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Brain cannabinoids in chocolate

SIR — Chocolate craving, common in western societies, is still incompletely understood. Although sensory components of the nervous system are likely to be essential[1], the association of chocolate craving with certain drug-induced psychoses[2] suggests that pharmacologically active substances could also be involved. Attention in this respect has been focused primarily on the methylxanthines[3], which are thought to act as competitive antagonists at adenosine receptors[4]. We report here on a novel group of pharmacological constituents of chocolate, whose main target may be the endogenous cannabinoid system of the brain.

Anandamide (*N*-arachidonoylethanolamine) is a brain lipid that binds to cannabinoid receptors with high affinity and mimics the psychoactive effects of plant-derived cannabinoid drugs[5]. It is released from neurons[6] and is rapidly broken down by a selective enzyme activity[7], suggesting that it may be an endogenous cannabinoid neurotransmitter or neuromodulator. We considered that chocolate, which is rich in fat, might contain lipids chemically and pharmacologically related to anandamide. To test this possibility, we subjected samples of cocoa powder or chocolate (50 mg), obtained from three manufacturers, to sequential fractionations by solvent extraction, column chromatography, high-performance liquid chromatography and gas chromatography/mass spectrometry (GC/MS). We isolated three compounds that eluted from the GC at the same retention times as anandamide, *N*-oleoylethanolamine and *N*-linoleoylethanolamine, and displayed electron-impact mass spectra characteristic of these *N*-acylethanolamines and identical to those of synthetic standards (Fig. 1, and data not shown). Among the samples we analysed, the concentration of total unsaturated *N*-acylethanolamines varies from about 0.5 to 90 μg g^{-1}. In most cases, the rank order is *N*-oleoylethanolamine> *N*- linoleoylethanolamine >anandamide = 0.05–57 μg g^{-1}. By contrast, we detected no unsaturated *N*-acylethanolamines in white chocolate (a milk- and cocoa-butter-containing sweet used as a control for chocolate in behavioural studies)[1] or in brewed espresso coffee (whose pharmacological effects are attributed to caffeine, another methylxanthine).

N-oleoylethanolamine and *N*-linoleoylethanolamine do not activate brain cannabinoid receptors and their biological actions have not been yet defined. We found that *N*-oleoylethanolamine and *N*-linoleoylethanolamine inhibit anandamide hydrolysis in rat brain microsomes, a reaction catalysed by anandamide amidohydrolase activity[7] (Fig. 2*a*). Moreover, *N*-linoleoylethanolamine produces a similar inhibitory effect in intact cells. Rat cortical astrocytes in culture hydrolyse exogenous [^{3}H]anandamide to ethanolamine and [^{3}H]arachidonate. Arachidonate is readily incorporated into phosphatidylcholine and phosphatidylethanolamine[6]. In the presence of *N*-linoleoylethanolamine, degradation of [^{3}H]anandamide by the astrocytes is strongly reduced, and the amount of residual [^{3}H]anandamide increased correspondingly (Fig. 2*b*). The concentration of *N*-linoleoylethanolamine that inhibits [^{3}H]anandamide hydrolysis by 50% is ~5 μM (Fig. 2*b*, inset).

Our results demonstrate that cocoa powder and chocolate contain three unsaturated *N*-acylethanolamines that could act as cannabinoid mimics either directly (by activating cannabinoid receptors) or indirectly (by increasing anandamide levels). Further experiments are necessary to determine whether the concentrations of unsaturated *N*-acylethanolamines measured in our study are sufficient to produce these biological effects *in vivo*.

How can activation of the endogenous cannabinoid system participate in the sub-

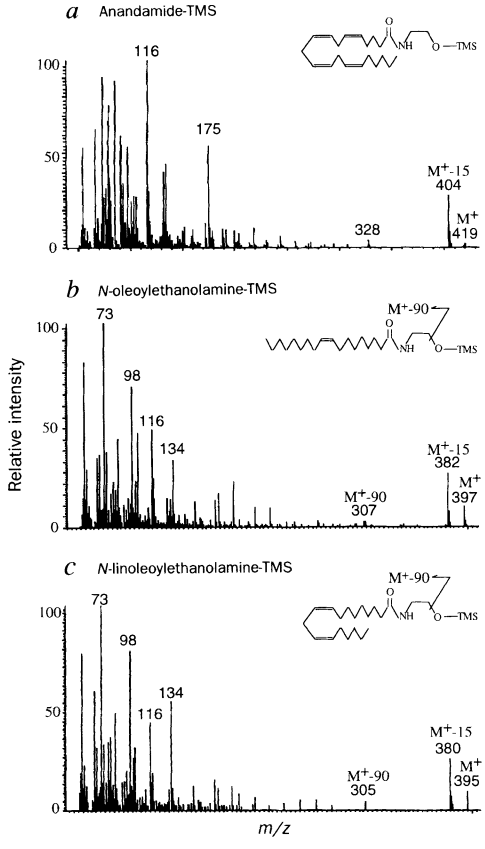

FIG. 1 Electron-impact mass spectra of the trimethylsilyl (TMS) ether derivatives of *a*, anandamide; *b*, *N*-oleoylethanolamine; *c*, *N*-linoleoylethanolamine, isolated from a 50-mg sample of commercial cocoa powder. The spectra are identical to those of synthentic standards (not shown).

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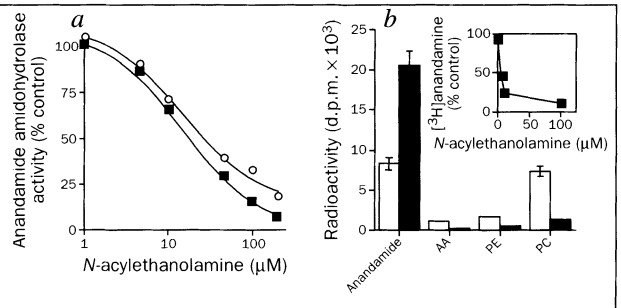

FIG. 2 *a*, Effects of *N*-oleoylethanolamine (circles) and *N*-linoleoylethanolamine (squares) on rat brain microsome anandamide amidohydrolase activity. *b*, Effect of *N*-linoleoylethanolamine on [^{3}H]anandamide degradation by rat cortical astrocytes in primary cultures. Control, open bars; *N*-linoleoylethanolamine (15 μM), filled bars. Inset: concentration-dependent effects of *N*-linoleoylethanolamine. Amidohydrolase activity was measured in rat brain microsomes (0.2 mg protein) incubated for 10 min at 37 °C in Tris buffer (pH 8) containing 100 nM [^{3}H]anandamide (NEN; 0.1 μCi ml^{-1}, 221 Ci $mmol^{-1}$). Astrocytes in culture[6] were incubated for 20 min with [^{3}H]anandamide (100 nM, 0.1 μCi ml^{-1}) in Tris-buffered Krebs' solution (pH 7.4). Reactions were stopped with cold methanol and the lipids extracted with chloroform. [^{3}H]anandamide and other lipids were purified by thin-layer chromatography and quantified by liquid scintillation counting. AA, arachidonate; PE, phosphateidylethanolamine; PC, phosphatidylcholine.

jective feelings associated with eating chocolate, and in chocolate craving? Cannabinoid drugs are known to heighten sensitivity and produce euphoria[8]. A possible effect of elevated brain anandamide levels could be to intensify the sensory properties of chocolate thought to be essential to craving. Alternatively, elevated anandamide levels could cooperate with other pharmacological components of chocolate (for example, caffeine, theobromine) to produce a transient feeling of well-being. Whether or not any of these speculations turn out to be correct, our results point to an unexpected link between non-drug craving and the endogenous cannabinoid system, which deserves further examination.

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Transcriptional activation by BRCA1

SIR — Mutations in the gene *BRCA1* may account for as much as 90% of inherited breast and ovarian cancers in families predisposed to both maladies. *BRCA1* encodes an 1,863-amino-acid protein containing a putative zinc-ring finger domain[1], suggesting that BRCA1 binds DNA. To date, no function has been ascribed to BRCA1. Here we report that the carboxy-terminal portion of BRCA1 acts as a strong transcriptional transactivator when fused to the GAL4 DNA-binding domain and that this activity is completely abolished in sequences corresponding to four different mutations found in *BRCA1* families.

We inserted portions of the *BRCA1* complementary DNA into the plasmid pSG424 to create in-frame fusions with the GAL4 DNA-binding domain. We co-transfected the resulting plasmids into 293T cells, a human kidney-derived cell line[2], together with the reporter-gene plasmid pGAL4BCAT, which contains 5 GAL4 binding sites and a minimal promoter linked to the chloramphenicol acetyltransferase gene.

We detected no activity for the GAL4 DNA-binding domain (DBD) alone, nor when it is fused to amino acids 1–1,652 of BRCA1. Three central portions of BRCA1 also fail to show activity (*a* in the figure). However, a C-terminal portion of BRCA1 (amino acids 1,528–1,863) shows significant transcription activation of the reporter when fused to the GAL4 DBD, whereas a smaller C-terminal fragment (1,760–1,863) shows moderate activity. We conclude that amino acids 1,760–1,863 constitute a minimal region required for transactivation, and that additional sequences N-terminal to this region are required for maximal activity.

More than 134 distinct mutations in *BRCA1* have been identified among families with a *BRCA1*-linked predisposition for breast and ovarian cancers[3]. Most of these are frameshift mutations which result in a truncated BRCA1 protein lacking C-terminal sequences. In addition, several *BRCA1* mutations have been described in which single amino-acid changes occur in the C-terminal portion of the protein.

To determine what effect such mutations might have on the transcriptional transactivation activity of BRCA1, we made GAL4–BRCA1 fusion proteins corresponding to four C-terminal mutations (*b* in the figure). The *BRCA1* point mutants A1708E (where the alanine residue at position 1,708 is changed to glutamate) and M1775R (methionine to arginine) are almost certainly associated with a predisposition to breast and ovarian cancers[1,3,4], whereas the disease association of the mutant P1749R (proline to arginine) is less certain, occurring in a patient with ovarian cancer but not in controls[5].

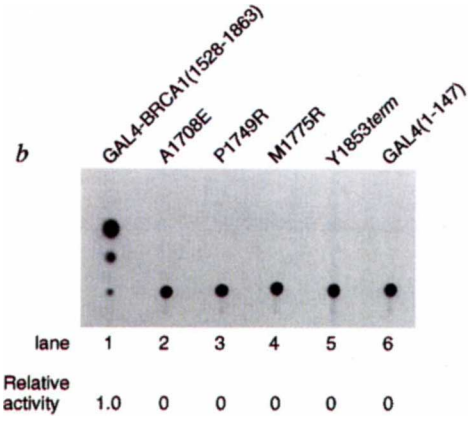
We also made a fourth mutant, Y1853*term*, in which the addition of a single nucleotide creates a termination codon at position 1,853, thereby deleting the final 11 amino acids of BRCA1. This *BRCA1* mutation has been found in several individuals with breast cancer, and is definitely associated with a predisposition to breast and ovarian cancer (ref. 6, and M.-C. King, personal communication). When each of these four *BRCA1* mutations is introduced into GAL4–BRCA1(1528–1863), we see no transactivation of the reporter gene (*b* in the figure), even when we used very high levels of the cell extract. In addition, when we introduced the mutation M1775R into the context of GAL4–BRCA1(1760–1863), this fusion construct showed about half the activity of its wild-type BRCA1 counterpart, GAL4–BRCA1(1760–1863) (data not shown).

When using western analysis with a polyclonal anti-GAL4 DBD antibody, we find no large differences in steady-state protein levels between the wild-type or mutant proteins in the GAL4–BRCA1-(1528–1863) context, although the levels of A1708E and Y1853*term* are somewhat reduced. Polyclonal anti-BRCA1 antibody detects the identical bands when the membranes are reprobed. Lack of transactivation by Y1853*term* mutant further defines the transactivation domain in BRCA1, in that the final 11 C-terminal amino acids are required for activity. It is somewhat surprising that mutants A1708E and P1749R show a complete lack of transactivation, because GAL4–BRCA1(1760–1863), which does not include these amino acids, still has some activity.

The involvement of *BRCA1* in familial

a

Construct	Relative activity
GAL4 (1–147)	0.0
GAL4–BRCAI(1–1652)	0.0
GAL4–BRCA1(303–1294)	0.0
GAL4–BRCA1(1313–1527)	0.0
GAL4–BRCA1(1558–1759)	0.0
GAL4–BRCA1(1528–1863)	1.0
GAL4–BRCA1(1760–1863)	0.2
GAL4–BRCA1(1528–1863)	1.0
GAL4–BRCAI(1528*1863)A1708E	0.0
GAL4–BRCAI(1528*1863)P1749R	0.0
GAL4–BRCAI(1528*1863)M1775R	0.0
GAL4–BRCAI(1528*1863)Y1853*term*	0.0



a, Transactivation by the C terminus of BRCA1. We fused portions of the *BRCA1* coding region in-frame to amino acids 1–147 of GAL4 (ref. 10) to create the GAL4–BRCA1 fusion proteins shown. We co-transfected fusion constructs with the reporter plasmid pGAL4BCAT (ref. 11) into 293T cells[2] and determined CAT activity at 24 h following transfection. Values represent at least three separate experiments. We included RSV-βgal plasmid for normalization of transfection efficiency. *b*, *BRCA1* mutants lack transactivation efficiency. Mutant BRCA1 sequences were made and fused to the GAL4 DNA-binding domain. 293T cells were transiently transfected and CAT assays done as in *a*.