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Dopamine activation of endogenous cannabinoid signaling in dorsal striatum

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We measured endogenous cannabinoid release in dorsal striatum of freely moving rats by microdialysis and gas chromatography/mass spectrometry. Neural activity stimulated the release of anandamide, but not of other endogenous cannabinoids such as 2-arachidonylglycerol. Moreover, anandamide release was increased eightfold over baseline after local administration of the D_2-like (D_2, D_3, D_4) dopamine receptor agonist quinpirole, a response that was prevented by the D_2-like receptor antagonist raclopride. Administration of the D_1-like (D_1, D_5) receptor agonist SKF38393 had no such effect. These results suggest that functional interactions between endocannabinoid and dopaminergic systems may contribute to striatal signaling. In agreement with this hypothesis, pretreatment with the cannabinoid antagonist SR141716A enhanced the stimulation of motor behavior elicited by systemic administration of quinpirole. The endocannabinoid system therefore may act as an inhibitory feedback mechanism countering dopamine-induced facilitation of motor activity.

The striatum is a key component of the forebrain system that controls planning and execution of motor behaviors. Excitatory signals generated in sensorimotor and limbic areas of the neocortex and in the thalamus converge on this region, where they are integrated and redistributed to other structures of the basal ganglia and to the substantia nigra[1]. How the striatum integrates these inputs, which are mediated by the fast neurotransmitter glutamate, is only partially understood. Nevertheless, it is generally agreed that slow-acting modulatory substances, including dopamine, acetylcholine and neuroactive peptides, participate in this process by influencing the excitability of striatal neurons[2]. In support of this idea, abnormalities in striatal neuromodulation have been linked to a spectrum of neuropsychiatric disorders, of which Parkinson's disease[3] and Tourette's syndrome[4,5] are two well-documented examples.

Cannabinoid receptors, the pharmacological target of the marijuana constituent Δ9-tetrahydrocannabinol (Δ9-THC), are densely expressed in striatum[6–8], where they are twice as numerous as D_1 dopamine receptors[8] and 12 times as numerous as μ opioid receptors[9]. Activation of cannabinoid receptors has profound consequences on the electrophysiological properties of striatal neurons[10], as well as on motor behaviors that are mediated by striatal projection systems[11]. Furthermore, clinical observations suggest that marijuana and Δ9-THC may be beneficial in psychomotor disorders associated with the basal ganglia, such as Tourette's syndrome[12,13], pointing to an involvement of cannabinoid receptors in abnormal striatal function. Interpreting these results is made difficult, however, by our inadequate knowledge of the intrinsic signaling system by which cannabinoid receptors are engaged. Indeed, although several endogenous cannabinoid (endocannabinoid) ligands, including anandamide[14,15] and 2-arachidonylglycerol (2-AG)[16–18], have been identified and their biosynthetic routes partially elucidated[15,18–21], the physiological mechanisms that regulate release of these compounds remain elusive.

Results

Anandamide release *in vivo*

We examined the occurrence and regulation of endogenous cannabinoid release in the dorsal striatum of freely moving rats by using microdialysis combined with isotope dilution gas-chromatography/mass-spectrometry (GC/MS)[22]. Microdialysis samples obtained during 30-min collections under baseline conditions contained detectable levels of anandamide (1.5 ± 0.3 pmol per sample, mean ± s.e.m, n = 60; Fig. 1), palmitylethanolamide (PEA), an acylethanolamide that activates peripheral CB2-like receptors[23,24] (0.7 ± 0.1 pmol/sample), and oleylethanolamide, the functions of which remain unknown[19,25] (1.5 ± 0.2 pmol per sample). By contrast, 2-AG was not detectable under these conditions (data not shown). No measure was taken in these analyses to prevent the uptake and enzymatic hydrolysis of anandamide and 2-AG[15,18,26–30]; thus the impact of these inactivation processes on endogenous cannabinoid levels remains to determined.

To test whether endogenous cannabinoids are released by neural activity, we perfused the striatum with artificial cerebrospinal fluid (ACSF) containing a depolarizing concentration

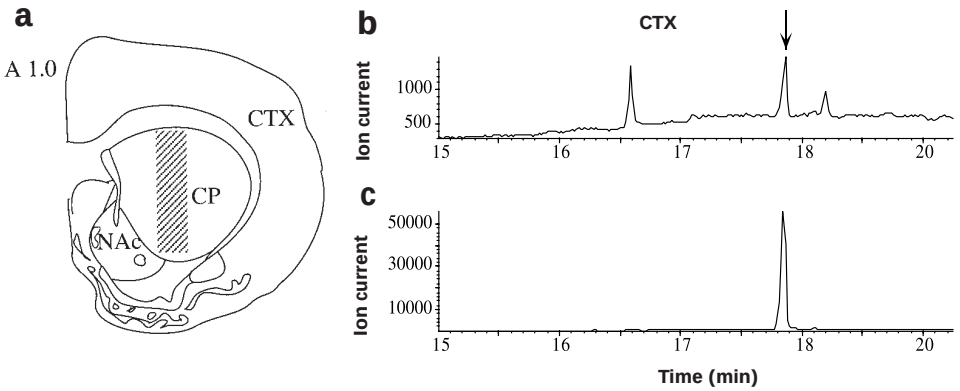

Fig. 1. Microdialysis of endogenous cannabinoids in rat brain. **(a)** Striatal location of the microdialysis probes. The stippled area indicates the approximate position of the probes in the 77 animals included in this study. CTX, cortex; CP, caudate-putamen; NAc, nucleus accumbens. **(b, c)** Identification by gas chromatography/mass spectrometry of anandamide in microdialysis perfusates of rat dorsal striatum. Anandamide and other endogenous cannabinoids were purified chromatographically from 30-min dialysate samples and analyzed simultaneously by selected ion monitoring GC/MS as bis trimethylsilylethers. For quantitation, synthetic deuterium-containing standards were added to all samples. Representative tracings for selected fragments characteristic of endogenous anandamide (**b**, mass-to-charge ratio, m/z = 404) and synthetic [$^2H_4$]-anandamide (**c**, m/z = 408). The arrow indicates the retention time of standard anandamide. Results are from one experiment and are typical of 77 independent experiments.

of KCl (60 mM). This high-K^+ pulse significantly increased anandamide outflow (Fig. 2a), whereas it had no effect on PEA, oleylethanolamide or 2-AG (Fig. 2c and data not shown; n = 13). After reinstatement of normal ACSF, anandamide levels rapidly returned to basal values (Fig. 2a). The overall time course of this response was identical to that of K^+-induced dopamine release, measured in parallel microdialysate samples (Fig. 2b). The effect of high K^+ on anandamide outflow was prevented either by the Na^+-channel blocker tetrodotoxin (1 μM) or by removal of Ca^{2+} ions, two treatments that alone had no significant effect on basal anandamide levels (Fig. 3). These results demonstrate that anandamide is released in the dorsal striatum of freely moving rats during neural activity, fulfilling an essential criterion for this lipid to be considered a neuromodulator in the central nervous system (CNS). Furthermore, the finding that 2-AG and PEA may not be released during neural activity indicates that in striatum such a role is specific to anandamide.

D_2 receptors stimulate anandamide release

The modulatory neurotransmitter dopamine regulates essential aspects of striatal physiology[2] by interacting with two pharmacologically distinct groups of G-protein-coupled receptors, D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4)[31,32]. To determine whether activation of dopamine receptors in striatum affects anandamide release, we locally applied by reverse dialysis selective D_1-like and D_2-like receptor ligands. Administration of the D_2-like agonist quinpirole resulted in an eightfold stimulation of anandamide outflow (Fig. 4a). Extracellular anandamide levels remained elevated for at least two hours after quinpirole administration (Fig. 4a), possibly as a result of slow clearance of the drug from its site of application. In support of this possibility, when the D_2-like antagonist raclopride (20 μM) was administered after quinpirole, baseline anandamide levels were reached within 30–60 min (data not shown). As with high K^+, the outputs of 2-AG, PEA and oleylethanolamide were not affected by quinpirole (data not shown).

To investigate the receptor mechanism underlying the response to quinpirole, we examined the effects of the D_2-like antagonist raclopride. Raclopride (20 μM) did not affect microdialysate anandamide concentrations when applied alone, but completely prevented the stimulatory effects of quinpirole (Fig. 4b). Furthermore, the D_1-like agonist SKF38393 (10 μM)

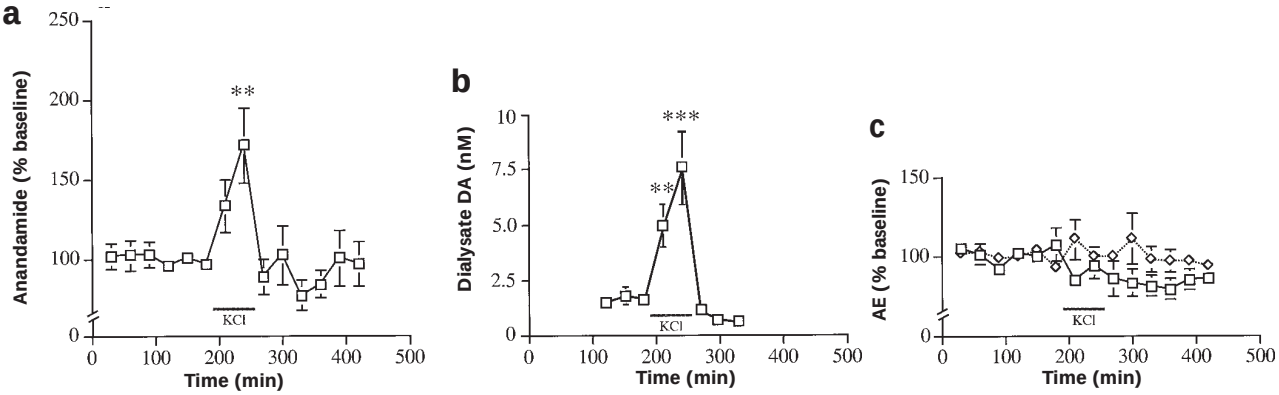

Fig. 2. Local application of a high KCl concentration (60 mM) stimulates anandamide release in dorsal striatum of freely moving rats. Effects of intrastriatal K^+ depolarization on dialysate levels of anandamide **(a)**, dopamine (DA; **b**) and other acylethanolamides (AE; **c**): palmitylethanolamide (squares) and oleylethanolamide (diamonds). Results are means ± s.e.m. (n = 13) of the amount of compounds present in 30-min dialysate samples, expressed as percent of baseline values **(a, c)** or as nmol per liter **(b)**. 2-AG was below the detection limit of this assay, which was approximately 1 pmol per sample. **p < 0.001, ***p < 0.0001.

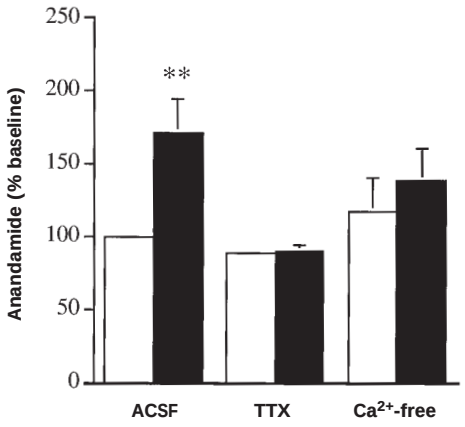

Fig. 3. K^+-stimulated anandamide release requires membrane depolarization and external Ca^{2+}. Effects of a high-K^+ pulse on the release of anandamide in artificial cerebrospinal fluid (ACSF, n = 13), ACSF containing tetrodotoxin (TTX, 1 μM, n = 6) and ACSF with zero Ca^{2+}(Ca^{2+}-free, n = 5). White bars, baseline anandamide release; black bars, K^+-stimulated release. Results are expressed as described in Fig. 2 legend.

did not change the basal outflows of anandamide (Fig. 4c), PEA, 2-AG or oleylethanolamide (data not shown). The lack of effect of SKF38393 underscores the differences between D_1-like and D_2-like receptor agonists with respect to anandamide release, but does not rule out the possibility that D_1-like receptors may regulate this process in other ways, for example by acting synergistically or antagonistically with D_2-like receptors.

Modulation of motor activity

The results of these neurochemical experiments indicate that dopamine acting at D_2-like receptors stimulates anandamide release in dorsal striatum, suggesting that the endocannabinoid system participates in dopaminergic regulation of striatal function. To test this possibility, we determined whether the behavioral response elicited by systemic administration of quinpirole in rats is affected by the CB1 receptor antagonist SR141716A[33]. In agreement with previous results[34], quinpirole (1 mg per kg) caused a biphasic motor response characterized by transient suppression of movement, which is thought to be caused by activation of presynaptic D_2-like receptors, followed by a longer-lasting hyperactivity, possibly due to activation of postsynaptic D_2-like receptors[34,35]. This response included changes in horizontal locomotion, time spent in immobility and sniffing frequency (Fig. 5). As previously reported[36], SR141716A had no overt effect on motor activity when given alone at a dose of 1 mg per kg (Fig. 5). Nevertheless, when SR141716A was injected at the same dose 60 min before quinpirole, the late phase of quinpirole-induced motor activation was markedly potentiated, whereas the initial phase of motor suppression remained unchanged (Fig. 5). Thus pharmacological blockade of CB1 receptors enhances the motor stimulation produced by activation of postsynaptic D_2-like receptors, but has little or no effect either on basal motor activity or on presynaptic D_2-receptor-dependent motor inhibition.

Discussion

In striatal and cortical neurons in primary culture, formation of anandamide is stimulated by membrane depolarization, suggesting that this compound may be produced during neural activity and participate in endocannabinoid signaling[15,18]. Here we used a combination of microdialysis and GC/MS techniques[22] to investigate the release of anandamide and other endogenous cannabinoid substances in the dorsal striatum of freely moving rats. We found that neural activity evoked by a localized pulse of high K^+ stimulates the outflow of anandamide, but not 2-AG and PEA. The possibility implied by these findings that anandamide acts as a neural mediator in striatum is supported by both anatomical and pharmacological evidence. GABAergic medium spiny neurons, which account for about 95% of the striatal neuron population and are the source of most striatofugal projections[1], contain large numbers of CB1 cannabinoid receptors[8]. Activation of these receptors causes presynaptic inhibition of GABA release *in vitro*[10] and profoundly affects motor behaviors *in vivo*[37,38]. Indeed, certain aspects of the motor inhibition produced by systemically administered cannabimimetic drugs, such as attenuation of stereotyped behaviors, may be mediated by their ability to activate striatal CB1 receptors[39]. By showing that anandamide is released in striatum during neural activity, our results point to this endogenous cannabinoid lipid as a primary component of the network of neurally active substances that regulate striatal function[2].

Brain tissue contains 2-AG in amounts 170 times greater than anandamide[18,40]. Thus, we were surprised to find that the extracellular levels of this compound in striatum are undetectable both under baseline conditions and during neural activity. Limitations of our isotope dilution assay are unlikely to account for this negative result, as this method provides very similar detection limits for 2-AG (1 pmol per sample) and anandamide (0.4 pmol per sample, see Methods). Differences in biological inactivation are also an improbable explanation, because 2-AG and anandamide are eliminated at comparable rates (M. Beltramo and D.

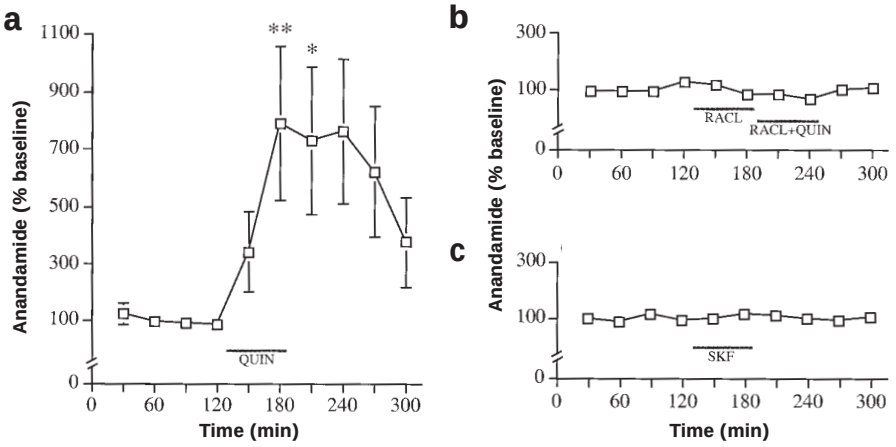

Fig. 4. D_2-like dopamine receptor activation evokes anandamide release in striatum. Effects on dialysate anandamide levels of intrastriatal administration of quinpirole (QUIN, 10 μM), a D_2-like agonist **(a)**, raclopride (RACL, 20 μM), a D_2-like antagonist applied alone or with quinpirole **(b)** or SKF38393 (SKF; 10 μM), a D_1-like agonist **(c)**. Results, expressed as described in Fig. 2 legend, are means ± s.e.m. of six experiments for each condition. *p < 0.05; **p < 0.001.

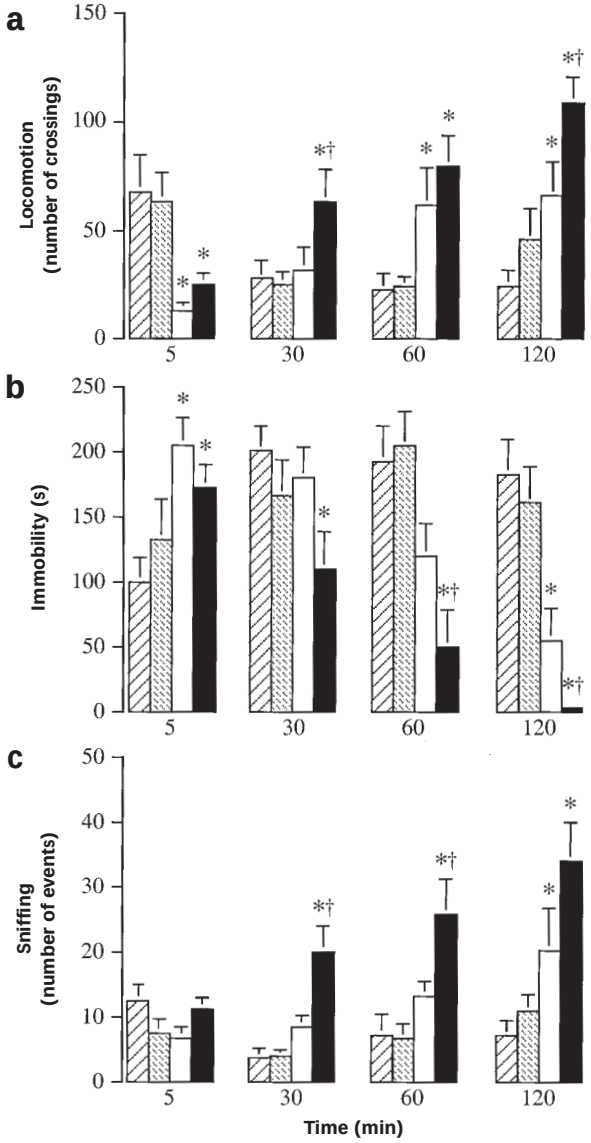

Fig. 5. The cannabinoid antagonist SR141716A potentiates quinpirole-evoked hyperactivity. Effects of systemic administration of vehicle (hatched bars), SR141716A (dotted bars; 1 mg per kg, i.p.), quinpirole (open bars; 1 mg per kg, s.c.) or quinpirole plus SR141716A (closed bars; 1 mg per kg each; SR141716 was injected 60 min before quinpirole) on horizontal locomotion **(a)**, immobility **(b)** and sniffing **(c)**. Results are means ± s.e.m. of 7–9 experiments. *$p < 0.05$ compared to vehicle; †$p < 0.05$ compared to quinpirole alone.

Piomelli, unpublished observations). Alternatively, anandamide and 2-AG may be produced under different physiological circumstances and/or in distinct regions of the CNS. Consistent with this view, in hippocampal slices, high-frequency stimulation of glutamatergic Schaffer collaterals selectively increases the accumulation of 2-AG, but not anandamide[18].

Although anandamide and PEA are formed through a common biosynthetic mechanism[15,19], PEA does not interact with either of the two cannabinoid receptor subtypes[41], CB1 and CB2, whose genes have been isolated thus far[42]. Pharmacological experiments indicate, however, that, in peripheral tissues, PEA activates a CB2-like receptor, which mediates antinociception and anti-inflammation[23,24]. Our results, showing that PEA may not be released extracellularly during neural activity in striatum, further highlight the peripheral roles of this compound.

Unlike neurotransmitters and neuropeptides, which are released from synaptic terminals via vesicle secretion, anandamide may be produced and released upon demand by a mechanism that involves phospholipase-mediated cleavage of the membrane phospholipid precursor *N*-arachidonylphosphatidylethanolamine[15,19–21,43]. Such nonvesicular release process suggests that anandamide may act in the CNS more as an autacoid (local mediator) substance than as a classical neuromodulator. Lipid autacoids such as the eicosanoids and platelet-activating factor are formed by receptor-mediated cleavage of phospholipids and act near their sites of production, where they are also rapidly inactivated (for review, see ref. 44). That anandamide may conform to this model is suggested by our finding that occupation of striatal D_2-like dopamine receptors dramatically stimulates anandamide outflow. A parsimonious interpretation of this result is that anandamide may be released from striatal neurons or nigrostriatal dopaminergic terminals, both of which bear D_2-like receptors, and may exert its effects within a confined volume of striatal tissue.

What, if any, is the physiological function of striatal anandamide release? We have begun to address this question by studying the effects of the selective CB1 receptor antagonist SR141716A on the behavioral responses produced by quinpirole in rats. The results show that, although SR141716A has no overt effect when administered alone, it enhances the motor activation elicited by quinpirole. The inverse agonist properties of SR141716A, which have been characterized *in vitro*[45], cannot account for such a differential effect; a more plausible interpretation that is also consistent with our neurochemical data is that pharmacological blockade of cannabinoid receptors increases quinpirole-induced hyperactivity by removing the inhibitory control of endogenously released anandamide. According to this hypothesis, occupation of D_2-like receptors by dopamine elicits the release of anandamide in striatum and possibly in other regions of the CNS that contribute to movement control. By engaging CB1 receptors, anandamide may act in turn to counter dopamine stimulation of motor activity, which is thought to be mediated by postsynaptic D_2-like and D_1-like receptors[46]. In further support of this hypothesis, anandamide inhibits movement when it is administered as a drug[47], and cannabimimetic agents attenuate amphetamine-evoked hyperactivity[48]. The functional interaction between anandamide and dopamine demonstrated in this study suggests a possible participation of the endogenous cannabinoid system in pathologies that involve dysregulated dopamine neurotransmission. Thus, our findings may have implications for neuropsychiatric disorders such as schizophrenia, Tourette's syndrome and Parkinson's disease and may point to novel therapeutic approaches for these conditions.

Methods

Drugs. SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide•HCl) was provided by RBI (Natick, Massachusetts) as part of the Chemical Synthesis Program of the NIMH (N01MH30003); all other drugs were from RBI or Sigma (St. Louis, Missouri).

Microdialysis. Male Wistar rats (Charles River, Holister, California) were anesthetized with halothane (1.0–1.5%), and stainless steel microdialysis guide cannulae (model CMA/10; Carnegie Medicine Apparatus, Solna, Sweden) were implanted in the caudate-putamen (from Bregma A + 1.0 mm, L ± 2.5 mm; from dura V – 2.8 mm). A recovery period of at least 5 days was allowed before the experiments. Approximately 12 hours before

the microdialysis sessions, animals were lightly anesthetized (1–2% halothane), and microdialysis probes (model CMA/10, Carnegie Medicine Apparatus; 4 mm active length) were inserted into the guide cannulae. Anesthesia was sufficiently brief so that animals regained movement within 3 min of probe insertion. An artificial cerebrospinal fluid (ACSF) consisting of 145 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.25 mM ascorbic acid and 5.4 mM D-glucose (pH 7.2–7.4) was used as perfusion medium; this solution was delivered at a flow rate of 0.2 µl per min until two hours before the start of the experiment when the flow rate was increased to 10 µl per min. Preliminary experiments indicated that this relatively high flow rate was necessary to collect a sufficient amount of endogenous cannabinoids for reliable detection under baseline conditions. *In vitro* recovery of [^{3}H]anandamide was 5.8 ± 0.2% (n = 3), which was comparable to that of dopamine under similar flow-rate conditions[49]. Dialysate concentrations were not corrected for recovery. Probe outlet tubing was modified to reduce back-pressure so that no ultrafiltration was observable across the dialysis membrane. The ACSF was delivered to the probes via a single-channel liquid swivel (Instech, Plymouth Meeting, Pennsylvania) attached to a balance arm above the animal cage to ensure freedom of movement during the experiment. Microdialysate samples were collected at 30-min intervals into glass vials containing internal standards for GC/MS analysis (1.2 nmol of each [$^2H_4$]acylethanolamide and 1.0 nmol of [$^2H_8$]-2-AG) in 1 ml methanol. At the end of each experiment, microdialysis probe placement was verified histologically[50].

Analytical procedures. Microdialysis samples were extracted with chloroform/methanol, fractionated by high-performance liquid chromatography (HPLC) and analyzed by GC/MS as described[22], (N. Stella and D. Piomelli, unpublished results). [$^2H_4$]acylethanolamides were prepared following standard procedures[22] and [$^2H_8$]-2-AG was purchased from Deva Biotech (Hartboro, Pennsylvania). The limit of detection, that is, the injected quantity that produced a signal corresponding to an average blank plus 3 standard deviations, was 0.4 pmol for anandamide, 0.1 pmol for PEA, 0.1 pmol for oleylethanolamide[22] and 1 pmol for 2-AG (N. Stella and D. Piomelli, unpublished data). Concentrations in microdialysis perfusates are expressed as percent of baseline values, which were calculated by averaging the first three samples collected before treatment. Dopamine was measured by HPLC and electrochemical detection[50]. Statistical significance was determined by one-way analysis of variance followed by Student-Newmann-Keuls multiple comparison test.

Behavioral testing. We studied the effects of pretreatment with SR141716A (1 mg per kg intraperitoneal, i.p., 60 min before) or vehicle (10% dimethylsulfoxide in water, i.p., 60 min before) on the acute effects of quinpirole (1 mg per kg subcutaneous, s.c.) on spontaneous behavior and horizontal locomotor activity. Spontaneous behavior was studied in a glass observation box (40 cm × 30 cm × 30 cm, one rat per box). Animals were placed in the box five minutes before the test, and tested during five minutes for various behaviors including sniffing frequency and time spent in immobility. This procedure was repeated for each animal 5, 30, 60 and 120 min after the administration of either vehicle or drugs. The animals were returned to their home cage at the end of each testing interval. The tests were conducted in a sound-isolated room, illuminated with an indirect halogen light (125 lux). The behavior was videotaped and scored by trained observers blind to experimental conditions. Locomotor activity was studied in an opaque open field (100 cm × 100 cm × 40 cm), the floor of which was marked with 20 cm × 20 cm squares. The field was illuminated using a ceiling halogen light that was regulated to yield 350 lux at the center of the field. The rats were habituated to the field of study for 10 min the day before testing. On the experimental day, the animals were placed in the center of the field and locomotor activity (number of lines crossed) scored during five minutes. Behavior was tested 5, 30, 60 and 120 minutes after the injection of either vehicle or drugs.

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