

γ -Aminobutyric Acid Amides of Nortriptyline and Fluoxetine Display Improved Pain Suppressing Activity

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The GABA amides of the antidepressants nortriptyline and fluoxetine, **1** and **2**, were compared to their respective parent compounds in rodent models of pain. The amides significantly reduced early nociceptive and late inflammatory responses compared to nortriptyline or fluoxetine, where **1** exhibited overall better efficacy than **2**. Amide **1** was most efficacious in lowering cytokine secretion, edema and hyperalgesia induced by formalin and λ -carrageenan, respectively. Thus, **1** is a promising candidate for the treatment of pain.

Introduction

Inadequate management of postoperative and trauma pain, cancer, and chronic noncancer pain is widely prevalent.^{1–4} Pain can be divided into two main categories: acute and chronic. Acute pain is a protective response to tissue injury or inflammation and is most often nociceptive, namely, is caused by or is a response to a tissue damaging stimulus. Chronic pain that may be nociceptive or neuropathic results from neuronal transmission of pain, either peripherally or in the central nervous system (CNS^a). The spinal column and the CNS are modulated by excitatory and inhibitory neurotransmitters. Norepinephrine and serotonin are inhibitors of pain transmission, whereas other neurotransmitters, e.g., glutamate and *N*-methyl-D-aspartate are excitatory.^{1,5,6} Antidepressant and antiepileptic drugs are thought to relieve neuropathic pain through interaction with specific neurotransmitters and ion channels.^{7,8} The tricyclic antidepressants amitriptyline and its metabolite nortriptyline, which show inhibition of norepinephrine and serotonin reuptake, were reported to be efficacious in the treatment of neuropathic and non-neuropathic pain syndromes.^{9–12} Serotonin inhibits the transmission of pain at the spinal cord and midbrain level. The efficacy of tricyclic antidepressants in the treatment of neuropathic pain is evident at lower dosages than those typically used to treat depression and is independent of their antidepressant effects.^{13,14} Experimental models of pain and clinical data

suggest that compounds with greater norepinephrine reuptake inhibitory (NRI) activity versus serotonin reuptake inhibitory (SRI) activity would be more effective in the treatment of pain than compounds with only SRI activity.¹⁵

γ -Aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the CNS, and its deficiency is responsible for many pain states.⁵ Neuropathic lesions of the type that evoke hyperalgesic states are also known to induce a loss of GABAergic inhibition in the spinal dorsal horn.^{16,17} This phenomenon could be explained by a decrease in dorsal horn levels of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) and associated neuronal apoptosis.¹⁸ Gabapentin and pregabalin were initially synthesized to mimic the chemical structure of GABA. However, they were found to act on voltage-gated N-type calcium ion channels in the central nervous system and not on the brain receptors of GABA.¹⁹

On the basis of the association between GABA deficiency and pain events and the described analgesic activity of NRI and SRI antidepressants, we synthesized new chemical entities composed of GABA amides of nortriptyline **1** (BL-1021) and of fluoxetine **2** (BL-1024)²⁰ and tested them in rodent models. The GABA amides of the antidepressants were originally designed on the basis of the analogous GABA ester of the typical antischizophrenic agent perphenazine (BL-1020), which we reported earlier.²¹ This ester was found to behave as a mutual prodrug that upon metabolic hydrolysis in the brain concomitantly releases perphenazine and GABA. In the present case, the GABA derivatives are amides. Although amides are significantly more stable than esters in biofluids, it is difficult to predict whether they will undergo metabolic hydrolysis or be active as the intact molecules. Herein, we describe the chemical and biological studies conducted with the GABA-antidepressant amides.

Results and Discussion

GABA amide of nortriptyline **1** and of fluoxetine **2** were prepared as shown in Scheme 1.

Effect of the Antidepressants Nortriptyline, Fluoxetine, and Their GABA Amides 1 and 2 on Pain Sensation Using the Hot Plate Test. Amides **1** and **2** were evaluated as analgesics in comparison to their parent antidepressants on the hot plate that measures the response to a noninflammatory acute

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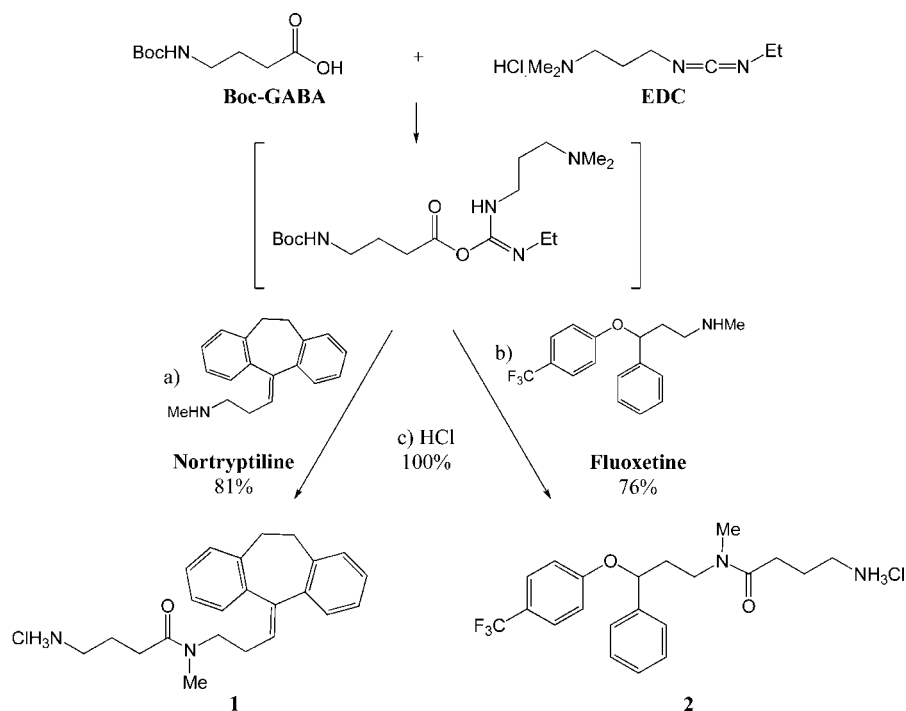
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^a Abbreviations: CNS, central nervous system; DDW, doubly distilled water; GAD, glutamic acid decarboxylase; GABA, γ -aminobutyric acid; NRI, norepinephrine reuptake inhibitory; SRI, serotonin reuptake inhibitory; PMSF, phenylmethylsulfonyl fluoride; eqM, equimolar; TNF- α , tumor necrosis factor- α ; INF- γ , interferon- γ ; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; [³H]CGP, ([*S*-(*R,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl]-[3,4-³H]cyclohexylmethyl)phosphonic acid.

Scheme 1. Synthesis of GABA Amides^a

^a (a) Nortriptyline/Boc-GABA/EDC/Et₃N ratio 1:1.2:1.2:1, CH₂Cl₂, room temp, 48 h; (b) fluoxetine/Boc-GABA/EDC/Et₃N ratio 1:1.2:1.2:1, CH₂Cl₂, room temp, 48 h; (c) 4 N HCl/EtOAc, room temp, 4 h.

nociceptive input used to study central antinociceptive activity.²² The latency of the withdrawal response evoked by exposing mice paws to a thermal stimulus (hot plate set to 52 ± 0.2 °C) determined the antinociceptive reaction. To assess the effect of nortriptyline, fluoxetine, and amides **1** and **2** on pain perception, mice were treated po with these drugs and their respective parent antidepressants. Oral administration of 0.25–4.5 mg/kg nortriptyline and equimolar (eqM) doses of **1** and 7.5–40 mg/kg of fluoxetine and eqM doses of **2** showed that after 240 min the activity of nortriptyline and **1** leveled off at 0.5 mg/kg and that of fluoxetine and **2** at 10 mg/kg and they were attenuated thereafter (Figure 1 A,C). Treatment with 0.25, 0.5, and 4.5 mg/kg nortriptyline or eqM doses of **1** led to a significant antinociceptive activity compared to vehicle treated mice, where compound **1** at 0.5 and 4.5 mg/kg doses imparted a significantly greater efficacy than nortriptyline at the corresponding doses ($p < 0.05$). Treatment with 7.5 mg/kg fluoxetine or an eqM dose of **2** did not impart a significant inhibition on the response time to heat sensation, while treatment with 10–40 mg/kg fluoxetine or eqM doses of **2** exhibited a significant increase in the response time compared to vehicle treated mice. Only 10 mg/kg eqM dose of **2** imparted a significant increase in the latency of the response compared to fluoxetine. At higher doses (eqM doses of 20 or 40 mg/kg fluoxetine) the activity of respective eqM dose **2** was attenuated and was similar to that of fluoxetine (Figure 1C). These results indicate that **1** is an effective antinociceptive agent at low eqM doses of 0.25–4.5 mg/kg while the activity of **2** peaked at 10 mg/kg and subsided at higher doses, demonstrating the advantage of the former amide. A time course study of the response to heat of mice treated po with 0.4 mg/kg nortriptyline, an eqM dose of **1**, 10 mg/kg fluoxetine, or an eqM dose of **2** showed that the response after 2–4 h following amide treatment was delayed significantly compared to that of the corresponding antidepressants or vehicle (Figure 1B,D). After 5 h the analgesic effect subsided.

To evaluate the effect of repeated drug treatment, mice were treated daily, po for 12 days, with nortriptyline (0.2 mg/kg) or an eqM dose of **1** and the effect on heat sensation was determined as indicated (Figure 2A). From the first day, 3–5 h after treatment, the latency time of the response to heat increased significantly in mice treated with nortriptyline (#, $p < 0.05$) or **1** compared to vehicle treated mice. From days 3 to 12, 3–5 h after treatment, **1** imparted a significantly better antinociceptive effect than nortriptyline (*, $p < 0.05$). Moreover, from day 10, even prior to the daily treatment, as well as 3–5 h after treatment, a significantly longer antinociceptive activity was noted in mice treated repeatedly with **1** compared to those treated with nortriptyline (*, $p < 0.05$) or vehicle (#, $p < 0.05$), indicating that repeated administration of **1** prolonged the duration of the analgesic effect (Figure 2A). Repeated dosing with **1** not only imparted longer lasting effects from days 10 to 12 but also significantly increased the latency time of the responses on days 10 and 12 compared to the latency time it induced on days 1–5 (@, $p < 0.05$). The effect of repeated administration of fluoxetine (po 10 mg/kg) or an eqM dose of **2** on days 1–11 was also evaluated. The analgesic effect of **2** 3–5 h after treatment, from day 1, was significantly greater than that of fluoxetine (*, $p < 0.05$). This advantage was maintained throughout the experiment except for one time point, on day 7, 5 h after the daily treatment (Figure 2B). It is noted that the extent of the delay in the response induced by **2** on day 11 prior to treatment was 2-fold longer than that induced on days 1–9 prior to the treatment, indicating a prolonged effect after repeated doses of **2**. A comparison of the latency induced by **1** on days 10 and 12 to that induced by **2** on day 11 shows that **1** induced longer latency period prior and 3–5 h after treatment; therefore, **1** is superior to **2**.

Chronic administration of GABA did not contribute to the antinociceptive or cumulative analgesic effects. Mice treated for 5 consecutive days with three oral doses of GABA (0.05,

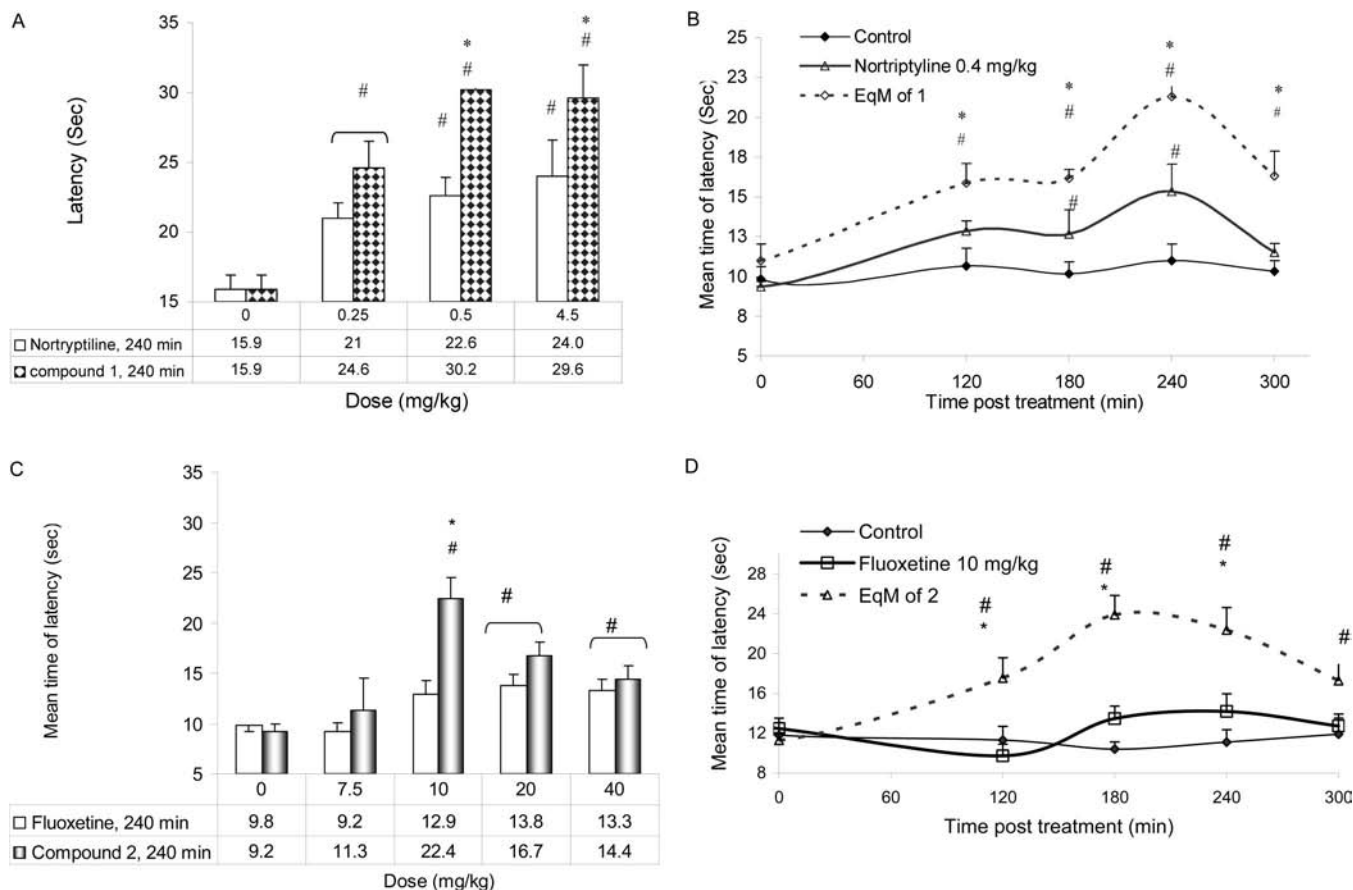


Figure 1. Effect of heat sensation on mice treated with nortriptyline, fluoxetine, and their GABA amides: dose response and time course. The latency of the reaction to heat (in s) using a hot plate set to 52 ± 0.2 °C was examined with male Balb-c mice (6/group). Dose–response of the animals after 240 min of po treatments with vehicle or the indicated doses of nortriptyline and fluoxetine or eqM doses of their respective amide **1** or **2** are shown (A, C). Time course of the heat response of mice treated with 0.4 mg/kg nortriptyline, 10 mg/kg fluoxetine, and eqM doses of their amides was determined (B, D). Results are expressed as the mean \pm SEM: (*) $p < 0.05$, significant vs nortriptyline (A, B) and vs fluoxetine (C, D); (#) $p < 0.05$, significant vs control (vehicle treated).

0.1, or 2.39 mg/kg) that corresponded to or exceeded the relative amounts of GABA in **1** or corresponded to the eqM dose of GABA in the efficacious dose of **2** did not exhibit a change in the response to heat (data not shown).

Effect of Nortriptyline, Fluoxetine, and Amides 1 and 2 in the Formalin Pain Model. A second model acceptable for nociception is the formalin induced pain test, characterized by two phases. The first phase is brief and can be detected immediately after formalin injection (0–5 min). The nociceptive response in this phase results from a direct activation of nociceptors and is similar to that of the hot plate test.²² The second phase can be measured 20–60 min after formalin injection and is attributed mainly to the development of an inflammatory reaction at the injection site as well as to increased synaptic transmission in the spinal cord. Animals treated with formalin exhibited a typical flinching behavior of the injected paw. In comparison to the hot plate test, which is indicative of acute pain, the formalin test can also be used to predict the potential of analgesic agents for the treatment of chronic pain due to inflammation.²³

Balb-c mice were treated 2 h prior to the formalin injection with nortriptyline (0.5 and 5 mg/kg), fluoxetine (10 and 30 mg/kg), and eqM doses of **1** and **2**. The mice in the control group were treated with the vehicle. Mice were then placed in an acrylic observation chamber, and the frequency of pain-related flinching was recorded during the early (0–5 min) and the late (25–35 min) phases following the injection of formalin. In mice

treated with nortriptyline and **1**, pain response was significantly ($p < 0.01$) inhibited in both phases compared to vehicle treated mice. In addition, **1**, in the two treatment doses and phases, was significantly ($p < 0.05$) more efficacious than the corresponding doses of nortriptyline (Figure 3A,B). In mice treated with fluoxetine, a significant reduction in the pain response in the neurogenic phase was observed only at 30 mg/kg compared to vehicle treated ones. However, treatment of mice with 10 and 30 mg/kg eqM doses of **2** imparted a significant reduction of pain responses compared to fluoxetine or vehicle. In the second inflammatory phase, 10 mg/kg fluoxetine and an eqM dose of **2** were equally effective in reducing the response; 30 mg/kg fluoxetine further attenuated it, but an eqM dose of **2** imparted a significantly greater inhibition than fluoxetine (Figure 3C,D). Taken together, these results indicate that **1** is effective at an over 20-fold lower dose than **2** and in both phases it exhibited a superior antinociceptive activity compared to nortriptyline.

Effect of Nortriptyline, Fluoxetine, and Their GABA Amides 1 and 2 on Sedation and Anxiety. Since it is important that analgesics should exert minimal sedation and anxiety, the effect of **1** and **2** on the behavior of mice in the open-field test was evaluated. This test is an acceptable model for psychiatric disorders, involving confrontation of rodents with an anxiety situation by a transfer to a new, large open field.²⁴ In addition to assessing anxiety, the test measures the parameters of motor activity and sedation (immobility). Table 1 shows the effect of

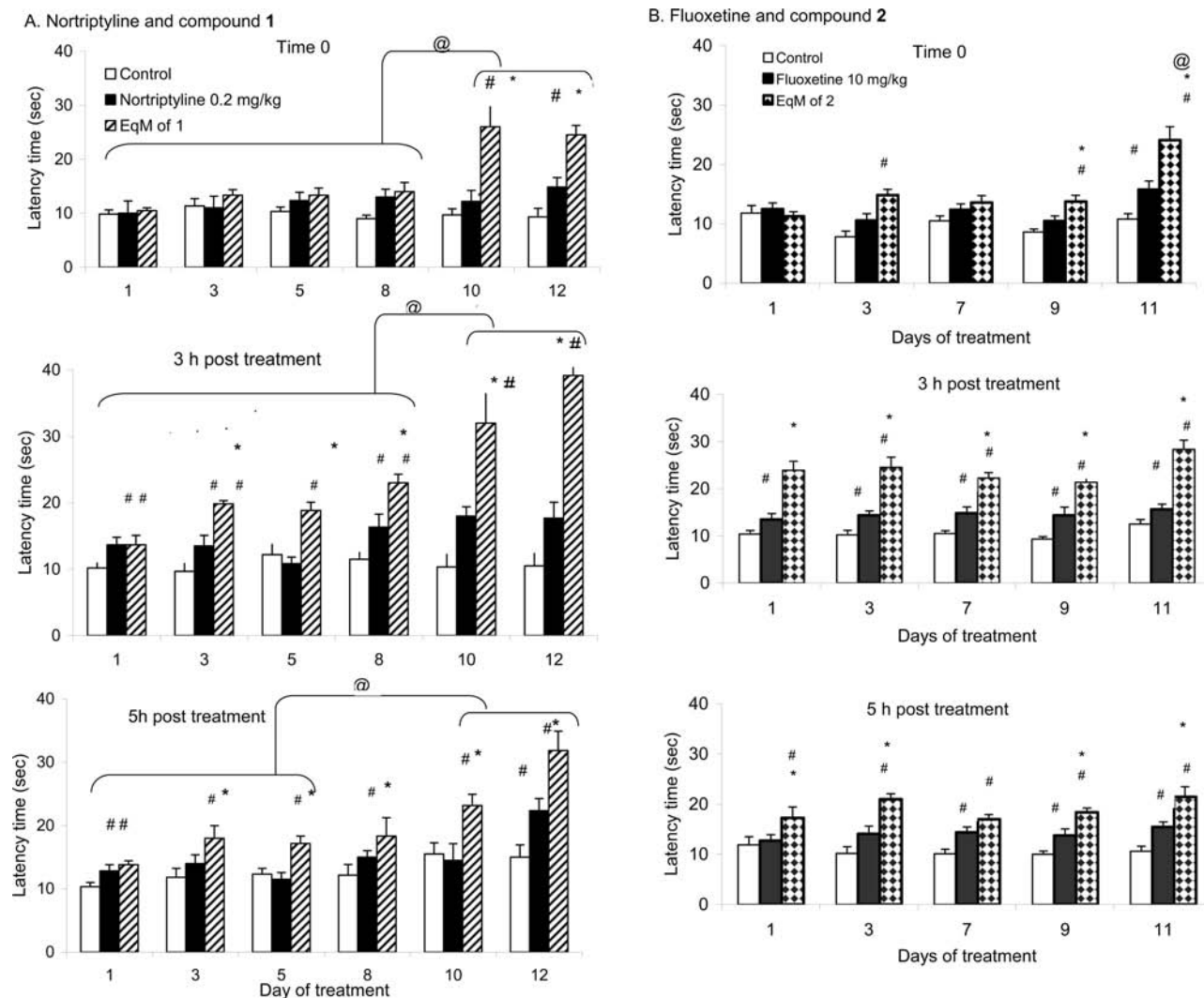


Figure 2. Effect of daily administration of nortriptyline, fluoxetine, and their GABA amides on latency of the nociceptive reaction to heat. Balb-c mice were treated daily with 0.2 mg/kg nortriptyline ($n = 6$), an eqM dose of **1** ($n = 6$), 10 mg/kg fluoxetine ($n = 10$), or an eqM dose of **2** ($n = 10$) at the indicated days of treatment, and their latency of reaction to heat (s) on the hot plate (52 ± 0.2 °C) was measured prior to treatment (time 0) and 3 and 5 h after treatment. Results are expressed as the mean \pm SEM: (#) $p < 0.05$ significant vs control; (*) significant vs eqM nortriptyline or fluoxetine; (@) $p < 0.05$ significant vs previous days.

nortriptyline (1, 5, and 15 mg/kg), fluoxetine (5, 10, and 20 mg/kg) and eqM doses of **1** and **2** on distance moved, velocity, and sedation in the open field on mice treated po with the drugs compared to those treated with vehicle. Nortriptyline at 1 and 5 mg/kg doses did not modify significantly the distance moved or velocity, while at 15 mg/kg a significant decrease was found in both parameters ($p < 0.05$ vs controls). At the same time, **1** at 15 mg/kg eqM dose did not significantly affect these parameters. At this dose a tendency toward increased immobility (more sedation) compared to control was observed in animals treated with both nortriptyline and **1**; however, the difference did not reach statistical significance. Mice treated with high doses of fluoxetine (20 mg/kg) and an eqM dose of **2** exhibited a significant ($p < 0.01$) reduction in distance moved and velocity. The immobility observed at these doses increased significantly with fluoxetine ($p < 0.05$), and a tendency ($p > 0.05$) to immobility was observed with **2**. These results show that fluoxetine at a high dose (20 mg/kg) significantly reduced distance moved and velocity and increased sedation, whereas an eqM dose of **2** decreased distance moved and velocity without affecting immobility. Compound **1** at 15- to 30-fold higher doses than its effective antinociceptive doses did not affect distance moved, velocity, and immobility, while **2** reduced motor activity

at about 2-fold of its effective pain suppressing dose. Therefore, a greater dose range of **1** can be used without imparting adverse effects, and its higher analgesic activity cannot be attributed to sedation.

Effect of Nortriptyline and Its Amide 1 on Inflammation. Inflammatory cytokines have been widely implicated in both the establishment and the perpetuation of the inflammatory process and neuropathic pain-related behavior in rodents.²⁵ The efficacy of **1** in the second phase of the formalin test led us to investigate its effect on cytokine secretion at the formalin-injection site and its anti-inflammatory activity against λ -carageenan-induced rat paw edema.

The tissue from the formalin injected site was analyzed after 24 h for the level of the proinflammatory cytokines interferon- γ (INF- γ) and tumor necrosis factor- α (TNF- α). Nortriptyline (po, 0.5 mg/kg), an eqM dose of **1**, gabapentin (ip, 50 mg/kg), and a mixture of nortriptyline (po, 0.5 mg/kg) and an eqM po dose of GABA significantly reduced the TNF- α levels compared to those observed in vehicle treated mice. However, **1** imparted a significantly ($p < 0.05$) greater reduction compared to nortriptyline, gabapentin, or the mixture of nortriptyline and GABA (Figure 4A). The levels of INF- γ were significantly (< 0.02) reduced only in mice treated with **1** (Figure 4B). These results

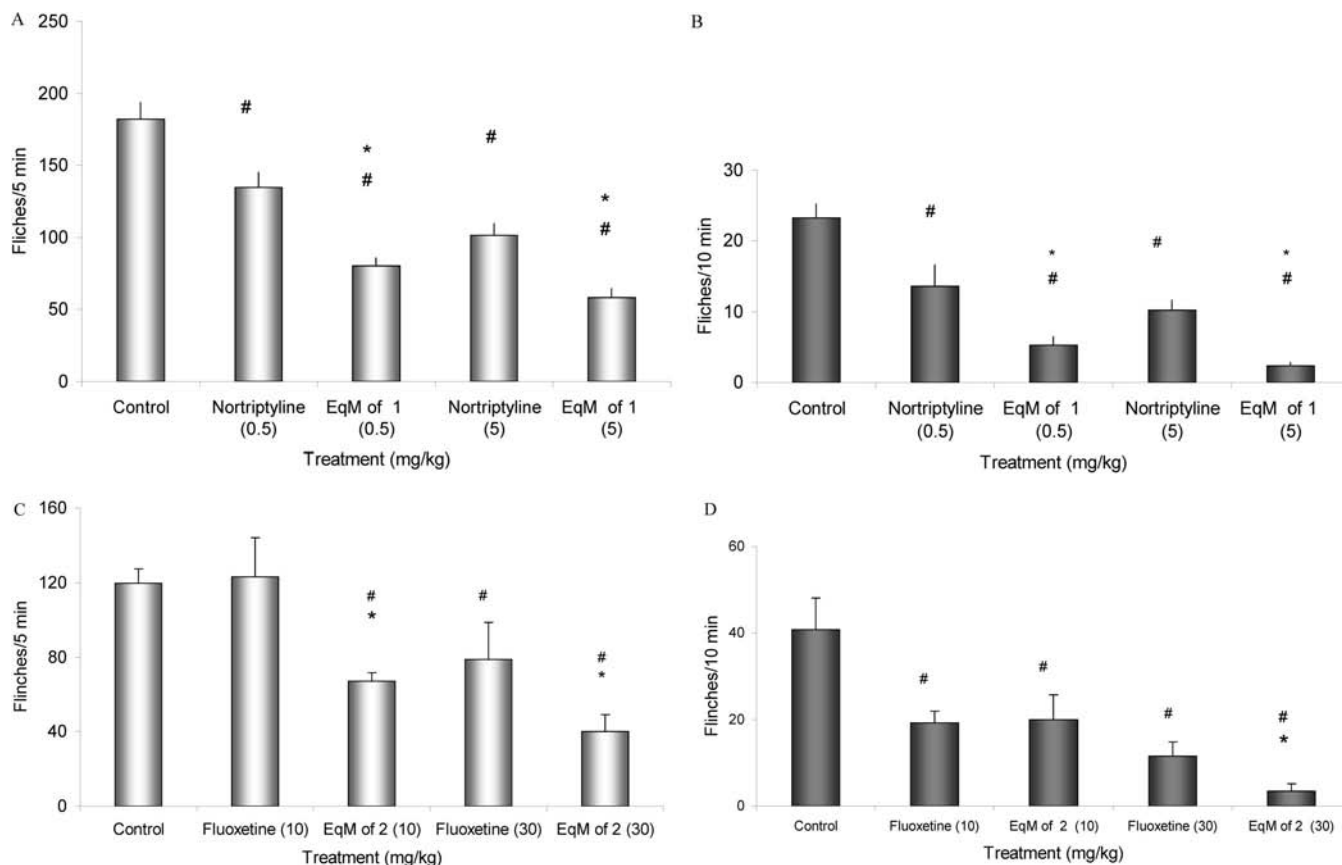


Figure 3. Effect of nortriptyline, fluoxetine, and their amides on neurogenic and inflammatory response to formalin. Balb/c mice (8/group) were treated with control vehicle, 0.5 or 5 mg/kg nortriptyline, and eqM doses of **1** at 2 h prior to 1% formalin (20 μ L) injection to the right hind paw (A, B). Similarly, Balb/c mice (5/group) were treated with control vehicle, fluoxetine 10 or 30 mg/kg, and eqM doses of **2** (C, D). Following the formalin injection, the animals were placed in an acrylic observation chamber, and the number of times that the mice licked, bit, or shook the injected paw was recorded as a quantitative indication of nociception. The early neurogenic phase (nociceptive response) was measured between 0 and 5 min (A, C), and the late phase (B, D) was measured between 25 and 35 min after the formalin injection. Results are expressed as the mean \pm SEM: (#) $p < 0.05$, significant vs vehicle control; (*) $p < 0.05$, significant vs nortriptyline (A, B) and vs fluoxetine (C, D).

Table 1. Effect of Nortriptyline and Fluoxetine and Their GABA Amides **1** and **2** on Distance Moved, Velocity, and Immobility on Male Balb/c Mice in an Open Field Test^a

drug	dose (mg/kg)	distance moved (cm)	velocity (cm/s)	immobility (s)
vehicle		8485 \pm 610	7.07 \pm 0.51	458.4 \pm 41.0
nortriptyline	1	8474 \pm 407	7.13 \pm 0.37	468.6 \pm 37.0
nortriptyline	5	7385 \pm 447	6.17 \pm 0.38	496.0 \pm 45.4
nortriptyline	15	6815 \pm 51*	5.68 \pm 0.38*	547.9 \pm 47.4
1	eqM 1	8053 \pm 379	6.72 \pm 0.32	447.2 \pm 16.7
1	eqM 5	8157 \pm 375	6.85 \pm 0.30	467.9 \pm 21.1
1	eqM 15	7413 \pm 860	6.18 \pm 0.72	572.8 \pm 86.7
fluoxetine	5	7485 \pm 506	6.24 \pm 0.42	552.2 \pm 44.7
fluoxetine	10	7970 \pm 413	6.64 \pm 0.37	558.0 \pm 42.0
fluoxetine	20	5783 \pm 43.3**	4.78 \pm 0.36**	709.3 \pm 42.9*
2	eqM 5	7767 \pm 371	6.47 \pm 0.31	501.5 \pm 21.6
2	eqM 10	8003 \pm 589	6.67 \pm 0.49	536.3 \pm 32.0
2	eqM 20	6462 \pm 506**	5.39 \pm 0.42**	639.9 \pm 50.5

^a Treatment vs control: (*) $p < 0.05$; (**) $p < 0.01$. Results are expressed as the mean \pm SEM of six to eight determinations. There were six mice in the groups treated with nortriptyline or **1** and eight mice in the groups treated with fluoxetine or **2**.

substantiate the observations obtained in the second phase of the formalin test and suggest the potential of **1** in alleviating pain induced by chronic inflammation.

The rat paw edema test, which measures the ability of a compound to reduce local edema induced upon injection of the irritant λ -carrageenan, is used for screening anti-inflammatory agents. The edema development depends on the participation of polymorphonuclear leucocytes with their pro-inflammatory

factors, e.g., prostaglandins.²⁵ Wistar rats were treated po with vehicle, 5 mg/kg nortriptyline, an eqM amount of **1**, or 50 mg/kg of gabapentin administered ip. Two hours later the rats were injected a solution of λ -carrageenan to the surface of their hind paw. The volume of the injected site was measured 4 and 24 h later, and the anti-inflammatory activity was expressed as the change in volume between the two measurements (Figure 5A). While nortriptyline did not reduce the edema, treatment with gabapentin or **1** led to a significant edema volume reduction compared to vehicle treatment ($p < 0.01$) and **1** to nortriptyline ($p < 0.05$). The mean increase in edema volume in rats treated with gabapentin was greater than that of rats treated with **1** (99 \pm 42 vs 32.2 \pm 23, respectively); however, this difference was not statistically significant.

The local production of the inflammatory mediators activates peripheral afferent fibers and leads to hyperalgesia and allodynia manifested by increased sensitivity to thermal and mechanical stimuli. To evaluate the effect of **1** on the heat sensation following the λ -carrageenan injection, the rats were tested on the hot plate. In rats treated with nortriptyline, an eqM dose of **1**, or gabapentin (50 mg/kg), a significant increase in the latency of the response 2–4 h after λ -carrageenan injection was observed (Figure 5B). Consistent with the superiority of **1** in the edema test, it also induced a significantly greater latency than nortriptyline or gabapentin 4 h after λ -carrageenan injection, demonstrating a significant inhibition of hyperalgesia in this test.

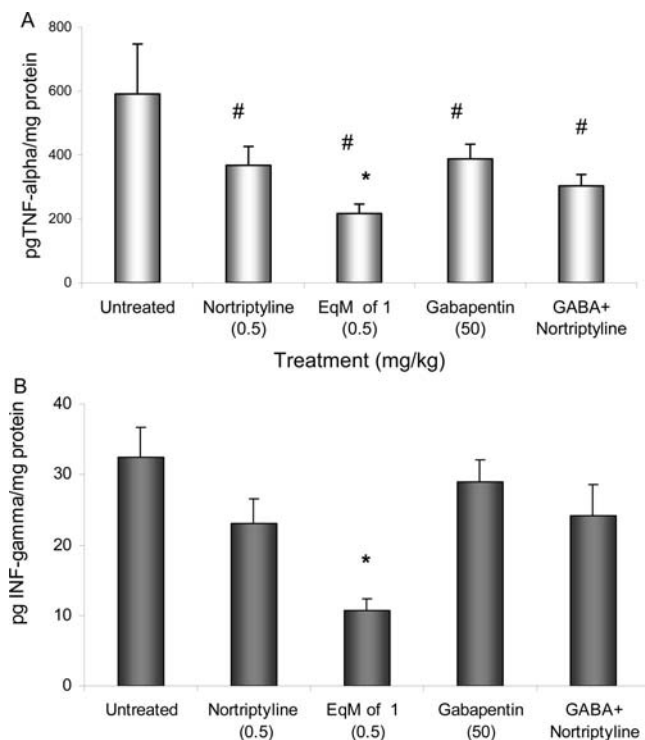


Figure 4. Compound **1** significantly reduced the level of interferon- γ and TNF- α at the formalin-injection site: (#) significant vs control; (*) significant vs all. Male Balb-c mice (8/group) were treated orally (po) as indicated 120 min prior to the injection of formalin (20 μ L of 1% formalin solution) to their right hind paws. The treatment groups were vehicle-treated control (po), 0.5 mg/kg nortriptyline and an eqM dose of **1** (po), 50 mg/kg gabapentin (ip), and a mixture of nortriptyline 0.5 mg/kg and an eqM dose of GABA (po). After 24 h (after the injection of formalin) the animals were sacrificed by CO₂ inhalation and the tissues from the injected site were removed, homogenized in 300 μ L of ice-cold PBS containing 0.4 mM NaCl, 0.05% Tween-20, 0.1 mM phenylmethylsulfonyl fluoride, and cocktail protease inhibitors. The homogenate was centrifuged at 10000g for 30 min at 4 °C, the supernatant was removed, and the protein content was determined and assayed for TNF- α and interferon- γ using commercial ELISA kits according to the manufacturer's instructions.

In plasma samples spiked with **1**, GABA was not detected as a metabolite (data not shown). In an in vitro receptor binding assay (performed by Cerep, France), the binding of an excess of **1** (10 μ M) to the GABA_A, GABA_B, and GABA-nonselective GABA receptors resulted in 11%, -27%, and 6% inhibition of control specific binding, respectively. Since the % inhibition was lower than 50%, the K_i values were not determined. Thus, it can be concluded that the in vitro binding affinity of **1** to GABA receptors is low. These findings are similar to those of the GABAergic drugs gabapentin and pregabalin that were initially synthesized as GABA mimetics, and their mechanism of action was found to be mediated through various mechanisms not directly involving the GABA receptors.²⁶ Since GABA is not a metabolite of **1** and no specific binding to the GABA receptors was observed, it is unlikely that GABA plays a part in the mechanism of action. It is thus proposed that **1** is a new chemical entity with analgesic activity superior to that of its constituents nortriptyline and GABA. The mechanism by which **1** elicits its analgesic activity is under investigation.

Conclusion

Amides **1** and **2** in animal models of pain exhibited significantly greater antinociceptive activity than their corresponding parent compounds nortriptyline and fluoxetine. Com-

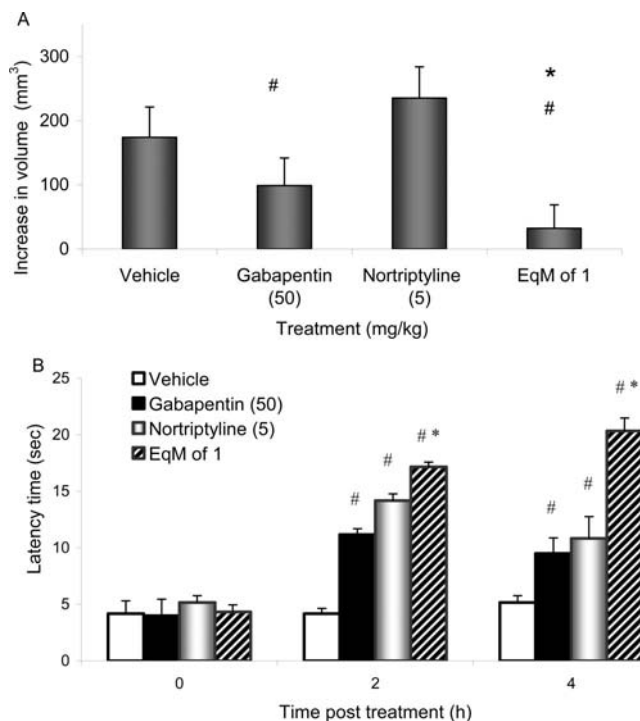


Figure 5. Compound **1** attenuated λ -carrageenan-induced paw edema: (#) significant vs control; (*) significant vs nortriptyline and gabapentin. Wistar rats (groups of 6) were treated po with vehicle, 5 mg/kg nortriptyline, an eqM amount of **1**, and ip with 50 mg/kg gabapentin. After 2 h, paw edema was induced by injecting 100 μ L of a solution of 2.5% λ -carrageenan into the plantar surface of the left hind paws of the rats. The area and the height of the induced edema were measured 4 and 24 h later using a caliper. The change in the volume of the injected site from the first measurement at 4 h, the second measurement at 24 h, after λ -carrageenan administration, for the different treatment groups is shown in (A). The latency of the reaction of the rats to heat using the hot plate set to 52 \pm 0.2 °C was tested at 0, 2, and 4 h after λ -carrageenan injection (B).

ound **1** was active at 20-fold lower doses than **2** without inducing sedation and reduced acute nociceptive sensation and chronic inflammatory reaction. Thus, **1** has a potential of alleviating acute and chronic pain.

Experimental Section

Chemistry. General Procedures. ¹H and ¹³C NMR spectra were obtained on Bruker AC 200 and DPX 300 MHz spectrometers. Chemical shifts are expressed in ppm downfield from Me₄Si (TMS) used as internal standard. The values are given in δ scale. Multiplets in quotation marks have second order characteristics. Low resolution mass spectra (LRMS) were obtained in a QToF microspectrometer in electrospray mode, and relative intensities of the measured peaks appear in parentheses indicated as %. High resolution mass spectra (HRMS) were obtained on an AutoSpec Premier (Waters, U.K.) spectrometer in CI (=chemical ionization), CH₄, mode. Progress of the reactions was monitored by TLC on silica gel (Merck, article 5554). The purity of the compounds was evaluated by HPLC, and compounds **1** and **2** were confirmed to be of \geq 95% purity. The HPLC (Agilent) system was used with a reverse phase Gemini 5 μ m, 110 Å, 250 mm \times 4.6 mm column at a flow rate of 1 mL/min, using two different solvent systems. Solvent system I was 0.1% formic acid in water and acetonitrile. Solvent system II was 0.3% TFA in water (A) and 0.3% TFA in acetonitrile (B). Commercially available compounds were used without further purification.

General Procedure I. To a solution of fluoxetine or nortriptyline (1 equiv) and 4-*tert*-butoxycarbonylamino-butyric acid (1.2 equiv) in anhydrous CH₂Cl₂, under N₂, were added 1-(3-dimethylamino-

propyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.2 equiv) and Et₃N (1 equiv). The mixture was stirred at room temperature for 48 h and then was washed with 1 N HCl (×3), 5% aqueous NaHCO₃ (×3), brine (×3), dried with MgSO₄, filtered, and evaporated to give the desired amide as a mixture of two rotamers.

Removal of *N*-Boc Group. General Procedure II. A solution of 4 N HCl in EtOAc was added to a solution of an *N*-Boc protected compound in EtOAc. The mixture was stirred for 4 h at room temperature. Evaporation of the solvent gave the crude product which was crystallized from methanol–ether.

10,11-Dihydro-5-(3-methylamonipropylidene)-5*H*-dibenzo-[1,4]cycloheptene 4-amino-*N*-butaneamide Hydrochloride, 1. *tert*-Butyl 3-(10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptene-5-ylidene)-*N*-methylcarbamoyl)propylcarbamate (*N*-*tert*-Boc-1), prepared as described in the general procedure I from nortriptyline, was obtained as a colorless oil (yield 81%) and was used as such without further purification. Its NMR spectrum indicated the presence of two rotamers in an approximate 1:1 ratio. ¹H NMR (300 MHz, CDCl₃) ppm δ 1.43 (s, 9H, CMe₃), 1.71/1.79 (“quint”, *J* = 7 Hz, 2H, CH₂CH₂CH₂), 2.22–2.50 (m, 4H, CH₂CO₂, CHCH₂CH₂), 2.80/2.84 (s, 3H, NCH₃), 3.06 (t, *J* = 6.5 Hz, 2H, Ar-CH₂CH₂-Ar), 3.13 (t, *J* = 6.5 Hz, 2H, Ar-CH₂CH₂-Ar), 3.28/3.45 (“t”, *J* = 7 Hz, 4H, CH₂NH, CH₂NMe), 5.75–5.88 (m, 1H, CH), 6.98–7.29 (m, 8H, Ar). ¹³C NMR (300 MHz, CDCl₃) ppm δ 25.2/25.3 (CH₂CH₂CH₂), 27.5/28.5 (CHCH₂CH₂), 28.4 (Me₃C), 30.0/30.8 (CH₂CO₂), 31.9/33.7 (Ar-CH₂CH₂-Ar), 32.0/33.7 (Ar-CH₂CH₂-Ar), 33.5/35.3 (NCH₃), 40.4 (CH₂NHCO₂), 47.4/49.4 (CH₂NMe), 78.7 (CMe₃), 125.8 (CH), 125.9 (CH), 126.1 (CH), 126.2 (CH), 127.1 (CH), 127.4 (CH), 127.5 (CH), 127.8 (2CH), 128.0 (CH), 128.1 (CH), 128.2 (CH), 128.3 (CH), 128.6 (CH), 130.0/130.2 (CH), 136.9/137.0 (C, Ar), 139.3/139.4 (C, Ar), 139.5/139.8 (C, Ar), 140.46/141.0 (C, Ar), 144.4/146.1 (C=CH), 156.0 (HNCO₂), 172.2 (MeNCO). MS (ES+): *m/z* 449 (MH⁺, 81%), 349 (MH⁺ – C₅H₈O₂, 100%). HRMS: calcd for C₂₈H₃₇N₂O₃ (M⁺, DCI/CH₄) 449.2823, found 449.2804. The *N*-Boc protective group of this compound was removed as described in general procedure II to give **1** in quantitative yield. The product was analyzed by HPLC using solvent system I. The purity of **1** was 98.3% (retention time, 9.56 min) with a 0.87% minor impurity identified as nortriptyline (retention time, 8.93 min). The NMR spectrum indicated the presence of two rotamers in an approximate 1:1 ratio. ¹H NMR (300 MHz, MeOD) ppm δ 1.81/1.90 (“quint”, *J* = 7 Hz, 2H, CH₂CH₂CH₂), 2.16–2.41 (m, 2H, CHCH₂CH₂), 2.45 (t, *J* = 7 Hz, 2H, CH₂CO₂), 2.69/2.83 (s, 3H, NCH₃), 2.83–3.0 (m, 4H, Ar-CH₂CH₂-Ar), 3.15–4.47 (m, 4H, CH₂NH₂, CH₂NMe), 5.76–5.84 (m, 1H, CH), 6.96–7.26 (m, 8H, Ar). ¹³C NMR (300 MHz, MeOD) ppm δ 23.7/23.8 (CH₂CH₂CH₂), 28.4/29.2 (CHCH₂CH₂), 30.8/31.5 (CH₂CO₂), 32.9/34.8 (Ar-CH₂CH₂-Ar), 33.0/34.8 (Ar-CH₂CH₂-Ar), 34.1/35.9 (NCH₃), 40.4 (CH₂NH₂), 48.6/50.3 (CH₂NMe), 126.8/127.0 (CH, Ar), 127.05/127.1 (CH, Ar), 127.8/128.2 (CH, Ar), 128.4/128.7 (CH, Ar), 128.8/128.9 (CH, Ar), 129.1 (CH, Ar), 129.2/129.3 (CH, Ar), 129.4 (CH, Ar), 131.0/131.2 (CH, Ar), 138.1 (C, Ar), 140.6/140.9 (C, Ar), 141.8/142.2 (C, Ar), 145.9/147.2 (C=CH), 173.55/173.7 (MeNCO). MS (ES+): *m/z* 349 (MH⁺, 100%), 332 (MH⁺ – NH₃, 73). Anal. Calcd for C₂₃H₂₉ClN₂O·0.5H₂O: C 70.12, H 7.68, N 7.11. Found C 69.89, H 7.93, N 7.24.

***N*-(3-(*p*-Trifluoromethyl)phenoxy)-3-phenylpropyl)-4-amino-*N*-ethylbutaneamide Hydrochloride, 2.** *tert*-Butyl 3-(*N*-(3-(4-trifluoromethyl)phenoxy)-3-phenylpropyl)-*N*-methylcarbamoyl)propylcarbamate (*N*-*tert*-Boc-2), prepared as described in general procedure I from fluoxetine, was obtained as a colorless oil (yield 76%) and was used as such without further purification. HPLC analysis of the product, using solvent system II, identified purity of 99.5% (retention time, 19.7 min). The NMR spectra of the compound indicated the presence of two rotamers in an approximate 3:2 ratio. ¹H NMR (300 MHz, CDCl₃) ppm δ 1.41 (s, 9H, CMe₃), 1.77 (“quint”, *J* = 7 Hz, 2H, CH₂CH₂CH₂), 2.11–2.28 (m, 4H, CH₂CO₂, CHCH₂CH₂), 2.92/2.95 (s, 3H, NMe, minor/major), 3.05/3.13 (t, *J* = 7 Hz, 2H, CH₂NH, minor/major), 3.38–3.69 (m, 2H, CH₂NMe), 5.14/5.21 (dd, *J* = 8.5, 4 Hz, 1H, OCH, minor/major), 6.89 (d, *J* = 8.5 Hz, 2H, Ar), 7.2–7.47 (m, 7H, Ar). ¹³C NMR

(300 MHz, CDCl₃) ppm δ 25.2/25.5 (CH₂CH₂CH₂, major/minor), 28.5 (Me₃C), 30.2/30.9 (CH₂CO, minor/major), 33.6/36.0 (NMe, minor/major), 36.4/37.5 (CHCH₂CH₂, major/minor), 40.5 (CH₂NH), 45.4/46.3 (CH₂NMe, major/minor), 77.4/78.4 (OCH, minor/major), 79.2 (CMe₃), 115.8 (2CH, Ar), 123.6 (CCF₃), 125.5 (CF₃), 125.7/125.8 (2CH, Ar, minor/major), 126.9 (2CH, Ar), 128.0/128.4 (CH, Ar, major/minor), 128.9/129.2 (2CH, Ar, major/minor), 140.8/143.3 (C, Ar, major/minor), 156.2 (NHCO₂), 160.4 (C, Ar), 172.6 (MeNCO). MS (ES+): *m/z* 517 (MNa⁺, 40%), 495 (MH⁺, 56%), 395 (MH⁺ – C₅H₈O₂, 100%). HRMS: calcd for C₂₆H₃₄N₂O₄F₃ (M⁺, DCI/CH₄) 495.2507, found 495.2471. HPLC of the products, using solvent system II, indicated that the purity of **2** was 97.7% (retention time, 19.7 min) with a minor impurity of 2.3% (retention time, 14.9 min). The *N*-Boc protective group of this compound was removed as described in general procedure II to give **2** in quantitative yield. The NMR spectra of the compound indicated the presence of two rotamers in an approximate 3:2 ratio. ¹H NMR (300 MHz, MeOD) ppm δ 1.90 (“quint”, *J* = 7 Hz, 2H, CH₂CH₂CH₂), 2.01–2.33 (m, 2H, CHCH₂CH₂), 2.46–2.60 (m, 2H, CH₂CO), 2.85–3.01 (m, 2H, CH₂NMe), 2.93/3.04 (s, 3H, NMe, minor/major), 3.43–3.73 (m, 2H, CH₂NH), 5.37/5.46 (dd, *J* = 8, 4 Hz, 1H, OCH, major/minor), 7.02 (“t”, *J* = 8.5 Hz, 2H, Ar), 7.2–7.52 (m, 7H, Ar). ¹³C NMR (300 MHz, MeOD) ppm δ 23.7/23.9 (CH₂CH₂CH₂, major/minor), 30.9/31.5 (CH₂CO, minor/major), 34.0/36.4 (NCH₃, minor/major), 37.0/37.8 (CHCH₂CH₂, major/minor), 40.4 (CH₂NH), 46.4/47.4 (CH₂NMe, major/minor), 78.5/79.2 (OCH, minor/major), 117.2 (2CH, Ar), 123.4 (CCF₃), 124.0 (CF₃), 127.1 (2CH, Ar), 127.7 (2CH, Ar), 128.9/129.1 (CH, Ar, major/minor), 129.7/129.9 (2CH, Ar, major/minor), 141.7/142.1 (C, Ar, minor/major), 161.7/161.9 (C, Ar, minor/major), 173.9/174.1 (NMeCO, minor/major). MS (ES+): *m/z* 395 (MH⁺). HRMS: calcd for C₂₁H₂₅F₃N₂O₂ ([MH⁺], DCI/CH₄) 395.1946, found 395.1906. Anal. Calcd for C₂₁H₂₅F₃N₂O₂·HCl·H₂O: C 56.19, H 6.29, N 6.24. Found C 56.45, H 6.82, N 5.96.

Solubilization of the Drugs for the Biological Studies. Fluoxetine, nortriptyline, **1**, **2**, and gabapentin (Sigma) were solubilized in doubly distilled water (DDW). Control animals received the same volume of DDW, of a maximum of 0.1 mL/kg weight. The drugs were administered to the animals by oral gavage (po) using 20-gauge curved needles (Pop-per, NY) except gabapentin that was given by ip injection.

Biology: Animals. Wistar male rats, 8–10 weeks old, and Balb-c male mice, 8–12 weeks old, (Harlan, Israel) were housed under conditions of controlled temperature (23 ± 3 °C) and humidity (55 ± 15%) with a 12 h light/12 h dark cycle and were acclimated at least 1 week prior to their use in the experiment. All animals were fed with a commercially available rodent diet (ad libitum), and free access to drinking water was available. All experiments were carried out in accordance with the ethical guidelines of the Committee on the Care and Use of Laboratory Animals of Tel Aviv University.

Hot Plate Test. The hot plate test used to measure latency in response to heat was carried out on the basis of the method described,²⁰ with the following modifications. The hot plate (MRC, model MH-4, 230 V/50 Hz, 750 W) was maintained at 52 °C. Balb-c mice or Wistar rats were placed on the heated surface, and the time of response to heat sensation was detected by the following reactions of raising or licking the paw, jumping, or running and was recorded as time (s) of response latency. Data were collected between 0 and 5 h after po administration of the specified drugs.

Formalin Test. The method used was based on that described.²² Balb-c mice, 5–10/group, were treated orally with the tested compounds, and 2 h later, 20 μL of a 1% formalin solution was injected sc into the dorsal surface of their right hind paws. The formalin induced a typical licking or biting of the injected paw (flinching behavior). The animals were placed in a transparent chamber, and the number of times that they licked or bit the injected paw during the first 5 min (phase I) and between 25 and 35 min (phase II) after the injection was counted.

Measurement of TNF-α and INF-γ in the Skin of the Paws of Mice. At the specified times shown after injection of the formalin, the animals were killed and the skin tissue was removed from the

injected site. The tissue was homogenized (Polytron; Kinematica, Lucerne, Switzerland) in 300 μL of ice-cold PBS containing 0.4 mM NaCl, 0.05% Tween-20, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). The homogenate was centrifuged at 10000g for 30 min at 4 °C. The supernatant was removed, and protein content was determined and assayed using BCA protein assay (Thermo Fisher Scientific Inc. Rockford, IL) mouse TNF- α ELISA kit (BD OptEIA, CA) and mouse INF- γ immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as picogram of TNF- α or INF- γ per milligram of protein.

λ -Carrageenan-Induced Paw Edema Test. Rats were marked with a permanent marker on the ankles of their left hind paws to define the area of the paws to be monitored. Paw edema was induced by injecting 100 μL of a 1% solution of λ -carrageenan (Sigma) in saline into the surface of the left hind paw. The change in height and volume of the paw after λ -carrageenan administration in vehicle-treated and drug-treated animals was measured using an electronic caliper and was calculated ($w^2 \times L/2$). Latency of response to heat of the treated rats was determined by the hot plate test, as described above.

Open Field Test for Anxiolytic and Motor Activity. Male Balb-c mice were placed in the testing room 12 h prior to the beginning of the test. The tested agents (vehicle, nortriptyline, fluoxetine, or the GABA amides **1** or **2** at eqM doses) were administered po 90 min prior to test. An individual mouse was placed in the novel environment of a square open field (50 cm \times 50 cm). The animal behavior in the open field was videotaped using a camera placed above the field for 20 min and subsequently analyzed digitally using Noldus (Noldus, The Netherlands) software for animal behavior. The measurements included distance moved, velocity, and immobility time.

Binding Profile of Compound **1 to GABA Receptors.** The in vitro ligand binding to the specified GABA receptors was performed by Cerep, France, according to their standard operating procedures. Binding to the GABA_A receptor was tested by incubation of 5 nM of the specific ligand [³H]muscimol (Perkin-Elmer) and 10 μM of nonradioactive **1** for 10 min at 4 °C with rat cerebral cortex. Binding to the GABA_B receptor was assayed by incubation of 2.5 nM [³H]CGP (American Radiolabeled Chemicals, Inc.) The receptor's specific ligand [³H]CGP and 10 μM unlabeled **1** were incubated for 60 min at 22 °C with HEK-293 cells, stably expressing the human recombinant GABA_B receptor. The binding to the nonselective GABA receptor was tested by incubation of 10 nM of the receptor's specific ligand [³H]GABA (Perkin-Elmer) and 10 μM unlabeled **1** for 60 min at 22 °C with rat cerebral cortex. The specific ligand binding to the receptors was defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess 10 μM of unlabeled **1** (in duplicate). The results were expressed as a % inhibition of control specific binding obtained in the presence of **1**.

Statistical Analysis. The data are expressed as the mean \pm SEM and were analyzed by the Student's *t* test. Values of *p* < 0.05 were considered statistically significant.

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