenergic agonists (24), and adenosine agonists (25), decrease Fos-IR in the spinal cord after formalin injection. Locally-administered capsaicin (26) and intraplantar lidocaine (27) and MK-801 (28) can also act peripherally to reduce Fos-IR in the spinal cord after noxious stimulation. Such studies indicate a general parallel between behavioral actions and expression of Fos-IR in the spinal cord. This study indicates that a new class of drugs, antidepressants, can be added to those that are able to act peripherally to suppress pain.

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