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9. Concentric dialysis probes were prepared with AN 69 fibers (Hospal Dasco, Italy) as described (*21*). All animal experimentation was conducted in accordance with European Economic Community guidelines for care and use of experimental animals. Male Sprague-Dawley rats (280 to 300 g; Charles River, Calco, Como, Italy) were anesthetized with ketamine (100 mg/kg ip) and placed in a stereotaxic apparatus. The skull was exposed and a small hole drilled to expose the dura on each side. Each rat was implanted with one dialysis probe on each side, aimed at the NAc shell on one side and at the core on the other side, according to the rat brain atlas of G. Paxinos and C. Watson [*The Rat Brain in Stereotaxic Coordinates* (Academic Press, Sydney, 1987)] [uncorrected coordinates: shell, A = +2.0, L = 1.4, V = 8.0; core, A = +1.4, L = 2.0, V = 7.8 (A, anterior; L, lateral; and V, ventral)]. Under halothane anesthesia, a polyethylene catheter was inserted into the right femoral vein and then tunneled subcutaneously to exit at the nape of the neck. A femoral vein was catheterized and experiments were performed 24 hours after implant of probes. Ringer's solution (147 mM NaCl, 2.2 mM $CaCl_2$, and 4 mM KCl) was pumped through the dialysis probes at a constant rate of 1 μl/min. Dialysate samples (10 μl) were taken every 10 min and injected without purification into a reversed-phase high-performance liquid chromatography apparatus (LC-18 DB, 15 cm, particle size 5 μm; Supelco) and a coulometric detector (ESA; Coulochem II, Bedford, MA) to quantify dopamine. The oxidation and reduction electrodes of the detector were set at +130 mV and –175 mV, respectively. The mobile phase contained 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM *n*-octyl sodium sulfate, and 18% (v/v) methanol (pH adjusted to 5.5 with Na_2HPO_4). The mobile phase was pumped with an LKB 2150 pump at 1.0 ml/min. Assay sensitivity for dopamine was 2 fmol per sample.
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12. Heroin (Salars, Milano, Italy) was dissolved in saline with the aid of a drop of glacial acetic acid and administered iv (1 ml/kg).
13. SR141716A was suspended in 0.3% Tween 80 in saline and administered ip (3 ml/kg). Naloxone (Sigma, Milano, Italy) was dissolved in saline and administered sc (1 ml/kg).
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15. Naloxonazine (RBI Chemicals) was infused bilaterally into the VTA through stainless steel cannulas (uncorrected coordinates: A = –3.0 from bregma, L ± 1.0, V = –8.5 from dura) [L. J. Pellegrino *et al.*, *A Stereotaxic Atlas of the Rat Brain* (Plenum, New York, 1979)].
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17. The observation that in the Sprague-Dawley rats naloxone reduces the effect of Δ^9-THC on dialysate DA in the NAc is consistent with observations in Lewis rats (*20*).
18. S. W. Johnson and R. A. North [*J. Neurosci*. **12**, 483 (1992)] provided electrophysiological evidence for the existence of non-DA neurons in the VTA that tonically inhibit the DA neurons and are depressed by μ-opiates such as morphine. This neural system might also be the substrate of the DA-stimulating action of Δ^9-THC. Δ^9-THC may promote the release of endogenous opioids in the VTA, but its primary site of action could be outside the VTA, on neurons projecting to the VTA.
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Activation of Corticotropin-Releasing Factor in the Limbic System During Cannabinoid Withdrawal

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Corticotropin-releasing factor (CRF) has been implicated in the mediation of the stress-like and negative affective consequences of withdrawal from drugs of abuse, such as alcohol, cocaine, and opiates. This study sought to determine whether brain CRF systems also have a role in cannabinoid dependence. Rats were treated daily for 2 weeks with the potent synthetic cannabinoid HU-210. Withdrawal, induced by the cannabinoid antagonist SR 141716A, was accompanied by a marked elevation in extracellular CRF concentration and a distinct pattern of Fos activation in the central nucleus of the amygdala. Maximal increases in CRF corresponded to the time when behavioral signs resulting from cannabinoid withdrawal were at a maximum. These data suggest that long-term cannabinoid administration alters CRF function in the limbic system of the brain, in a manner similar to that observed with other drugs of abuse, and also induces neuroadaptive processes that may result in future vulnerability to drug dependence.

Cannabis continues to be a major drug of abuse, and as many as 9% of *Cannabis* users may meet criteria for substance dependence (*1*). Short-term exposure to *Cannabis* derivatives (hashish, marijuana) produces subjective emotional responses ranging from mild relaxation to panic reactions (*1, 2*); long-term use of *Cannabis* may result in mental lethargy and anhedonia (*3*). A clear-cut abstinence syndrome is rarely reported, presumably because of the long half-life of cannabinoids, which precludes the emergence of abrupt abstinence symptoms (*1*), although nervousness, tension, restlessness, sleep disturbances, and anxiety have been described in humans, monkeys, and rats after termination of long-term cannabinoid administration (*4*). A distinct abstinence syndrome can, however, be elicited in animals treated with cannabinoids over a long period (*5*) by administering a competitive cannabinoid antagonist (*6*). This antagonist-precipitated withdrawal may unmask the development of underlying neuroadaptive processes that contribute to the development of cannabinoid dependence. The neurobiological substrates of cannabinoid-induced emotional responses remain to be elucidated, although they are likely to be mediated by activation of CB_1 cannabinoid receptors, which are present in the limbic system and brain nuclei that have been implicated in stress responses (*7*). Psychotropic cannabinoids are potent activators of the hypothalamic-pituitary-adrenal (HPA) axis (*8*), and this property may contribute to the unpleasant side effects described by users of *Cannabis*.

A common element of withdrawal from drugs of abuse is a negative affective state that is characterized in humans by dysphoria and anxiety and in animals by a reward deficit and enhanced behavioral reactivity to stressors (*9*). We report here that cannabinoid withdrawal, induced by administration of a cannabinoid CB_1 antagonist, re-

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sults not only in enhanced behavioral responses to stressors but also in increased release of CRF and induction of c-*fos* in the central nucleus of the amygdala. Our data reveal an unambiguous neurochemical response in the limbic system, attributable to long-term cannabinoid exposure, similar to that produced by other major drugs of abuse (*9, 10*). This finding supports the hypothesis that cannabinoids can set in motion neuroadaptive processes in the brain that contribute to the development of substance dependence.

Among the various brain neurochemical and neuroendocrine systems that participate in the mediation of motivational aspects of drug dependence, CRF may hold a prominent position (*11*). Recent evidence suggests that hypothalamic and extrahypothalamic CRF systems have a role in mediating cannabinoid-induced anxiety (*12*). Short-term treatment with the CB_1 cannabinoid receptor agonist (−)-Δ^8-tetrahydrocannabinol dimethyl heptyl (HU-210) activates the HPA axis in rats (*12*). In addition to activation of the pituitary-adrenal axis by hypothalamic CRF neurons (*13*), brain CRF systems, particularly in the central nucleus of the amygdala, appear to mediate behavioral responses to stressors. CRF neurons and receptors in the central nucleus of the amygdala participate in the arousal-enhancing properties of psychostimulants as well as in behavioral sensitization (*14*) and play a key role in anxiety reactions observed during ethanol withdrawal (*10, 11*). A CRF antagonist, α-helical CRF(9–41), can also attenuate the anxiogenic behavioral effects of HU-210. Moreover, studies with intracranial microdialysis indicate that immobilization stress as well as ethanol and cocaine withdrawal result in elevated extracellular concentrations of CRF in the central nucleus of the amygdala (*10*).

We exploited the availability of a CB_1 cannabinoid receptor antagonist, SR 141716A, to evaluate the role of the central amygdaloid CRF system in the effects of short-term and long-term cannabinoid exposure as well as in cannabinoid withdrawal (*15*). We used intracranial microdialysis (*16, 17*) to examine changes in extracellular CRF in the rat central nucleus of the amygdala in response to a single administration of either HU-210 or SR 141716A (Fig. 1D). Release of CRF was also monitored in rats after long-term (2 weeks) exposure to HU-210 and during a behavioral withdrawal syndrome induced by injection of SR 141716A after long-term exposure to HU-210. We studied the temporal profile of the abstinence syndrome as well as anxiety-like responses in different experimental groups with observational measures (*18*) and a defensive withdrawal test (*19*) previously characterized for use with cannabinoids (*12*). In addition, we examined activation of the HPA stress response by measuring plasma corticosterone concentrations (*20*). Finally, we examined the anatomical distribution of cannabinoid-responsive brain areas by analyzing the appearance of Fos immunoreactivity (*21*), which has been an effective tool for mapping neural activity after stress (*22, 23*), CRF administration (*24*), and drug withdrawal (*25*).

A single injection of HU-210 decreased

Table 1. Defensive withdrawal test results (seconds spent in the chamber; mean ± SEM) and plasma corticosterone concentrations (mean ± SEM) in rats after a single dose of HU-210 or SR 141716A and after long-term (14 days) exposure to HU-210 alone or followed by a single dose of SR 141716A.

	Single dose			Long term	
	Vehicle	HU-210	SR 141716A	HU-210	HU-210 + SR 141716A
Defensive withdrawal					
Time (s)	460 ± 41	693 ± 82*	751 ± 97*	518 ± 50	780 ± 75*
N	11	9	8	9	9
Plasma corticosterone					
Concentration (ng/ml)	175 ± 29	345 ± 40*	185 ± 34	249 ± 39	325 ± 45*
N	9	10	9	11	9

*$P < 0.05$ versus vehicle.

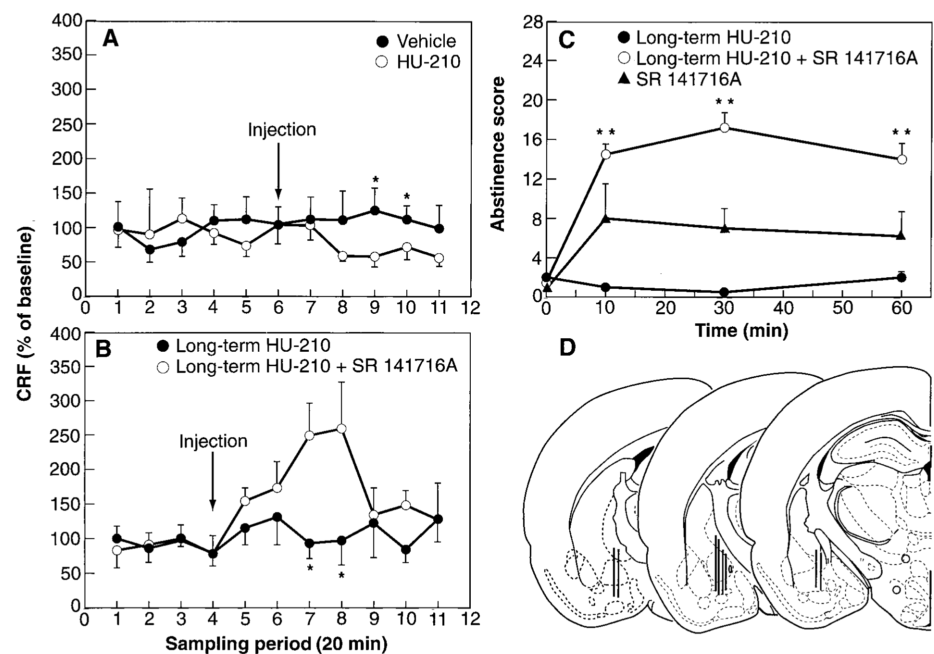

Fig. 1. (**A**) Effects of a single injection of HU-210 (100 μg/kg) on CRF release from the central nucleus of the amygdala. Statistical analysis (one-way analysis of variance for repeated measures) revealed that HU-210 lowered CRF release [$F_{11,66} = 1.99$, $P < 0.05$ (*), $N = 7$]. Vehicle injections did not alter CRF efflux ($F_{11,55} = 0.45$, $P = 0.93$, $N = 6$). Administration of SR 141716A did not modify CRF release (*31*). (**B**) Effects of SR 141716A (3 mg/kg) on CRF release from the central amygdaloid nucleus in animals pretreated for 14 days with HU-210 (100 μg/kg). Cannabinoid withdrawal induced by SR 141716A was associated with increased CRF release [$F_{11,77} = 3.54$, $P < 0.005$ (*), $N = 8$]. Vehicle injections did not alter CRF efflux ($F_{11,66} = 0.69$, not significant, $N = 7$). Data in (A) and (B) were standardized by transforming dialysate CRF concentrations into percentages of baseline values based on averages of the first four fractions. (**C**) Mean ± SEM of summed cannabinoid withdrawal scores 0, 10, 30, and 60 min after injection of SR 141716A in rats treated for 14 days with HU-210 or its vehicle. The cannabinoid antagonist induced a mild behavioral syndrome in drug-naïve rats receiving long-term pretreatment with vehicle (SR 141716A) and a clear withdrawal syndrome in animals pretreated with HU-210 (long-term HU-210 + SR 141716A) [$F_{2,18} = 33.49$, $P < 0.0001$ (**)]. Rats pretreated with cannabinoid (long-term HU-210) that received vehicle on the test day did not exhibit withdrawal signs. Drug-naïve control animals that received vehicle injections were indistinguishable from the long-term HU-210 treatment group, and cannabinoid-naïve rats did not exhibit observable changes in behavior after a single injection of HU-210 (*31*). (**D**) Anatomical location of the active region of microdialysis probes (outer diameter, 0.5 mm) in animals subjected to SR 141716A–induced cannabinoid withdrawal.

Table 2. Distribution of Fos-immunopositive cells after a single dose of HU-210 or SR 141716A and after long-term (14 days) exposure to HU-210 alone or followed by a single dose of SR 141716A. Each group contained three or four animals. Number of immunopositive cells is indicated as follows: 0 (−), 1 to 10 (+), 11 to 20 (++), 21 to 30 (+++), 31 to 50 (++++), >50 (+++++).

	Single dose			Long term	
	Vehicle (*N* = 4)	HU-201 (*N* = 3)	SR 141716A (*N* = 4)	HU-210 (*N* = 3)	HU-210 + SR 141716A (*N* = 4)
Cortex					
Piriform cortex	−	−	−	−	+++++
Hippocampus	−	+	+	−	+++
Basal ganglia					
Caudate-putamen	−	−	−	−	+++
Ventral pallidum	−	−	−	−	+++
Accumbens core	+	++	−	−	−
Extended amygdala					
Accumbens shell	+	++++	++	+	++++
Central amygdala	+	++++	+	+	++++
BNST (medial anterior)	−	+	−	−	+++
BNST (lateral ventral)	−	++	−	−	++
BNST (lateral dorsal)	−	++++	−	−	−
Thalamus					
Paraventricular anterior	+++	++++	+++	++	++++
Hypothalamus					
Supraoptic	−	++	−	+	++
Suprachiasmatic	−	+	+	−	++
Paraventricular	−	++++	++	−	++
Brainstem					
Substantia nigra	+	−	+	−	++
VTA	+	+++	+	+	++++
Locus coeruleus	−	+	−	−	+++
Central gray	+	+	++	−	++
Solitary tract	+	++++	+	−	+++++
Area postrema	−	+	+	−	+++

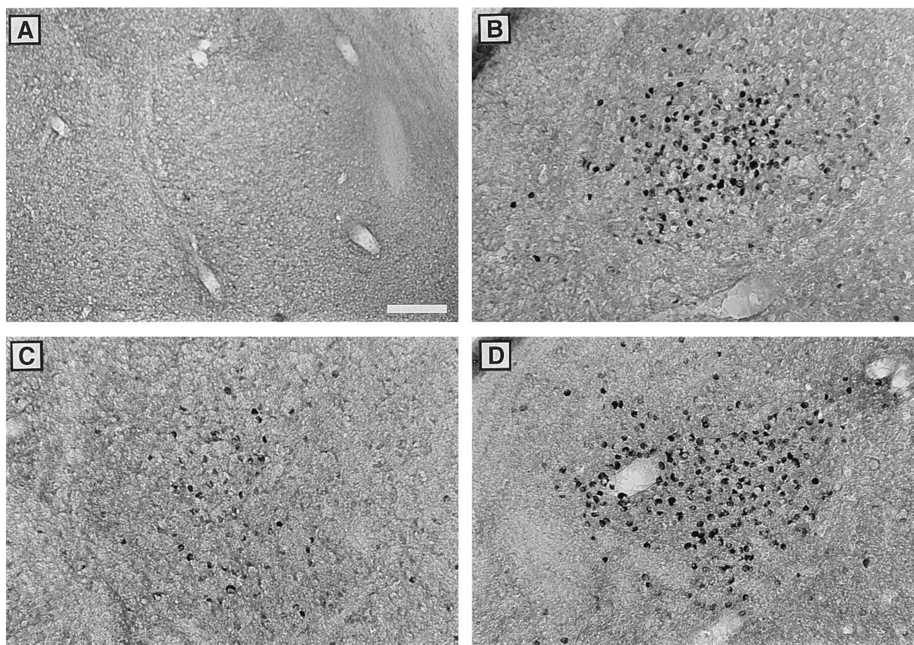

Fig. 2. Brain sections showing Fos immunoreactivity in cell nuclei of the central amygdaloid nucleus of rats after a single injection of vehicle (**A**), HU-210 (**B**), or SR 141716A (**C**) and during SR 141716A–induced withdrawal in rats receiving long-term HU-210 pretreatment (**D**). Fos immunoreactivity in rats undergoing long-term HU-210 treatment that received vehicle was indistinguishable from saline controls in (A) (*31*). Fos-immunopositive cell count (mean ± SEM) was as follows: 1.7 ± 1.7 [vehicle (*N* = 4)], 36.7 ± 6.7 [HU-210 (*N* = 3)], 8.0 ± 4.6 [SR 141716A (*N* = 4)], and 32.5 ± 4.4 [SR 141716A after long-term HU-210 (*N* = 4)]. Scale bar, 50 μm.

the amount of CRF released from the central nucleus of the amygdala (Fig. 1A). The inhibitory effects of HU-210 on CRF release were still apparent 24 hours after completion of the long-term cannabinoid treatment regimen (*26*). In contrast, induction of withdrawal by SR 141716A after long-term exposure to HU-210 had the opposite effect and increased CRF efflux (Fig. 1B). The increase in extracellular CRF concentration paralleled the progression of behavioral withdrawal symptoms over time (Fig. 1C). The behavioral changes after administration of the cannabinoid antagonist in animals receiving long-term treatment with HU-210 were also reflected in anxiety-like responses in the defensive withdrawal test (*27*) and activation of the HPA axis as revealed by increased plasma corticosterone concentrations (Table 1). In addition, the changes in extracellular CRF concentration were accompanied by the appearance of increased Fos immunoreactivity in the central nucleus of the amygdala (Table 2 and Fig. 2).

The distribution of Fos immunoreactivity indicated that induction of withdrawal by SR 141716A activated not only the central nucleus of the amygdala but also other stress-responsive brain sites that receive projections from this nucleus, such as the shell of the nucleus accumbens, the bed nucleus of the stria terminalis (BNST), the paraventricular nucleus of the hypothalamus (PVN), and brainstem structures involved in autonomic responses to stress, including the nucleus of the solitary tract (NTS). Cannabinoid withdrawal was associated with activation of stress-related mid- and hindbrain nuclei, including the ventral tegmental area, locus coeruleus, central gray, NTS, and, especially, the area postrema (Table 2), brain regions that are also activated during opiate withdrawal (*25*) and are recognized as critical for conveying stress information to the PVN, which ultimately triggers the HPA response (*28*).

Cannabinoid withdrawal was also associated with increased Fos immunoreactivity in extrapyramidal motor regions that are rich in CB_1 receptors (*7*), such as the caudate-putamen, ventral pallidum, and substantia nigra. This profile of neural activation is consistent with the dominant behavioral symptoms of cannabinoid withdrawal in rats (compulsive grooming and scratching, forepaw treading, and rubbing of the face), and it points toward involvement of the basal ganglia in the motor component of cannabinoid withdrawal. The greatest Fos immunoreactivity was found in the piriform cortex, a cortical area involved in limbic kindled seizures (*29*), which were observed in 2 of 10 animals.

Blockade of CB_1 receptors with SR

141716A in cannabinoid-naïve rats did not alter release of CRF in the amygdala and produced a different pattern of neuronal activation than in animals undergoing long-term HU-210 treatment. The antagonist increased Fos immunoreactivity mainly in the PVN, accumbens shell, and central gray matter, and it induced anxiety-like responses in both the defensive withdrawal test (Table 1) and the elevated plus-maze tests (*30*). However, SR 141716A did not increase plasma corticosterone concentrations in drug-naïve animals (Table 1). In conjunction with earlier findings that SR 141716A can increase arousal and disrupt the sleep-waking cycle in rats (*6*), this observation suggests that endogenous "cannabinoid tone" may have a role in normal behavioral function without affecting HPA activity.

Together, our results provide in vivo neurochemical, endocrinological, and immunocytochemical evidence that long-term exposure to cannabinoids leads to neuroadaptive changes that result in enhanced release of CRF in the central amygdala as well as activation of stress-responsive nuclei during cannabinoid withdrawal. These changes are consistent with the irritability and discomfort that have been described to occur after cessation of long-term consumption of marijuana (*4*). Moreover, the neurobiological consequences of cannabinoid withdrawal, in particular the alteration in amygdaloid CRF function (*9, 10*), are similar to those observed during withdrawal from ethanol, cocaine, and opiates as well as during exposure to environmental stressors. Thus, activation of the amygdaloid CRF system may have a motivational role of mediating the stress-like symptoms and negative affect that accompany withdrawal and, therefore, may be a common element in development of dependence on drugs of abuse. The demonstration that long-term exposure to a cannabinoid agonist evokes neuroadaptive processes in the limbic system that resemble those associated with other major drugs of abuse may provide a neurobiological basis for the gateway hypothesis. Cannabinoid abuse, by activating CRF mechanisms, may lead to a subtle disruption of hedonic systems in the brain that are then "primed" for further disruption by other drugs of abuse (*9*).

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15. Male Wistar rats (250 to 300 g) were used. HU-210 was provided by R. Mechoulam (Hebrew University of Jerusalem). SR 141716A was obtained through SANOFI Recherche (Montpellier, France). Both drugs were prepared in a vehicle solution of saline, propylene glycol, and Tween 80 (90:5:5). Doses were selected on the basis of full dose-response studies (*6, 12*). Drugs were administered intraperitoneally in a volume of 1 ml per kilogram of body weight. Animals assigned to the cannabinoid withdrawal condition received daily injections of HU-210 (100 μg/kg) for 14 days.
16. Intracranial microdialysis for CRF was performed as described (*10*). Fractions of the perfusate were collected at 20-min intervals in polyethylene tubes on ice. Five fractions were collected for determination of basal CRF efflux. Animals were then injected with either HU-210 (100 μg/kg), SR 141716A (3 mg/kg), or vehicle. Rats that had been pretreated with HU-210 (100 μg/kg) for 14 days (the "cannabinoid withdrawal" group) received either SR 141716A (3 mg/kg) or vehicle. Sampling continued for six to eight fractions after drug treatments were terminated, at which time the microdialysis probes were perfused for 60 min with artificial cerebrospinal fluid containing the depolarizing agent 4-aminopyridine (5 mM) (4-AP, Sigma) to confirm the neurogenic origin of CRF. At the end of each experiment, rats were injected with a lethal dose of pentobarbital; their brains were perfused and fixed in 4% paraformaldehyde and then frozen, sectioned, and stained with cresyl violet.
17. Dialysate fractions were analyzed for CRF-like immunoreactivity (CRF-IR) by radioimmunoassay as described [W. Vale *et al.*, *Methods Enzymol.* **103**, 565 (1983)] and adapted for application with microdialysis [E. Merlo Pich *et al.*, *Neuroscience* **55**, 695 (1993)]. Sensitivity of the assay was 0.35 fmol of CRF per 50 μl. Perfusion with 4-AP increased CRF concentration in the dialysates of all groups ($F_{1,29}$ = 11.75, $P < 0.002$ compared with basal concentrations) except after single treatments with HU-210 or SR 141716A. Means ± SEM of basal CRF concentrations versus CRF concentrations after treatment with 4-AP (fmol of CRF-IR per 50 μl) were as follows: 0.99 ± 0.22 versus 2.32 ± 0.9 (vehicle); 1.2 ± 0.29 versus 1.57 ± 0.45 (HU-210); 1.07 ± 0.19 versus 1.75 ± 0.56 (SR 141716A); 0.85 ± 0.2 versus 1.51 ± 0.21 (long-term HU-210); and 0.69 ± 0.12 versus 1.30 ± 0.19 (SR 141716A after long-term HU-210).
18. Cannabinoid withdrawal signs were measured with counted signs (such as wet-dog shakes, compulsive grooming, and scratching sequences) and observed signs (such as ptosis, piloerection, teeth chattering, salivation, and diarrhea). Counted signs (total number of events) were summed with observed signs (events observed over a specified observation time) and subjected to parametric statistical analysis, because sums are normally distributed.
19. The defensive withdrawal test was conducted as described [L. K. Takahashi, N. H. Kalin, J. A. van de Burgt, J. E. Sherman, *Behav. Neurosci.* **103**, 648 (1989)]. The apparatus consisted of an illuminated (350 lux) opaque open field (100 by 100 by 40 cm) marked with squares (20 by 20 cm). The field contained a cylindrical chamber (17 by 10 cm) open at one end and positioned 20 cm from one corner of the field. Rats were placed inside the chamber for 15 min and scored for (i) latency to leave the chamber (emergence latency), (ii) total time spent in the chamber, (iii) mean time spent in the chamber per entry, and (iv) motor activity (rearing and square crossings outside the chamber). Drugs were injected 30 min before the test. All animals were habituated to the apparatus before testing.
20. Plasma corticosterone concentration was monitored in separate groups of rats that were exposed to the same treatment as the animals in the microdialysis experiments. Rats were decapitated 3 hours after treatment. Corticosterone was measured by radioimmunoassay (*8*) with a specific antibody from Bio Clin (Cardiff, England). The detection limit was 62 pg/ml.
21. Three hours after cannabinoid treatments, rats were quickly perfused with 0.9% saline followed by 2% paraformaldehyde in isotonic sodium phosphate buffer (PBS, pH 7.4). Brains were removed, fixed in the perfusion buffer for 24 hours, stored for 3 to 7 days in a 30% solution of sucrose in PBS, sliced in 40-μm sections (Cryocut 1800; Leica, Foster City, CA), and collected in PBS. The Fos protein was quantified by immunohistochemistry analysis with affinity-purified rabbit antibodies to a peptide corresponding to human Fos amino acid residues 3 to 16 (Santa Cruz Biotechnology, Santa Cruz, CA) that was not reactive to Fos-B and Fra-1 proteins. Sections were incubated with goat antiserum to rabbit antibody in 0.3% Triton X-100 in PBS solution for 2 hours at room temperature, followed by Fos antiserum (diluted 1:1000) in 0.3% Triton X-100 containing 0.1% bovine serum albumin in PBS for 20 hours at 4°C [A. E. Ryabinin, K. R. Melia, M. Cole, E. E. Bloom, M. C. Wilson, *J. Neurosci.* **15**, 721 (1995)].
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26. Long-term exposure to HU-210 produced a trend toward lower mean (±SEM) basal CRF concentrations (fmol per 50 ml) in the central nucleus of the amygdala [vehicle, 1.31 ± 0.18 (N = 19); HU-210, 0.90 ± 0.12 (N = 15); $F_{1,32}$ = 3.08, $0.05 < P < 0.1$].
27. In the defensive withdrawal test, a single dose of HU-210 in drug-naïve rats also produced an anxiety-like effect. An important factor in the subjective reac-

tion to cannabinoids is dosage (*1*). Low doses of HU-210 abolish the behavioral response to novelty and inhibit the HPA stress response, whereas higher doses, particularly under conditions of novelty, have the opposite effect (*12*), as in the test described here. Comparative analysis of the patterns of Fos expression in the withdrawal and short-term HU-210 treatment conditions demonstrated an overlap as well as a dissociation of affected brain regions (Table 2), implicating the involvement of different neural substrates in the anxiety-like response induced by a single high dose of cannabinoid as opposed to withdrawal from long-term cannabinoid exposure. In the central amygdala, Fos expression appeared dispersed after a single injection of HU-210, whereas after antagonist-induced withdrawal Fos-positive nuclei were densely distributed (Fig. 2). In the BNST, immunopositive cells were found in a more medial-anterior gradient during cannabinoid withdrawal, whereas Fos activation was more prominent in the lateral dorsal region after short-term cannabinoid exposure (Table 2). In the hypothalamus, the PVN exhibited less Fos immunoreactivity during cannabinoid withdrawal compared with the effects of a single treatment with cannabinoid agonist. Thus, HPA activation after a single exposure to HU-210 in drug-naïve rats appears to be mediated directly by the PVN, whereas the increase in plasma corticosterone concentrations during withdrawal may involve activation of the central amygdala, transmitted to the PVN through its direct connections or by the BNST, which, in turn, may also activate the PVN. Because both the central amygdala and PVN are thought to be involved in anxiety-like behavioral responses to stress (*13, 14*), these observations suggest that the balance between the contributions of both structures after acute cannabinoid treatment or antagonist-induced withdrawal may result in the particular behavioral reactivity to the novelty condition in the defensive withdrawal test.

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Receptor-Mediated Activation of a MAP Kinase in Pathogen Defense of Plants

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Parsley cells recognize the fungal plant pathogen *Phytophthora sojae* through a plasma membrane receptor. A pathogen-derived oligopeptide elicitor binds to this receptor and thereby stimulates a multicomponent defense response through sequential activation of ion channels and an oxidative burst. An elicitor-responsive mitogen-activated protein (MAP) kinase was identified that acts downstream of the ion channels but independently or upstream of the oxidative burst. Upon receptor-mediated activation, the MAP kinase is translocated to the nucleus where it might interact with transcription factors that induce expression of defense genes.

Plants react to pathogen attack with a variety of defense responses, including transcriptional activation of defense genes, accumulation of phytoalexins and pathogen-related (PR) proteins, and impregnation of the cell wall with phenolic substances and specific proteins (*1*). Infection of parsley leaves with spores from the soybean pathogen *Phytophthora sojae* leads to small necrotic lesions resulting from hypersensitive cell death, incorporation of phenolic compounds into, and apposition of callose onto, cell walls at the infection site, as well as local and systemic activation of defense-related genes and secretion of fouranocoumarin phytoalexins into the infection droplet (*2, 3*). Cultured parsley cells show most of these defense reactions when treated with elicitor preparations from the fungus and have been used as a model system to study the plant-pathogen interactions (*4–7*). An extracellular 42-kD fungal glycoprotein was identified in these preparations as the principal elicitor of the multicomponent defense response in parsley cells (*6*). An oligopeptide fragment of 13 amino acids in length (Pep13) within this glycoprotein is necessary and sufficient to induce the same reactions as the intact glycoprotein (*7, 8*). Pep13 specifically interacts with a plasma membrane target site in the plant and initiates a signal transduction cascade leading to the transient activation of plant defense genes and the accumulation of phytoalexins (*7*).

Elicitor signal transduction in parsley cells involves Ca^{2+}-dependent transient changes in protein phosphorylation, suggesting the participation of protein kinases in defense gene activation (9). To detect specific protein kinases that catalyze such reactions, we treated cultured parsley cells with Pep25, a larger fragment of the elicitor that includes the Pep13 sequence and induced an identical response but was more stable in the culture medium than Pep13 (*7*). A protein kinase that phosphorylated myelin basic protein (MBP) was activated within 5 min after elicitor treatment (Fig. 1A). From its relative mobility on SDS-polyacrylamide gels, the apparent molecular mass of this enzyme was estimated to be ~45 kD, similar to that of known plant mitogen-activated protein (MAP) kinases (*10–15*).

To determine whether the elicitor-activated protein kinase might belong to the class of MAP kinases, we incubated the same cell extracts used for activity assays with three different antisera—M7, M11, and M14—that were raised against synthetic peptides representing the COOH-terminal 10 amino acids of the alfalfa MMK4, MMK2, and MMK3 MAP kinases, respectively (*12*). Elicitor treatment exclusively activated a protein kinase that was immunoprecipitated by the M7 antiserum (Fig. 1B). The similarity of the activation kinetics in the kinase and immunoprecipitation assays indicate that elicitor treatment activates a specific MAP kinase pathway in parsley cells.

Because the M7 antiserum specifically recognized the elicitor-responsive MAP kinase from parsley, a radiolabeled fragment of the alfalfa *MMK4* gene was used to screen a cDNA library prepared from RNA isolated from cultured parsley cells. A 1.6-kb cDNA fragment was isolated that contained an open reading frame of 1113 nucleotides potentially encoding a protein of 371 amino acids and a molecular mass of 43 kD. The deduced amino acid sequence is most similar to those of the MAP kinases from *Arabidopsis* (MPK3, 83%) (*11*), alfalfa (MMK4, 81%) (*12*), and tobacco (WIPK, 83%) (*13*). The overall structure of the parsley, tobacco, *Arabidopsis*, and alfalfa kinases is highly conserved (Fig. 2). DNA gel blot analysis of parsley cells with the radiolabeled kinase cDNA fragment under high-stringency hybridization conditions revealed the parsley kinase to be present as a single-copy gene (*16*). RNA gel blot analysis of cultured parsley cells with radiola-

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