

Behavioral Evidence for the Interaction of Oleamide with Multiple Neurotransmitter Systems

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ABSTRACT

While the endogenous fatty acid amide oleamide has hypnotic properties, neither the breadth of its behavioral actions nor the mechanism(s) by which these behaviors may be mediated has been elucidated. Therefore, the effects of oleamide on the performance of rats in tests of motor function, analgesia, and anxiety were investigated. Oleamide reduced the distance traveled in the open field ($ED_{50} = 14, 10\text{--}19\text{ mg/kg}$, mean, 95% confidence interval), induced analgesia and hypothermia, but did not cause catalepsy. Moreover, a dose of oleamide without effect on motor function was anxiolytic in the social interaction test and elevated plus-maze. These actions of a single dose of oleamide lasted for 30 to 60 min. While rats became tolerant to oleamide following 8 days of repeated administration, oleamide is a poor inducer of physical dependence. Pretreatment with antagonists of the serotonin ($5HT$)_{1A}, $5HT$ _{2C}, and vanilloid re-

ceptors did not modify oleamide's effects. However, the cannabinoid receptor antagonist SR 141716A inhibited oleamide-induced analgesia in the tail-flick assay, the γ -aminobutyric acid (GABA)_A receptor antagonist bicuculline reversed the analgesia and hypothermia, and the dopamine D_2 receptor antagonist L 741626 blocked oleamide's locomotor and analgesic actions. Interestingly, oleamide analogs resistant to hydrolysis by fatty acid amide hydrolase (FAAH) maintained but did not show increased behavioral potency or duration of action, whereas two FAAH inhibitors produced analogous behavioral effects. Thus, oleamide induces behaviors reminiscent of the actions of endogenous cannabinoids, but the involvement of GABAergic and dopaminergic systems, either directly or indirectly, in the actions of oleamide cannot be ruled out.

Unsaturated fatty acid amides, such as arachidonoyl ethanolamide (AEA, e.g., anandamide; Devane et al., 1992), can potentially alter neuronal function and suppress neurotransmitter release, in part through their interactions with the cannabinoid (CB₁) receptor (Cadogan et al., 1997; de Miguel et al., 1998; Gifford et al., 1999). Another member of this family was isolated from the cerebrospinal fluid of sleep-deprived cats, with subsequent structural analysis revealing this compound to be *cis*-9-octadecenamide, or oleamide (Cravatt et al., 1995). Oleamide is environmentally ubiquitous, being found in a variety of vegetable oils, and is used as an industrial lubricant in polyolefin manufacturing (Molnar, 1974; Cooper et al., 1995). However, accumulating evidence supports a role for oleamide as an endogenous signaling factor. Its catabolic enzyme, fatty acid amide hydrolase (FAAH), has been localized in the liver (Cravatt et al., 1996) and several brain regions (Thomas et al., 1997b) and was

subsequently isolated, cloned, and expressed. There is also evidence for the *de novo* synthesis of oleamide from brain microsomes (Sugiura et al., 1996), although its synthetic pathway remains to be defined. It has been proposed that oleamide can competitively inhibit FAAH catabolism of the endogenous CB receptor agonist AEA (Mechoulam et al., 1997), thereby acting through cannabinergic systems to produce its physiological and behavioral actions even though there is no evidence for potent interactions with CB receptors (Boring et al., 1996). In addition, several reports indicate that oleamide potently (10^{-9} – 10^{-7} M) modulates gap junction currents (Guan et al., 1997; Boger et al., 1998) and currents gated by serotonin ($5HT$)_{1A}, $5HT$ _{2C} (Huidobro-Toro and Harris, 1996), and GABA_A receptors (Yost et al., 1998) as well as increasing $5HT$ ₇-activated adenylate cyclase activity (Thomas et al., 1997a). These *in vitro* observations provide ample evidence that neurotransmitter systems other than the cannabinergic system may be involved in mediating oleamide-induced behaviors.

Oleamide clearly has hypnotic properties and may be in-

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ABBREVIATIONS: AEA, arachidonoyl ethanolamide; CB, cannabinoid; FAAH, fatty acid amide hydrolase; $5HT$, serotonin; GABA, γ -aminobutyric acid; DMSO, dimethyl sulfoxide; MPE, maximum possible effect; CI, confidence interval; ANOVA, analysis of variance.

volved in endogenous processes related to the induction of sleep in mammals (Cravatt et al., 1995; Basile et al., 1999). Sleep induction time is reduced by oleamide without altering the duration of rapid eye movement sleep (Basile et al., 1999; Mendelson and Basile, 1999). Moreover, the absolute levels of oleamide (Hanuš et al., 1999) increase in the cerebrospinal fluid of rats following 6 h of sleep deprivation (Basile et al., 1999). Currently, it is not clear whether oleamide acts solely as a hypnotic. Many commonly recognized hypnotics, such as the benzodiazepines, also have significant anxiolytic actions. If the primary mechanism of oleamide is to indirectly enhance cannabinergic signaling by increasing AEA levels through competitive inhibition of FAAH then oleamide should induce locomotor depression, hypothermia, and analgesia (Chaperon and Thiebot, 1999). Alternatively, the involvement of other neurotransmitter systems might be indicated by the suppression of oleamide-induced behaviors by the administration of appropriate receptor antagonists. The current study investigates in greater detail some of the behavioral actions of oleamide with the intent of elucidating its mechanisms of action.

Materials and Methods

General

Male Sprague-Dawley rats (150–250 g; Taconic Laboratories, Frederick, MD) were housed and fed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility and maintained on a standard 12-h light/dark cycle (lights on, 6:00 AM; lights off, 6:00 PM). All described procedures were approved by the institute Animal Care and Use Committee. The drugs to be tested were dissolved in a vehicle consisting of 5% dimethyl sulfoxide (DMSO)/20% Alkamuls/75% water, which was injected intraperitoneally, and the treated rats tested as early as 15 min after injection. For the antagonist studies, rats were first injected with the antagonist, allowed to wait 15 min before injecting oleamide or an oleamide analog, and then allowed an additional 15 min to rest before testing. After preliminary tests indicated that there were no performance differences during day or night periods, all testing was conducted during the light phase (6:00 AM–6:00 PM) of the light/dark cycle and, to facilitate habituation, animals were transported to the testing room and left undisturbed for at least 1 h before testing. All apparatus used in the study were thoroughly cleaned between subjects using soapy water followed by 40% ethanol.

The drugs tested included oleamide (synthesized at the National Institutes of Health); SR 141716A (obtained through the National Institute on Drug Abuse Research Resources Drug Supply System and synthesized by the Research Triangle Institute); WIN 52466, bicuculline, SB 242084, SCH 23390, L 741626, and capsazepine (Tocris, Ballwin, MO); and WAY 100135 (Sigma/RBI, St. Louis, MO).

After administration of the test compounds, rats were then tested in the following behavioral paradigms. Each rat was tested only once per drug or drug/antagonist combination. Typically, rats underwent the following motor function and analgesia tests in sequence, ending with rectal measurements of body temperature using a lubricated thermistor probe connected to a digital thermometer.

Motor Function Tests

Open Field Performance. Rats were placed into the center of an open field apparatus (Columbus Instruments, Columbus, OH) under subdued lighting. Parameters of their motor activity (distance traveled, number of vertical movements, number of stereotypic movements, time ambulating, time resting) were then monitored and recorded over 20 min (Kelley, 1993).

Catalepsy/Inclined Grid Test. Immobility/catalepsy in rats was investigated using the inclined grid test. Rats were placed on a 30- × 30-cm grid inclined 60°, and the time the rat remained immobile measured for 2.5 min. The degree of catalepsy was scored from 0 to 5 based on the amount of time the animal remained immobile (min): 0 = 0 to 0.08; 1 = 0.09 to 0.35; 2 = 0.36 to 0.8; 3 = 0.81 to 1.42; 4 = 1.42 to 2.24; and 5 > 2.25 min (Ahlenius and Hillegaart, 1986).

Tests of Analgesia

Tail-Flick Test. Rat tails were placed under the focused beam of a halogen projection lamp (D'Amour and Smith, 1941). The intensity of the lamp was adjusted so that the unrestrained tail of a normal rat did not remain under the beam for longer than 4 s, with a cutoff time of 10 s. The latency to remove the tail from the beam path (s) following treatment was recorded and processed to yield the percentage of maximum possible effect: %MPE = $100 \times [(test - control) / (10 - control)]$ (Harris and Pierson, 1964).

Hot-Plate Test. Rats were placed in a plastic cylinder atop a hot-plate uniformly regulated to 55°C over the entire surface (Columbus Instruments). The amount of time before the rat showed evidence of thermal discomfort (e.g., licking a paw) was recorded and the test ended (Eddy and Leimbach, 1953).

Tests of Anxiety

Social Interaction Test. The social interaction test was conducted under bright light in an unfamiliar environment. Two previously unacquainted male rats were placed into a 1- × 1-m² arena of white acrylic. The following behaviors were registered and classified over a 15-min period as active social interactions: sniffing, following, grooming, biting, boxing, and crawling over or under the cohort (Ramos and Mormede, 1998).

Elevated Plus-Maze. The elevated plus-maze was composed of two open (70- × 15-cm) and two enclosed (70- × 15- × 15-cm) arms constructed of black acrylic radiating from a central platform to form a plus sign. The entire apparatus was elevated to a height of 1.2 m above floor level by a single central support. Testing commenced by placing a rat on the central platform of the maze facing an open arm. A 5-min test duration was used and the following parameters evaluated: the number of open and closed arm entries (arm entry = all four paws into a maze arm) and the time spent in various sections of the maze (including the central platform) (Rodgers and Cole, 1994).

Induction of Tolerance/Dependence and Scoring of Withdrawal Symptoms

Tolerance to immobility, hypothermia, and analgesia were determined after administering 20 mg/kg oleamide i.p., daily for 3 days, followed by the administration of 30 mg/kg oleamide twice daily for the next 5 days. Studies of the potential of oleamide to induce physical dependence were performed after 10 days of administering oleamide, 30 mg/kg twice daily, at 10:00 AM and 4:00 PM. Abstinence behaviors were measured over a 1-h period after spontaneous withdrawal (observation starting 16 h after the last dose) or after withdrawal was precipitated with 4.5 mg/kg SR 141716A. These behaviors included scratching, wet-dog shakes, head shakes, back arching, and teeth chattering. The occurrence of these behaviors was converted into an abstinence score reflecting the average number of times any withdrawal-associated behaviors were observed over a 1-h period (Costa et al., 2000).

Compound Synthesis

Compound 1 (*N*-7(*Z*)-hexadecenyl)-urea was synthesized as follows. 7-(Triphenylphosphonium)heptanenitrile bromide (Kishore et al., 1991) underwent a Wittig reaction upon treatment with potassium *tert*-butoxide and nonyl aldehyde to give 7(*Z*)-hexadecenitrile. This was reduced with lithium aluminum hydride to give 7(*Z*)-hexadecenylamine, which was converted to 1 (Boger et al., 1998). Compounds 2 to 4 (2: 2-methyl-9(*Z*)-octadecenamide; 3: 1-(2-benzox-

azolyl)-1-oxo-9(*Z*)-octadecene; and 4: 1,1,1-trifluoro-2-oxo-10(*Z*)-nonadecene) were synthesized as previously described (Patterson et al., 1996).

Compounds 5 to 7 were synthesized as follows. 7-Bromoheptanol was protected by treatment with *tert*-butyldimethylsilyl chloride and imidazole in *N,N*-dimethylformamide to form 1-*tert*-butyldimethylsiloxy-7-bromoheptane. This was reacted with 1-decyne acetylide (formed by treatment with *n*-butyllithium in hexamethylphosphoramide) to yield 1-*tert*-butyldimethylsiloxyheptadec-8-yne. The silyl protecting group was subsequently removed with Amberlyst 50 in methanol to quantitatively yield heptadec-8-yn-1-ol, which was reduced to the *cis*-olefin 8(*Z*)-heptadecen-1-ol by hydrogenation with nickel boride generated in situ. Misunobu substitution using triphenylphosphine/diisopropylazodicarboxylate/thioacetic acid in tetrahydrofuran converted the alkenol to 1-(acetylthio)heptadec-8(*Z*)-ene (compound 5), which was deacylated under Zemplén conditions and methylated using methyl sulfate to afford 1-(methylthio)heptadec-8(*Z*)-ene. The sulfide was then oxidized with sodium periodate in tetrahydrofuran/water to produce 1-(methylsulfinyl)heptadec-8(*Z*)-ene (compound 6), which was then reacted with *m*-chloroperoxybenzoic acid in ether to give 1-(methylsulfonyl)heptadec-8(*Z*)-ene (compound 7).

FAAH Assay

The FAAH inhibition studies were performed via a [14 C]oleamide-based assay. Rat liver plasma membranes were used as the source of FAAH, and were prepared from 15 livers disrupted in 80 ml of buffer (10% glycerol, 1% Triton X-100, 1 mM EDTA, 20 mM HEPES, pH 7.2) using a Dounce homogenizer (Patterson et al., 1996). The homogenate was stirred at 4°C for 2 h then centrifuged (145,000g, 1 h, 0–4°C). The supernatant was retained for use in the assay. All reactions were conducted in glass vials with a final volume of 200 μ l. [14 C]Oleamide (specific activity of 51 μ Ci/ μ mol) was diluted to 5 mM in ethanol and 4 μ l added to an assay vial using a glass syringe. The compound under investigation was dissolved in either DMSO or ethanol at 40 $\times$ the final concentration and 5- μ l aliquots added to the assay vial along with 91 μ l of reaction buffer (123 mM Tris, 1 mM EDTA, pH 9.0). DMSO or ethanol without the compound under investigation was used for control reactions. The assay was initiated by adding 100 μ l of FAAH preparation (4 μ l of liver supernatant in 96 μ l of reaction buffer) and the mixture incubated at room temperature for 5, 10, and 15 min. At each time point, a 50- μ l aliquot of the reaction mixture was removed using a glass syringe and the reaction terminated in 600 μ l of 0.07 N HCl. This was followed by extraction with approximately 1 ml of ethyl acetate, followed by removal of the organic layer and evaporation under a stream of N₂. The products were resuspended in 8 μ l of ethanol, spotted on thin-layer chromatography plates and developed in 1:1 ethyl acetate/hexane. The developed thin-layer chromatography plates were subsequently placed on a phosphorimaging plate for 2 to 12 h, and the conversion of oleamide to oleic acid measured using a phosphorimager (Packard Instruments, Meriden, CT). Relative rates of hydrolysis (R^2 values > 0.97) of oleamide were determined in the presence of 3–4 inhibitor concentrations. K_i values were determined by the Dixon method, using the x -intercept of a weighted linear fit of ligand concentration versus 1/rate at a constant substrate concentration. The formula ($K_i = -x_{int}/[1 + [S]/K_m]$) was used to derive the K_i values.

Results

Oleamide dose dependently influenced rat performance in tests of motor function and analgesia. As previously reported (Basile et al., 1999), oleamide suppressed every parameter of motor activity in the open field almost completely, with an ED₅₀ for suppression of distance traveled of 14, 10 to 19 mg/kg [mean, 95% confidence interval (CI); Fig. 1A]. In addition, oleamide showed some analgesic properties, signifi-

cantly increasing the latency in the tail-flick test from 4.0 $\pm$ 0.3 to 9.5 $\pm$ 0.5 s (mean $\pm$ S.E.M.), with an ED₅₀ of 66, 40 to 109 mg/kg (mean, 95% CI; Fig. 1B). However, oleamide (10–75 mg/kg) was not consistently effective in the hot-plate assay, although 75 mg/kg oleamide significantly increased latency by 170% (5.2 $\pm$ 0.3 versus 9.0 $\pm$ 1.0 s, vehicle versus 75 mg/kg oleamide, P < 0.01, n = 8 and 4, respectively). In contrast with its relatively low analgesic potency, oleamide decreased rat body temperature from 37.3 $\pm$ 0.07°C to a maximum of 35.3 $\pm$ 0.12°C, with an ED₅₀ of 14, 12 to 17 mg/kg (mean, 95% CI; Fig. 1C), comparable with its potency in suppressing locomotion. While rats developed a significant immobility as measured on the inclined grid catalepsy test in response to administration of the CB₁ receptor agonist WIN 52466 (5 mg/kg, immobility score = 3.3 $\pm$ 0.3, P < 0.01, n = 6; Fig. 1D), oleamide at doses up to 100 mg/kg had no significant effect on the immobility score (0.2 $\pm$ 0.2 versus 0.8 $\pm$ 0.2, vehicle versus 100 mg/kg oleamide, n = 5). Finally, oleamide was active in two behavioral tests of anxiety (Fig. 1, E and F). A dose of oleamide that had no effect on open field activity (5 mg/kg) increased the number of social interactions under high-light conditions almost 2-fold (280 $\pm$ 15 versus 540 $\pm$ 50, vehicle versus oleamide, P < 0.01, n = 9). This dose of oleamide also increased the time spent in the open arms of the plus-maze (1 $\pm$ 1 versus 130 $\pm$ 12 s, vehicle versus oleamide, P < 0.05, n = 5) in 5 of 11 rats tested. Furthermore, an enhancement in exploratory behavior in the plus-maze was observed in those rats responding to oleamide, as evidenced by an increase in the number of entries into the closed (1.2 $\pm$ 0.2 versus 2.1 $\pm$ 0.3 entries, vehicle versus oleamide, P < 0.05, two-way ANOVA, Bonferroni's post hoc comparison matrix, n = 5) as well as open arms (0 $\pm$ 0 versus 2.2 $\pm$ 0.4, vehicle versus oleamide, P < 0.05).

The duration of oleamide's locomotor, analgesic, and hypothermic actions was also studied. Oleamide (20 mg/kg) significantly suppressed the distance traveled in the open field (Fig. 2A) within 15 min of administration, an effect lasting 60 min. Tail-flick latency was enhanced by 30 min after administration (Fig. 2B), lasting up to 60 min. In contrast, significant decreases in body temperature (Fig. 2C) and increases in hot-plate latency (data not shown) were observed only at 15 and 30 min after oleamide administration.

The development of tolerance to, and dependence upon, oleamide was investigated using tests of locomotion, analgesia, and hypothermia. After 3 days of oleamide administration (see *Materials and Methods*), the ability of a challenge dose of oleamide (20 mg/kg) to decrease the distance traveled and increase tail-flick latency was unchanged compared with vehicle-treated rats (Fig. 3, A and B). However, the hypothermia induced by a challenge dose of oleamide declined by an average of 1.8°C (Fig. 3C). The dosing schedule was continued an additional 5 days (see *Materials and Methods*) and the animals subjected to another challenge dose of oleamide. After 8 days of exposure to oleamide, the challenge dose did not significantly reduce body temperature (37.7 $\pm$ 0.15°C, n = 6, Fig. 3C), nor did it significantly increase latency in the tail-flick (9.9 $\pm$ 6.1% MPE, n = 6, Fig. 3B). While the distance traveled in the open field was significantly decreased from vehicle control values (6200 $\pm$ 210 versus 4400 $\pm$ 330 cm/20 min, n = 6, P < 0.01, ANOVA, Bonferroni's post hoc comparison matrix), it was significantly higher than the distance

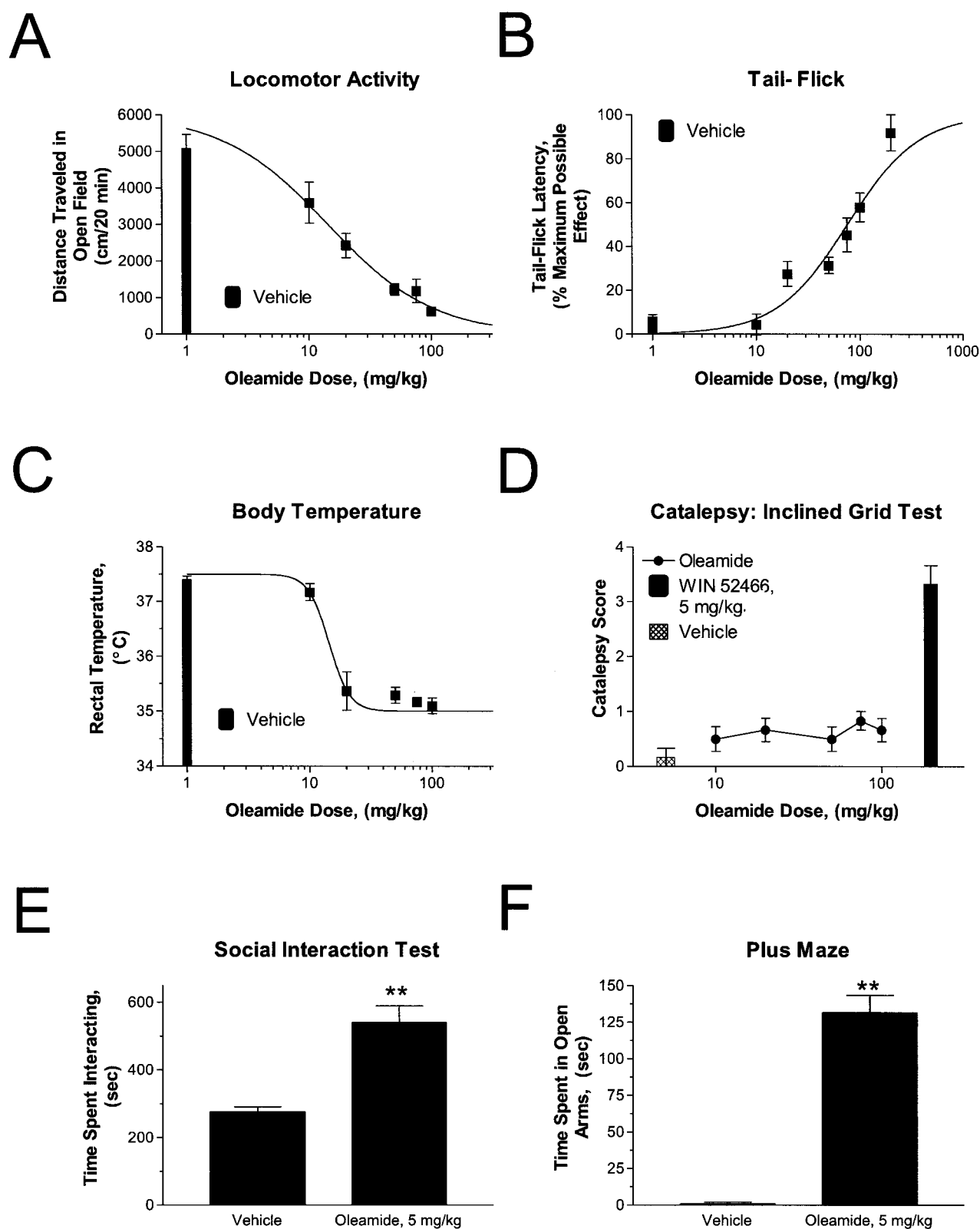


Fig. 1. Dose-response curves of the effects of oleamide on locomotor activity (A), analgesia (B), body temperature (C), and catalepsy (D), as well as oleamide's effects on two tests of anxiety (E and F). Oleamide (10–100 mg/kg i.p.) significantly suppressed locomotion in the open field, as evidenced by a decrease in distance traveled with increased dose (A). Oleamide (10–200 mg/kg) also exhibited analgesic activity, increasing the latency in the tail-flick test (B). Oleamide administration (10–100 mg/kg) resulted in hypothermia (C), but had no noticeable cataleptic actions as measured in the inclined grid test (D). Finally, a dose of oleamide (5 mg/kg) that had no significant effect on body temperature or locomotion showed significant anxiolytic effects in both the social interaction test (E) and plus-maze performance (F), increasing both the number of entries into the open arm, as well as the time spent in the open arm (F). **, significantly different from vehicle, $P < 0.01$, t test. Rats were concurrently tested in the open field, followed by tail-flick test and measures of body temperature (except for the 200 mg oleamide dose in tail-flick). Each data point represents the mean $\pm$ S.E.M., $n = 8$, except for E and F, where $n = 9$.

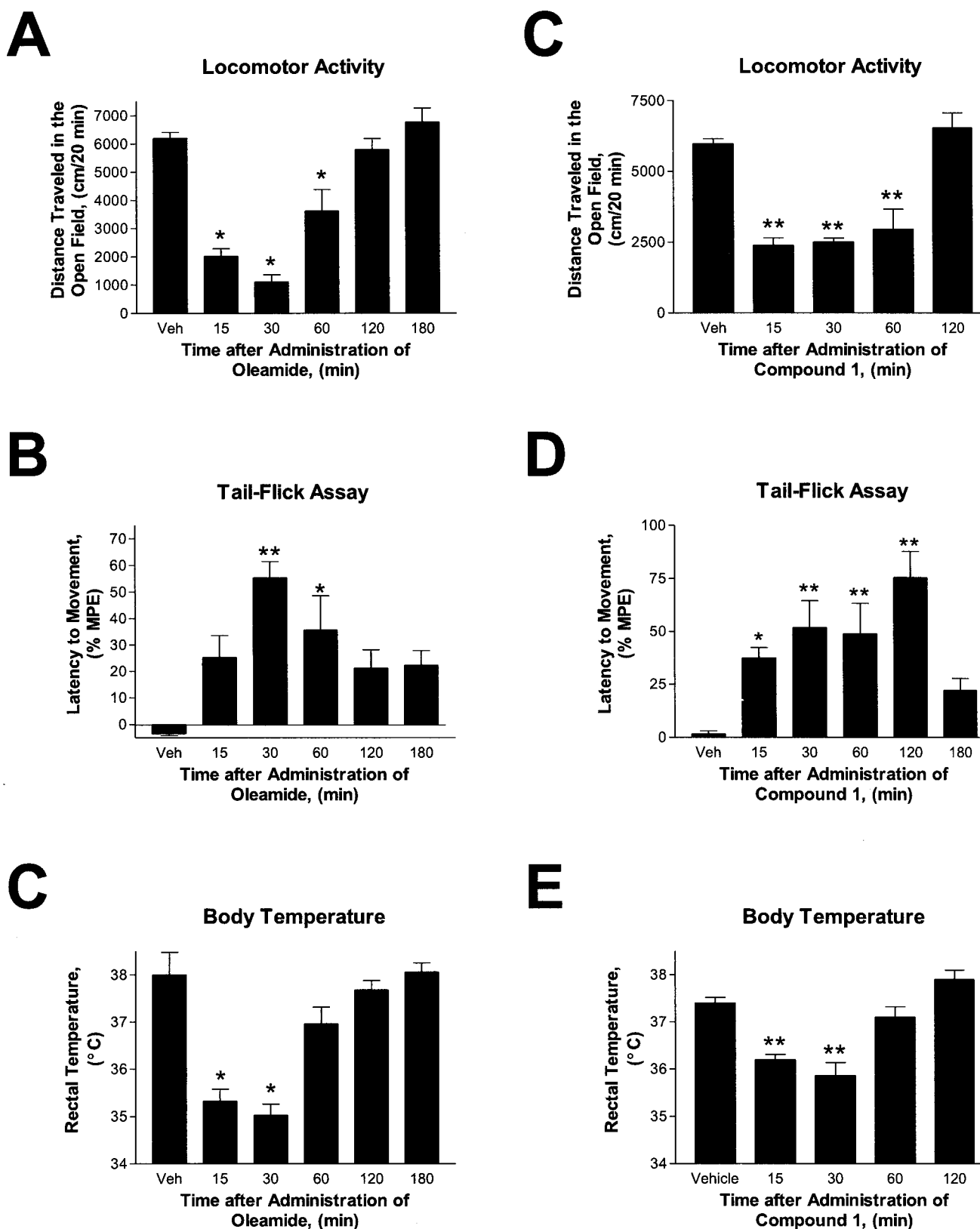


Fig. 2. Duration of the locomotor depressant, analgesic, and hypothermic actions of oleamide and an analog (compound 1). The oleamide-induced (20 mg/kg) decrease in the distance traveled in the open field was maximal at 30 min after administration and lasted for at least 60 min (A). The analgesic properties of oleamide were also maximal at 30 min and lasted for at least 1 h (B). In contrast, the hypothermic actions of oleamide, while maximal at 30 min, were gone by 60 min after administration i.p. Similarly, the 1-induced (20 mg/kg) decrease in the distance traveled in the open field (D) and hypothermia (F) were evident by 15 min after administration, but no longer apparent after 60 min (D). In contrast, 1-induced analgesia was manifested by 15 min after administration, but lasted at least 120 min (E). *, **, significantly different from zero time value, $P < 0.05$, 0.01, ANOVA followed by Dunnett's post hoc comparison matrix. Each value represents the mean $\pm$ S.E.M. of observations from five rats.

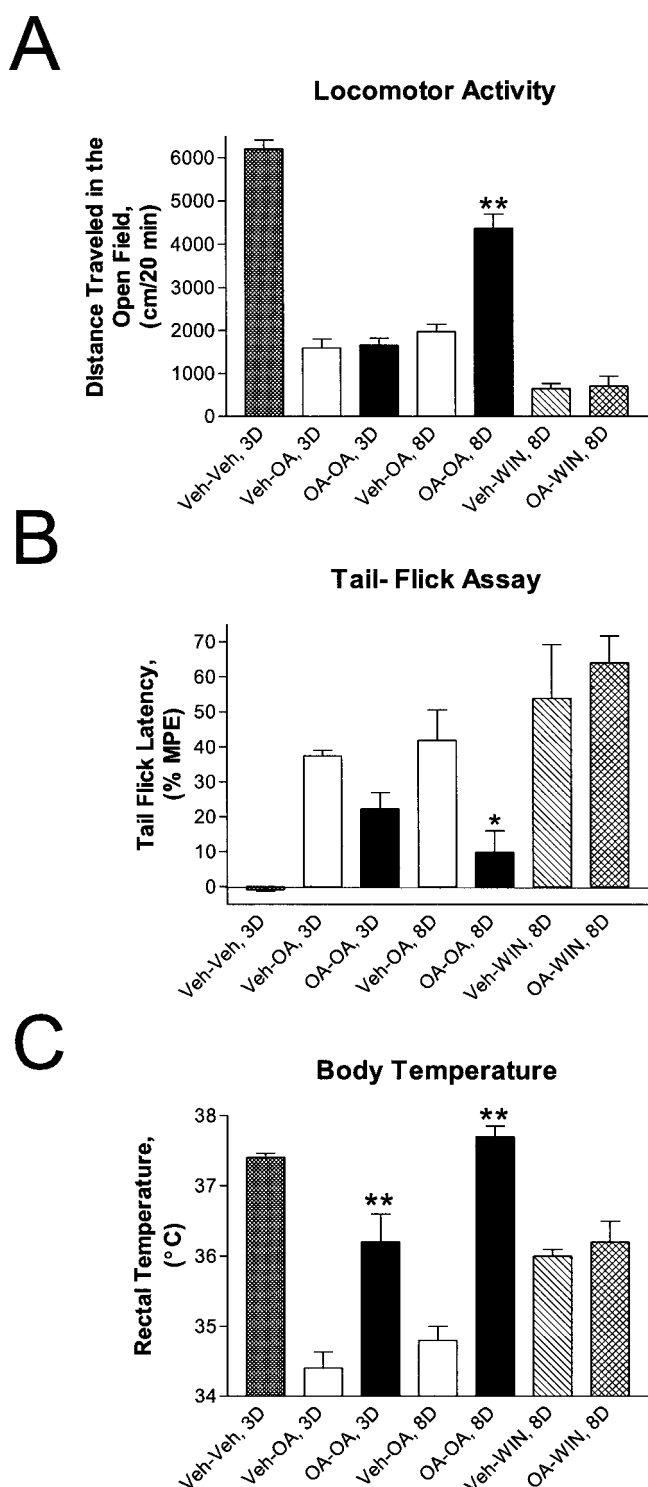


Fig. 3. Tolerance develops to the motor, analgesic, and hypothermic actions of oleamide. Three days of oleamide administration (see *Materials and Methods* for complete protocol) did not produce tolerance to any of its actions following a challenge dose [20 mg/kg, oleamide (OA)-OA 3D] except for hypothermia (C). In contrast, after 8 days of treatment, administration of a challenge dose of oleamide (OA-OA, 8D) had no effect on the distance traveled in the open field (A), tail-flick latency (B), or body temperature (C). Furthermore, there was no cross-tolerance to the actions of WIN 52466 in any of these assays (Veh-WIN, OA-WIN, 8D, hatched and cross-hatched bars in each panel). *, **, significantly different from corresponding vehicle treated group (Veh-Veh, Veh-OA, 3D, 8D) $P < 0.05$, 0.01 , ANOVA followed by Bonferroni's post hoc comparison matrix. Each column represents the mean $\pm$ S.E.M. of data obtained from six rats.

traveled by rats treated with vehicle for 8 d after a challenge dose of oleamide (1700 ± 170 cm). Despite evidence for the development of tolerance to challenge doses of oleamide, there was no cross-tolerance to the actions of the CB receptor agonist WIN 52466 (Fig. 3). Administration of WIN 52466 (5 mg/kg) to rats treated for 8 days with oleamide significantly depressed the distance traveled in the open field (700 ± 230 cm/20 min, Fig. 3A) relative to vehicle-treated controls ($P < 0.01$, ANOVA followed by Bonferroni's post hoc comparison matrix), while increasing tail-flick latency ($64 \pm 7.7\%$ MPE, Fig. 3B, $P < 0.01$, ANOVA followed by Dunnett's post hoc comparison test). However, no significant decrease in body temperature was observed after the administration of this dose of WIN 52466 ($36.2 \pm 0.3^\circ\text{C}$, Fig. 3C).

This evidence suggests that tolerance develops to the behavioral effects of oleamide. The possibility that physical dependence upon oleamide could also develop was therefore investigated. Oleamide administration was stopped after 10 days of administration (see *Materials and Methods*) and the presence of spontaneous withdrawal signs observed over a 1-h period beginning 16 h after the last oleamide dose. The type (Fig. 4A) and number (Fig. 4B) of observed withdrawal behaviors occurring spontaneously after cessation of oleamide administration were not different from those observed in vehicle-treated rats (1.0 ± 0.45 versus 3.0 ± 0.78 , cumulative abstinence score, vehicle versus oleamide). However, when withdrawal was precipitated by the administration of 4.5 mg/kg of the CB₁ receptor antagonist SR 141716A, the type of behaviors (Fig. 4C) and the amplitude of the abstinence scores were significantly different from those observed in vehicle-treated rats (6.8 ± 1.6 versus 17 ± 1.0 , vehicle versus oleamide, $P < 0.01$, t test, $n = 5$, Fig. 4D).

Insights into the potential mechanism of action of oleamide were provided by the administration of selected receptor antagonists (Table 1). The doses of these agents were chosen to have little or no activity in the behavioral assays used. The primary exception to this was the use of SR 141716A at a dose of 10 mg/kg, which reduced both the distance traveled in the open field (5700 ± 200 versus 3600 ± 250 cm, $P < 0.01$, ANOVA, Bonferroni's multiple comparison matrix, $n = 5$) and tail-flick latency ($-25.3 \pm 1.3\%$ MPE data, $P < 0.01$, ANOVA followed by Dunnett's post hoc test). This dose was used after no effect on oleamide's actions were observed at 1, 4, and 8 mg/kg SR 141716A. Modulation of the locomotor actions of oleamide (20 mg/kg) was observed after pretreatment with the 5HT_{1A} antagonist WAY 100135 (0.1 mg/kg s.c.), and the D₂ antagonist L 741626 (0.1 mg/kg i.p.). These modulations were in opposite directions and consisted of an additional 70% decrease (WAY 100135), or a 77% increase (L 741626) in the distance traveled relative to that induced by oleamide alone. In contrast, the oleamide-induced increase in tail-flick latency was suppressed by SR 141716A (57% decrease), bicuculline (67% decrease), and L 741626 (59% decrease). Body temperature was significantly increased only by bicuculline, although there was a trend toward blockade of hypothermia by L 741626. The 5HT_{2C} antagonist SB 242084 (0.5 mg/kg i.p.), the D₁ antagonist SCH 23390 (0.05 mg/kg i.p.) and the vanilloid receptor antagonist capsazepine (25 mg/kg s.c.) were without effect on oleamide-induced behaviors.

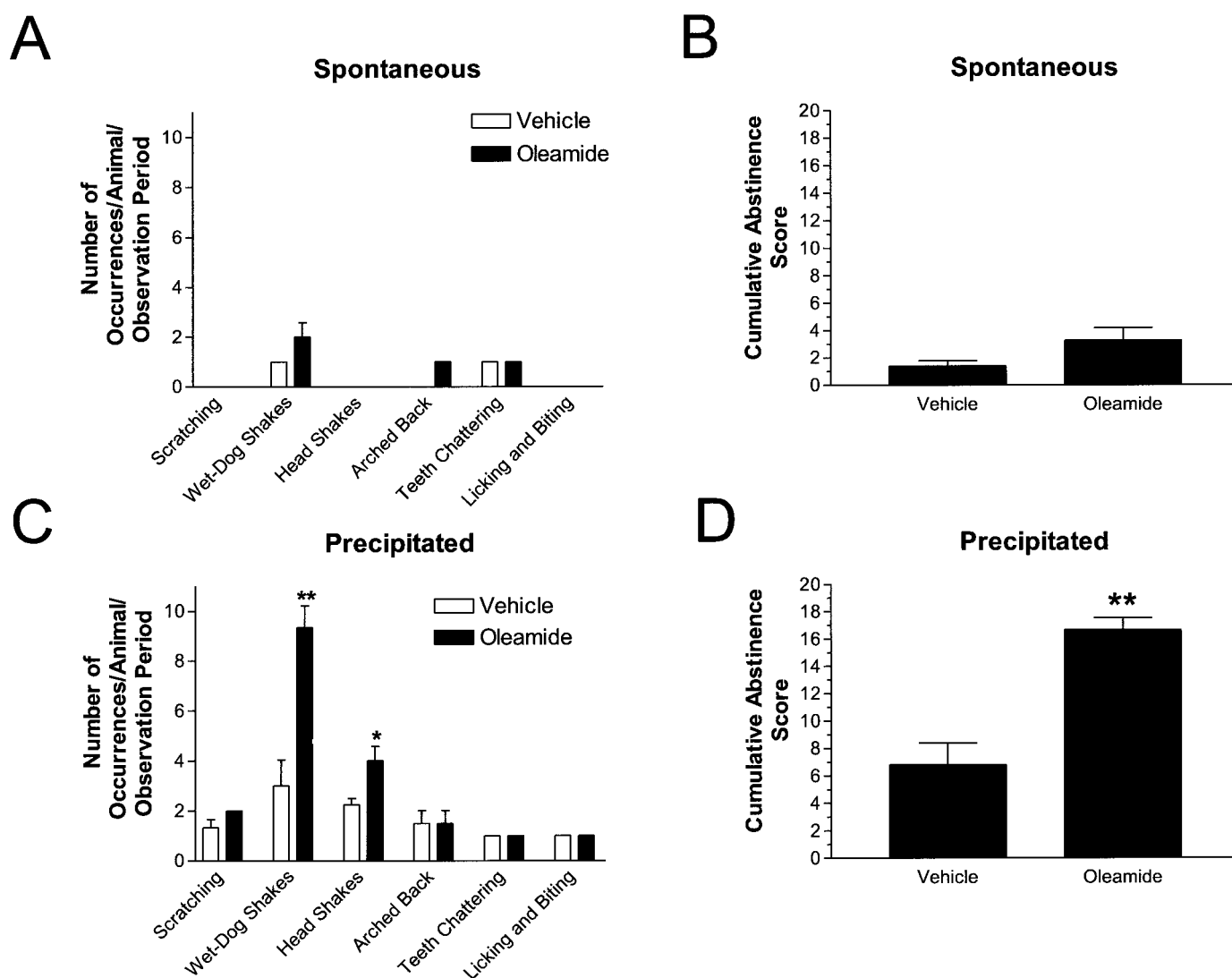


Fig. 4. Characterization of behavioral manifestations of oleamide "withdrawal". Rats were chronically treated with oleamide then allowed to either withdraw spontaneously (A and B) or have withdrawal precipitated with SR-141716A (4.5 mg/kg). Rats undergoing spontaneous withdrawal manifested few of the behavioral actions characteristic of abstinence (A and B), acting very much like vehicle-treated rats. However, when withdrawal was precipitated with SR 141716A, more withdrawal behaviors, particularly wet-dog shakes, were observed (C). Moreover, the cumulative abstinence score for oleamide-treated rats with precipitated withdrawal was significantly higher than vehicle-treated animals (D). **, significantly different from vehicle, $P < 0.01$, t test. Each value represents the mean $\pm$ S.E.M. of observations from six rats.

It has been previously suggested (Mechoulam et al., 1997) that oleamide's effects result from competitive inhibition of FAAH catabolism of AEA, causing an indirect activation of cannabinergic pathways in the brain. In an attempt to provide further insights into oleamide's mechanism of action, oleamide analogs were synthesized that either have very high affinity or are not substrates for FAAH (Table 2). Compounds 1 to 4 bind with high affinity to FAAH but are not substrates for degradation. However, 2 was devoid of behavioral activity at a dose of 20 mg/kg, while 3 and 4 reduced open field activity at 50 mg/kg. The sulfur-based analogs (5–7) of oleamide were inactive as FAAH inhibitors, but 5 was behaviorally inactive. Compounds 6 and 7 were about equally potent in reducing open field activity, although not to the same extent as oleamide. Compounds 1, 6, and 7 increased latency in the tail-flick and hot-plate assays, and induced hypothermia. Compound 1 appeared to be significantly more potent than oleamide in decreasing open field

activity ($ED_{50} = 6.7$, 4.1–9.1 mg/kg, mean, 95% CI), albeit less efficacious ($E_{max} = 3000 \pm 320$ cm at 40 mg/kg). Similarly, 1 was more potent than oleamide in the tail-flick assay ($ED_{50} = 9.3$, 3.9–25 mg/kg, mean, 95% CI), with a maximum latency of $88.7 \pm 17.4\%$ MPE at 40 mg/kg.

Because 1 appeared to be more potent than oleamide in a number of behavioral assays, it was characterized further. Following the administration of 20 mg/kg of 1, the distance traveled in the open field was significantly suppressed for up to 60 min (Fig. 2D), while the hypothermic actions of 1 lasted only 30 min (Fig. 2E). However, the analgesic actions of 1 were sustained for up to 120 min (Fig. 2F). Moreover, the ability of 1 (20 mg/kg) to increase tail-flick latency was inhibited by SR 141716A (1 mg/kg, Fig. 5B), while the 5HT_{1A} antagonist WAY 100135 (0.3 mg/kg s.c.) had no effect (Fig. 5B). The locomotor and hypothermic actions of 1 were unaffected by either antagonist. In addition, 40 mg/kg of 1 was not orally active in any of the three tests.

TABLE 1

Antagonism of oleamide actions

Oleamide (OA) (20 mg/kg i.p.) was injected 15 min after pretreatment with one of the receptor antagonists. Oleamide alone was administered to separate groups of animals in conjunction with each antagonist. Because the results from the individual oleamide groups were statistically identical, the data were pooled for presentation in this table. All antagonists were administered in the same vehicle as oleamide.

Compound	Receptor Pharmacology	Treatment	Distance Traveled	Tail-Flick Latency	Body Temperature
		<i>n, dose</i>	<i>cm/20 min</i>	<i>% MPE</i>	<i>°C</i>
Oleamide		Veh (6, 0.1 ml i.p.)	5700 ± 200	3.7 ± 0.15 (s)	37.4 ± 0.06
		OA (26, 20 mg/kg i.p.)	1100 ± 58	51 ± 5.4	35.3 ± 0.12
SR 141716A	CB ₁ antagonist	SR, (5, 10 mg/kg i.p.)	3600 ± 250	25 ± 1.3	37.3 ± 0.11
		SR + OA (5)	870 ± 210	5.1 ± 6.7*	35.2 ± 0.20
Bicuculline	GABA _A antagonist	BIC (4, 0.5 mg/kg i.p.)	6200 ± 200	-15 ± 0.30	37.4 ± 0.05
		BIC + OA (6)	1200 ± 240	13 ± 3.8**	36.7 ± 0.25**
WAY 100135	5HT _{1A} antagonist	WAY (6, 0.1 mg/kg s.c.)	5000 ± 160	16 ± 0.11	37.5 ± 0.10
		WAY + OA (7)	400 ± 83**	41 ± 4.3	35.1 ± 0.20
SB 242084	5HT _{2C} antagonist	SB (6, 0.5 mg/kg i.p.)	5500 ± 150	-1.5 ± 1.4	37.6 ± 0.13
		SB + OA (6)	1000 ± 220	34 ± 6.9	35.6 ± 0.40
SCH 23390	D ₁ antagonist	SCH (4, 0.025 mg/kg i.p.)	5300 ± 198	16 ± 3.3	38.1 ± 0.16
		SCH + OA (7)	760 ± 26	47 ± 8.6	34.7 ± 0.23
L 741626	D ₂ antagonist	L (6, 0.1 mg/kg i.p.)	600 ± 140	7.1 ± 1.5	37.5 ± 0.13
		L + OA (4)	2300 ± 300**	6.0 ± 6.1**	36.4 ± 0.39
Capsazepine	Vanilloid antagonist	Cap (6, 25 mg/kg s.c.)	5300 ± 220	0.16 ± 1.8	37.4 ± 0.16
		Cap + OA (4)	1100 ± 261	35.2 ± 3.1	35.2 ± 0.31

*, ** $P < 0.05, 0.01$, ANOVA followed by Bonferroni's post hoc comparison matrix.

Discussion

Oleamide is an endogenous, neuroactive fatty acid amide, as are the other members of this structural family, AEA, 2-arachidonoyl glycerol, and 2-arachidonoyl glyceryl ether (Hanuš et al., 2001). While CB receptors are the primary mediators of the actions of AEA and 2-arachidonoyl glycerol, the primary site of action of oleamide in the central nervous system remains unclear. Oleamide interacts with a number of receptor systems in vitro, including GABA_A (Yost et al., 1998), 5HT_{1A}, 2A, 2C, 7 (Huidobro-Toro and Harris, 1996; Thomas et al., 1997a), G proteins (Thomas et al., 1997a; Boring et al., 1996), and gap junctions (Boger et al., 1998). However, it does not interact directly with CB₁ receptors (Boring et al., 1996). The current behavioral studies were performed to provide insight into the molecular mechanism of oleamide action.

Parenteral administration of oleamide induced a number of behaviors in common with CB₁ receptor agonists. As previously reported, oleamide reduced motor behavior in the open field (Basile et al., 1999), but had no effect on performance in the inclined grid test, unlike the CB₁ agonist WIN 52466, which induced significant catalepsy. Oleamide also induced hypothermia and had moderate, but significant analgesic actions in the tail-flick test, while eliciting a less robust effect in the hot-plate test. Cannabinoids are effective analgesics in both tail-flick and hot-plate tests, but endogenous opioids appear to be involved in cannabinoid actions in the hot-plate test (Manzanares et al., 1999). The inability of oleamide to consistently induce analgesia in the hot-plate test may reflect an inability to activate these opioid effectors. Finally, low doses of oleamide were anxiolytic, as indicated by both the social interaction test and elevated plus-maze. While oleamide was effective in less than 50% of the rats tested in the elevated plus-maze, those actions were pronounced in the responding animals. This was indicated by dramatic increases in both the time spent in the open arm and overall exploratory behavior, reflected in the total number of arm entries. These actions of oleamide took 15 to 30 min to become manifested and lasted approximately 1 h. Full

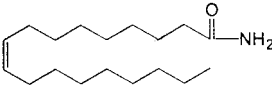
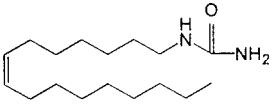
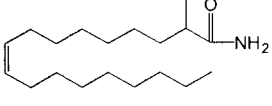
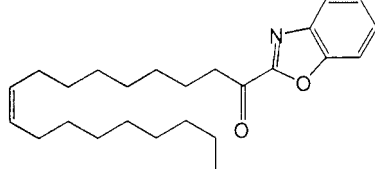
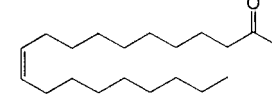
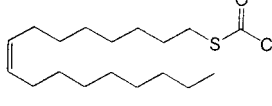
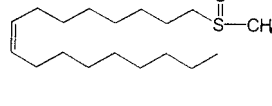
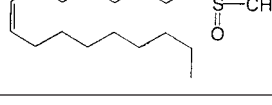
tolerance to the motor, analgesic, and hypothermic actions of oleamide developed after 8 days of administration, but cross-tolerance to the exogenous CB₁ agonist WIN 52466 was not observed. Whether tolerance also develops to the hypnotic actions of oleamide is unknown, but could be a critical factor influencing the utility of oleamide as a sleep-inducing agent. Despite the development of tolerance, oleamide is a very weak inducer of physical dependence. The abstinence score after spontaneous withdrawal of oleamide was not significantly different from that of vehicle-treated rats. This lower score may reflect the lack of severity of the withdrawal syndrome. However, longer observation times may be necessary, because the accumulation and redistribution of oleamide from depots formed during the chronic administration protocol may prolong and subdue the manifestations of withdrawal. Nonetheless, while the abstinence syndrome observed following withdrawal precipitated by SR 141716A was more pronounced than that noted during spontaneous withdrawal, the severity of the syndrome was far less than that observed following precipitated withdrawal by opiate-dependent animals (Matthes et al., 1996), or AEA (Costa et al., 2000), lacking many of the peripheral manifestations (diarrhea) as well as some of the more severe, central nervous system-mediated effects (myoclonus).

Because the general behavioral profile of oleamide's actions resembled those of CB receptor agonists, the efficacy of CB receptor antagonists to suppress oleamide-induced behaviors was also investigated. Despite the ability of the CB₁ antagonist SR 141716A to reverse the hypnotic (Mendelson and Basile, 1999) and analgesic actions of oleamide, as well as precipitating a withdrawal-like syndrome in oleamide-dependent rats, SR 141716A was ineffective in blocking the motor-depressant and hypothermic actions of oleamide. This may be due to the difficulty of consistently blocking the actions of endogenous CB receptor ligands with SR 141716A (Chaperon and Thiebot, 1999), but the possible involvement of other neurotransmitter systems in mediating these behaviors cannot be ruled out. Therefore, the effectiveness of antagonists of several other neurotransmitter systems impli-

TABLE 2

Behavioral actions of oleamide analogs

All compounds were tested for resistance to degradation by, or inhibition of FAAH. They were subsequently administered to rats ($n = 7-10$) in a single dose (all doses, 20 mg/kg except for compounds 3 and 4, which were tested at 50 mg/kg).

Compound	Structure	Relative Rate of FAAH Hydrolysis	Distance Traveled in Open Field	Tail-Flick Latency	Body Temperature
Vehicle		N/A	<i>cm/20 min</i> 6500 ± 490	<i>% MPE</i> 3.9 ± 0.3 (s)	<i>°C</i> 37.3 ± 0.10
Oleamide		1	2400 ± 330**	63 ± 12*	35.4 ± 0.35**
Compound 1		0.001 ^a	3000 ± 470**	74 ± 5.9**	35.5 ± 0.35**
Compound 2		0.09 ^b	7500 ± 1440		37.1 ± 0.14
Compound 3		$K_i = 0.5 \mu\text{M}^{c,d}$	4600 ± 350**		
Compound 4		$K_i = 0.0012 \pm 0.0004 \mu\text{M}^{c,d}$	3900 ± 670*		
Compound 5		>100 μM^e	5000 ± 530*	-1.6 ± 0.16	37.3 ± 0.11
Compound 6		>100 μM^f	3600 ± 460**	28.0 ± 2.4*	35.4 ± 0.28**
Compound 7		>100 μM^f	3700 ± 520**	33.4 ± 2.0*	35.1 ± 0.34**

^a Compound 1 stable to degradation by FAAH. May act as intrinsic agonist or weak competitive inhibitor of oleamide hydrolysis by FAAH.

^b Compound 2 stable to degradation by FAAH but lacks intrinsic agonist properties.

^c Compounds 3 and 4 bind with relatively high affinity to FAAH and act as competitive inhibitors of oleamide hydrolysis by FAAH.

^d From Patterson et al. (1996).

^e Compound 5 is inactive as FAAH inhibitor and is devoid of agonist properties.

^f Compounds 6 and 7 are inactive as FAAH inhibitors but show intrinsic agonist properties.

*, ** Significantly different from vehicle-treated values, $P < 0.05$, 0.01, t test. $n = 7$ for all drug groups, 14 for vehicle.

cated in the actions of oleamide were investigated, with mixed results. While serotonin-regulated behaviors were not specifically studied, antagonists of 5HT receptor subtypes had no effect on the oleamide-induced behaviors investigated. Despite the evidence for interaction of AEA with vanilloid receptors (Smart and Jerman, 2000), the vanilloid receptor antagonist capsazepine was also without effect. In

contrast, doses of bicuculline that were below the threshold for inducing seizures or hypolocomotion effectively blocked both the analgesic and hypothermic effects of oleamide. These observations are consistent with the involvement of GABA_A receptors in the actions of oleamide, but may be mediated through a direct interaction with the receptor, as opposed to an indirect mechanism involving AEA, which

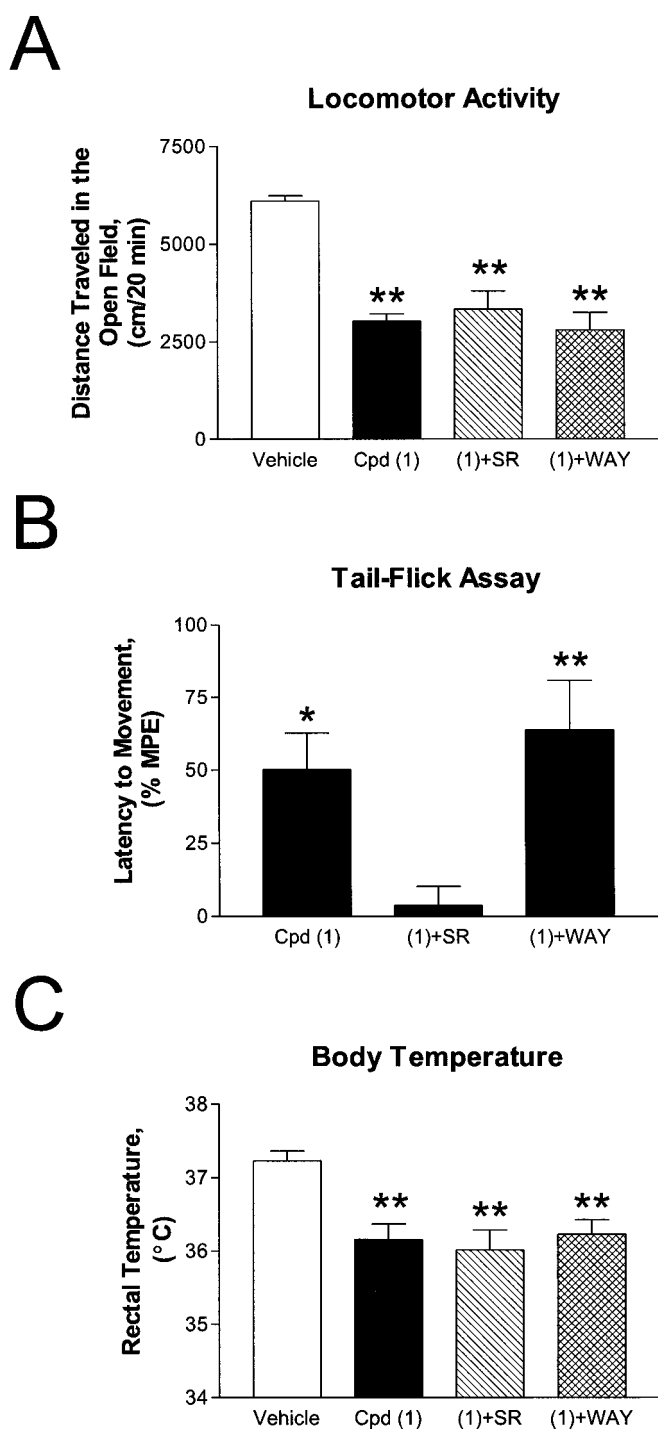


Fig. 5. Effects on compound 1-induced behaviors by CB and 5HT_{1A} receptor antagonists. The actions of a dose of 1 that reduced open field activity (A), induced analgesia (B), and lowered body temperature (C) were unaffected by the 5HT_{1A} antagonist WAY 100135 (cross hatches). However, the cannabinoid receptor antagonist SR 141716A blocked the analgesic actions of 1 (B, hatches). *, **, significantly different from vehicle, $P < 0.05$, 0.01 , ANOVA followed by Dunnett's post hoc test. Each column represents the mean $\pm$ S.E.M. of data obtained from seven rats.

suppresses GABA release in several brain regions (de Miguel et al., 1998; Gifford et al., 1999) and would be expected to complement the actions of bicuculline. Interestingly, a D₂ receptor antagonist was effective in reversing both the oleamide-induced analgesia and depression of motor activity.

Although the multiple behavioral actions of oleamide may appear to be disparate, they bear some consistency with the hypothesis that the cannabimimetic actions of oleamide result from increasing the levels of the endogenous CB receptor ligand AEA (Mechoulam et al., 1997). Furthermore, many of the actions of AEA are dissociable from those of exogenous CB receptor agonists, such as Δ^9 -tetrahydrocannabinol and WIN 52466. This hypothesis is supported by the relatively incomplete behavioral profile of oleamide (and AEA) relative to exogenous CB₁ agonists (no catalepsy, inconsistent hot-plate activity) (Chaperon and Thiebot, 1999); the long lag time for the onset of oleamide activity, suggesting that endogenous concentrations of AEA must increase to effective levels; the minor physical dependence induced by oleamide relative to exogenous CB agonists (Aceto et al., 1998; Cook et al., 1998; Costa et al., 2000); and the difficulty of CB antagonists to reverse the behavioral actions of oleamide (Chaperon and Thiebot, 1999; B. Martin, personal communication). Finally, the ability of a D₂ receptor antagonist to block the locomotor and analgesic actions of oleamide are consistent with the involvement of dopamine receptors in cannabinoid-induced behaviors (Castellano et al., 1997; Nava et al., 2000) and their ability to increase AEA levels (Giuffrida et al., 1999).

While it has been suggested that the cannabimimetic actions of oleamide may be mediated indirectly through elevations in endogenous AEA levels, whether this mechanism is operative in the current study and how it occurs is unclear. It has been proposed that oleamide suppresses AEA catabolism by serving as a competitive "decoy" substrate for FAAH (Mechoulam et al., 1997). For this reason, we tested a number of analogs for their ability to bind with high affinity to, while inhibiting or resisting catabolism by, FAAH. These analogs were designed with the intention of increasing oleamide's potency and duration of action, or potentiating the effect of endogenous oleamide. One of the high-affinity FAAH ligands tested was compound 1. While it was behaviorally active, it was no more active than oleamide itself. Compound 1 was highly resistant to FAAH catabolism and could serve as a stable oleamide agonist. However, it was behaviorally only twice as potent as oleamide and not as efficacious. Moreover, the duration of action of 1 was not significantly greater than that of oleamide, with the possible exception of an increase in the duration of analgesic activity. Similarly, compounds 6 and 7, which were not FAAH inhibitors, mimicked but did not surpass the activity of oleamide despite their resistance to degradation by FAAH. Finally, two potent, competitive inhibitors of FAAH (3 and 4) had behavioral effects analogous to oleamide, possibly by increasing endogenous concentrations of either oleamide and/or AEA. The relative lack of difference in the behavioral activity of agents that differed greatly in their affinity for and sensitivity to degradation by FAAH suggests that oleamide-induced behaviors may result not only from the involvement of other neurotransmitter systems (e.g., GABA_A receptors) but also through increases in the levels of endogenous AEA resulting from alternative mechanisms, such as the blockade of uptake pumps.

In summary, these results indicate that oleamide and its analogs have significant hypnotic, analgesic, and anxiolytic actions as well as a very low dependence liability. This contrasts with exogenous cannabinoid agonists, which not only

induce a greater range of locomotor impairments but also carry the potential for inducing a more severe physical dependence syndrome. While we provide evidence for the interaction of oleamide with other neurotransmitter-receptor systems (e.g., GABA), many of oleamide's behavioral effects are consistent with its being an indirect cannabimimetic, increasing either the levels or activity of endogenous cannabinoids (e.g., AEA). The mechanism by which this occurs remains unclear and may include the suppression of AEA uptake, because oleamide analogs resistant to catabolism by FAAH were no more effective than the parent compound.

Acknowledgments

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