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Leptin-regulated endocannabinoids are involved in maintaining food intake

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Leptin is the primary signal through which the hypothalamus senses nutritional state and modulates food intake and energy balance¹. Leptin reduces food intake by upregulating anorexigenic (appetite-reducing) neuropeptides, such as α -melanocyte-stimulating hormone^{2,3}, and downregulating orexigenic (appetite-stimulating) factors, primarily neuropeptide Y⁴. Genetic defects in anorexigenic signalling, such as mutations in the melanocortin-4 (ref. 5) or leptin receptors⁶, cause obesity. However, alternative orexigenic pathways maintain food intake in mice deficient in neuropeptide Y⁷. CB1 cannabinoid receptors⁸ and the endocannabinoids anandamide and 2-arachidonoyl glycerol are present in the hypothalamus⁹, and marijuana¹⁰ and anandamide^{11,12} stimulate food intake. Here we show that following temporary food restriction, CB1 receptor knockout mice eat

less than their wild-type littermates, and the CB1 antagonist SR141716A reduces food intake in wild-type but not knockout mice. Furthermore, defective leptin signalling is associated with elevated hypothalamic, but not cerebellar, levels of endocannabinoids in obese *db/db* and *ob/ob* mice and Zucker rats. Acute leptin treatment of normal rats and *ob/ob* mice reduces anandamide and 2-arachidonoyl glycerol in the hypothalamus. These findings indicate that endocannabinoids in the hypothalamus may tonically activate CB1 receptors to maintain food intake and form part of the neural circuitry regulated by leptin.

CB1^{−/−} and CB1^{+/+} mice were made as described previously^{13,14} and were maintained on a reverse light/dark cycle for measurement of food intake. After 18 h of fasting, the animals received a single intraperitoneal injection of vehicle or drug 10 min before the beginning of the dark cycle, and food intake was measured for the indicated period of time. After vehicle treatment, food intake was significantly lower in CB1^{−/−} than in CB1^{+/+} mice. When CB1^{+/+} mice were treated with 3 μ g g^{−1} of the selective CB1 receptor antagonist SR141716A (ref. 15), food intake was significantly reduced to the same level as in vehicle-treated CB1^{−/−} mice, whereas in CB1^{−/−} mice SR141716A did not affect food intake (Fig. 1). These results indicate that endogenous cannabinoids acting at CB1 receptors may be involved in maintaining food intake in mice made hyperphagic by brief food deprivation.

As leptin is known to downregulate the expression in the hypothalamus of orexigenic peptides such as neuropeptide Y (NPY)⁴, orexins¹⁶ and melanin concentrating hormone¹⁷, we investigated whether it may similarly regulate hypothalamic endocannabinoids. A single intravenous injection of 125 or 250 μ g of recombinant mouse leptin into normal Sprague–Dawley rats resulted in around 40–50% reductions in the hypothalamic levels of both anandamide and 2-arachidonoyl glycerol (2-AG) within 30 min, compared with levels in vehicle-treated controls (Fig. 2). By contrast, defective leptin signalling in obese Zucker rats was associated with elevated 2-AG levels in the hypothalamus compared with non-obese controls. The hypothalamic levels of anandamide and palmitoyl ethanolamide (PEA) were not significantly different in Zucker rats and their controls (Fig. 3a). In young (6–8-week-old) obese *db/db* mice with defective leptin receptors, hypothalamic levels of both 2-AG and anandamide were higher than in their

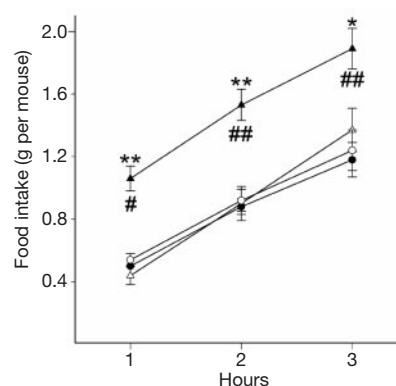


Figure 1 Food intake in CB1^{−/−} mice and their CB1^{+/+} littermates in the absence and presence of the CB1 receptor antagonist SR141716A. Cumulative food intake was measured in 18-h food-restricted CB1^{−/−} (circles) and CB1^{+/+} mice (triangles). The animals received a single intraperitoneal injection of vehicle (filled symbols) or 3 μ g g^{−1} SR141716A (open symbols) 10 min before the start of the testing period. Means $\pm$ s.e., $n = 9$ CB1^{+/+} and 5 CB1^{−/−} mice. Significance of difference from corresponding values in the presence of SR141716A in the same group of animals (* $P < 0.05$, ** $P < 0.01$), or from corresponding values in vehicle-treated CB1^{−/−} mice (# $P < 0.05$, ## $P < 0.01$) calculated by analysis of variance followed by Tukey's test. Food intake in non-fasted animals was much lower; it was similar in CB1^{+/+} (0.19 $\pm$ 0.05, 0.33 $\pm$ 0.06, 0.46 $\pm$ 0.06 g per mouse, $n = 8$) and CB1^{−/−} mice (0.16 $\pm$ 0.02, 0.33 $\pm$ 0.04, 0.45 $\pm$ 0.05 g per mouse, $n = 8$), at 1, 2 and 3 h, respectively.

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C57BL/6J controls, whereas the levels of PEA were similar in the two groups (Fig. 3b). In young *ob/ob* mice, which lack leptin, hypothalamic levels of 2-AG, but not anandamide, were higher than in their C57BLKS/J controls (Fig. 3c). In *ob/ob* mice treated with leptin ($2 \mu\text{g g}^{-1}$ intravenously) hypothalamic 2-AG levels were similar to levels measured in lean controls, and anandamide declined to undetectable ($<1.0 \text{ pmol g}^{-1}$) levels. These findings strongly suggest negative regulation of endocannabinoids by leptin, which appears to be specific for endocannabinoids and the hypothalamus, as the hypothalamic levels of PEA were similar and there was no difference in the cerebellar levels of anandamide (18.2 ± 1.0 versus $16.1 \pm 0.5 \text{ pmol g}^{-1}$, $P > 0.2$) or 2-AG (7.5 ± 0.3 versus $8.1 \pm 0.6 \text{ nmol g}^{-1}$, $P > 0.4$) between *db/db* mice and their controls, respectively.

In the absence of a neuronal storage mechanism for endocannabinoids, the effect of leptin on endocannabinoid levels in the hypothalamus may reflect decreased synthesis, increased degradation or both. Hypothalamic activity of the enzyme fatty acid amide hydrolase (FAAH), which hydrolyses both anandamide and 2-AG¹⁸, was unaffected by leptin (1.76 ± 0.10 versus $1.73 \pm 0.04 \text{ nmol per min per mg protein}$ in preparations from three control and three leptin-treated rats; $P > 0.5$). On the other hand, the hypothalamic levels of the direct biosynthetic precursor of anandamide, N-Ar-PE (ref. 19), increased significantly from 81 ± 8 to $221 \pm 8 \text{ pmol g}^{-1}$ ($n = 3$, $P < 0.001$) following treatment with leptin.

This indicates that leptin may downregulate the activity of the phospholipase D (PLD) enzyme that catalyses the conversion of N-Ar-PE into anandamide^{19,20}, leading to its accumulation. However, the activity of the N-Ar-PE-specific PLD in the hypothalamus was not significantly altered by treatment with $250 \mu\text{g}$ leptin (11.2 ± 2.2 versus $14.9 \pm 3.1 \text{ pmol per min per mg protein}$, $n = 6$, $P > 0.3$). Leptin may trigger or inhibit the release of putative soluble inhibitors or activators, respectively, of the N-Ar-PE-specific PLD, and such soluble substances may be lost in the *in vitro* assay system used to measure enzyme activity. For example, leptin acutely decreases low-glucose-induced mobilization of intracellular calcium in the arcuate nucleus²¹, and may thereby reduce the activity of the N-Ar-PE-specific PLD in a way that could not be reproduced in a cell-free system *in vitro*.

We also investigated the effect of leptin on the biosynthesis of 2-AG. We assayed the activity of diacyl glycerol (DAG) lipase, the enzyme responsible for the generation of 2-AG from DAG, in hypothalamic homogenates by the conversion of 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol to [^{14}C]2-AG in the absence and presence of a DAG lipase inhibitor²². Like that of PLD, DAG lipase activity was similar in preparations from rats pretreated with a

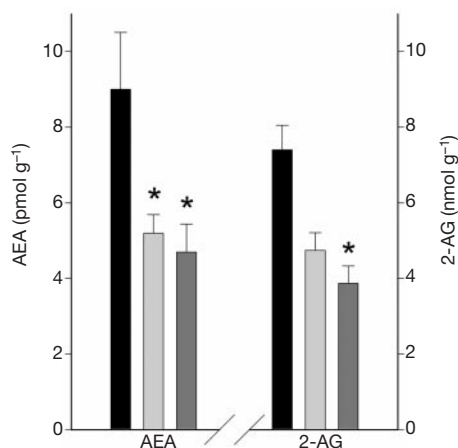


Figure 2 Hypothalamic levels of anandamide and 2-arachidonoyl glycerol (2-AG). Black, control rats; light grey, rats treated with $125 \mu\text{g}$ leptin; dark grey, rats treated with $250 \mu\text{g}$ leptin (means $\pm$ s.e. from six separate experiments; asterisk, $P < 0.05$).

single intravenous injection of saline or $250 \mu\text{g}$ leptin (3.12 ± 0.22 versus $4.27 \pm 0.48 \text{ pmol per min per mg protein}$, $n = 3$, $P > 0.1$). We also measured the hypothalamic levels of *sn*-2-arachidonate-containing DAGs, the immediate precursors of 2-AG. They were markedly reduced by leptin treatment, from 67.5 ± 10.6 to $19.6 \pm 3.3 \text{ nmol per g wet weight}$. This indicates that, unlike the situation with anandamide, the target of leptin may be upstream from the generation of DAG, possibly at the level of a phosphatidylinositol-specific PLC, which are calcium sensitive. The exact mechanisms by which leptin reduces endocannabinoid levels in the hypothalamus therefore remain to be clarified.

The ability of SR141716A to reduce food intake in rodents has been reported^{23,24}. However, the well-documented inverse agonist activity of SR141716A (ref. 25) and its possible nonspecific effects²⁶ make it difficult to conclude from those results that food intake is tonically stimulated by endocannabinoids. Unambiguous evidence for such a conclusion is provided by our findings that food intake is significantly lower in CB1 receptor-deficient mice than in their wild-type controls, and that SR141716A can reduce food intake in wild-type but not in CB1 receptor-deficient mice. The finding that the much lower food intake in animals with unlimited access to food is similar in CB1^{+/+} and CB1^{-/-} mice (Fig. 1) is compatible with the lack of difference in body weight between the two groups¹³ and suggests that, in the absence of food deprivation, other orexigenic signals may compensate for the loss of endocannabinoid signalling.

Genetic defects in anorexigenic signalling pathways, such as the absence of leptin or melanocortin-4 receptors, lead to obesity^{5,6}. However, the lack of hypophagia and of a lean phenotype in NPY-deficient animals⁷ indicates that there is more redundancy in

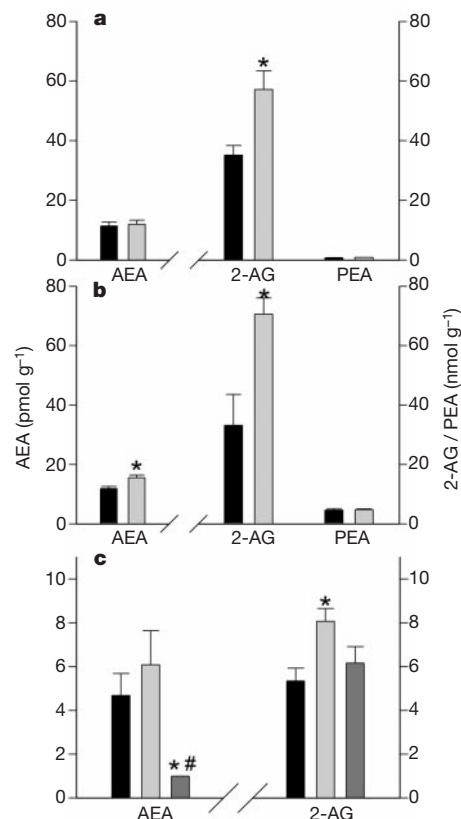


Figure 3 Hypothalamic levels of anandamide (AEA), 2-arachidonoyl glycerol (2-AG) and palmitoyl ethanolamide (PEA). **a**, Obese Zucker (*fa/fa*) rats (grey, $n = 12$) and lean controls (black, $n = 9$); **b**, obese *db/db* mice (grey, $n = 4$) and lean controls (black, $n = 4$); **c**, obese *ob/ob* mice treated with vehicle (light grey, $n = 8$) or $2 \mu\text{g g}^{-1}$ leptin intravenously (dark grey, $n = 5$), and lean controls (black, $n = 8$). Means $\pm$ s.e.; significance of difference from the corresponding values in lean controls (* $P < 0.05$) or from vehicle-treated *ob/ob* mice (# $P < 0.05$) is shown.

orexigenic signalling. We tested whether endocannabinoids may be the main factors compensating for the lack of NPY by comparing the ability of SR141716A to inhibit food intake in food-restricted NPY-deficient mice and their wild-type littermates. As illustrated in Fig. 4, a single intraperitoneal injection of $3 \mu\text{g g}^{-1}$ SR141716A 10 min before the dark cycle caused a marked reduction in food intake, which was similar in the two groups. This may indicate that endocannabinoids are involved in maintaining food intake in both the absence and the presence of NPY, and that the two systems operate essentially independently of one another. Simultaneous blockade of more than one orexigenic pathway may be necessary to achieve clinically relevant, long-term reductions in food intake in obese individuals.

The increased hypothalamic levels of endocannabinoids in genetically obese animals may indicate that these substances contribute to the hyperphagia that leads to obesity. We measured the effect of SR141716A on food intake in 5–6-week-old obese *db/db*

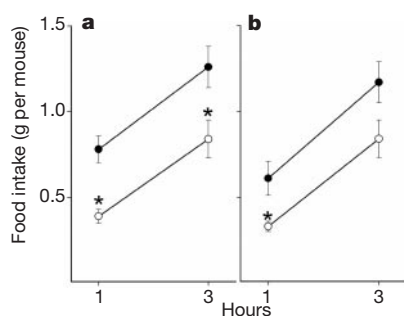


Figure 4 Effect of SR141716A on food intake in NPY-deficient mice. Food intake in NPY-deficient mice (a) and their controls (b) after acute treatment with vehicle (filled circles) or $3 \mu\text{g g}^{-1}$ SR141716A intraperitoneally (open circles). Mice kept under normal light/dark cycle were food deprived for 24 h before testing during the beginning of the dark phase. Means $\pm$ s.e. from eight animals in each treatment group. Asterisk, significant difference ($P < 0.05$) from vehicle-treated group.

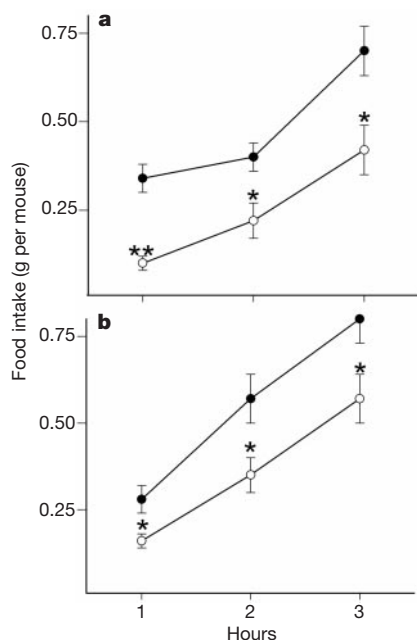


Figure 5 Effect of SR141716A on food intake in obese mice. Food intake in non-fasted *db/db* (a) and *ob/ob* mice (b) after acute treatment with vehicle (filled circles) or $3 \mu\text{g g}^{-1}$ SR141716A intraperitoneally 10 min before the test period. Means $\pm$ s.e. from 6–12 animals in each group. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$) from the vehicle-treated group.

and *ob/ob* mice with unrestricted access to food. Intraperitoneal injection of $3 \mu\text{g g}^{-1}$ SR141716A caused a significant, acute reduction in food intake in both types of obese mice (Fig. 5). Furthermore, daily treatment of four *db/db* mice with the same dose of SR141716A for seven days resulted in a reduction in body weight compared with four vehicle-treated *db/db* mice. On day four of the treatment, body weight in the two groups was $98.1 \pm 1.1\%$ versus $102.0 \pm 0.8\%$ of day 0 values, respectively ($P < 0.05$), but by day 6 catch-up growth in the SR141716A-treated animals had eliminated the difference. This may indicate that endocannabinoids, with other leptin-regulated orexigenic signals, contribute to overeating in the development of obesity.

Hypothalamic endocannabinoids appear to be under negative control by leptin and may be considered to belong to the growing family of orexigenic mediators, including NPY⁷, the orexins^{16,27} and melanin concentrating hormone^{17,28}. However, the cell groups in which endocannabinoids are synthesized and their neuronal connections with other hypothalamic nuclei that form the circuitry controlling food intake and satiety remain to be clarified. □

Methods

Animals

We bred CB1 receptor-deficient (*CB1*^{-/-}) mice and their homozygote littermates (*CB1*^{+/+} mice) as described^{13,14}. Details about the NPY-deficient mice and their controls have been reported⁷. Obese *db/db* and *ob/ob* mice with their respective C57BL/6J and C57BLKS/J controls and obese Zucker (*fa/fa*) rats with their controls were obtained from Jackson Laboratories. Sprague–Dawley rats were from Harlan.

Food intake measurement

We maintained male and female mice on a reverse light/dark cycle (lights on 21:00 to 09:00, lights off 09:00 to 21:00), with one animal per cage. The animals were fasted for 18 h before testing, except when noted otherwise. NPY-deficient mice and their controls were tested during their normal dark cycle following a 24-h fast. Ten minutes before the start of the test period, the animals received an intraperitoneal injection of vehicle or $3 \mu\text{g g}^{-1}$ SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl). Ten minutes later a food tray was placed in their cage, and cumulative food intake was measured by weighing the residual food in the tray at the indicated intervals and correcting for spillage, which was minimal.

Measurement of endocannabinoids in the brain

Animals were anaesthetized with ether and decapitated, and the brain was removed. We dissected the hypothalami by parallel coronal cuts rostrally at the edge of the optical chiasm and caudally through the mamillary bodies, then sagittal cuts 2 mm lateral from the midline (1.5 mm in mice), and finally a horizontal cut at the top of the third ventricle. The cerebellum was also removed and both tissues were immediately frozen in liquid nitrogen, within 1 min of decapitation. The hypothalami from two rats or four mice were pooled and extracted with 3 vol of chloroform/methanol 2:1 (v/v) containing 1 nmol *d*₈-anandamide (Cayman Chemicals), 2 nmol of *d*₈-2-AG (Deva Biotech) and 10 nmol of *N*-palmitoyl-phosphatidylethanolamine (Sigma) as external standards. The extracts were fractionated by chromatography on silica gel mini-columns eluted with chloroform/methanol 9:1 (v/v), as described⁹. We analysed pre-purified lipids by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (HPLC–APCI–MS). HPLC separation was through a Supelcosil LC-18 column (150 mm $\times$ 4.6 mm, 5 μm particle size), using a mobile phase of methanol/water/acetic acid 85:15:0.2 (v/v/v) at a flow rate of 1 ml min⁻¹. The MS was equipped with a Z-Spray APCI source operating in the (+) APCI mode (source temperature 120 °C, probe temperature 110 °C). N₂ was used as both the drying and nebulizing gas, with flow and probe position adjusted daily for optimum sensitivity. Both the column and the samples were maintained at 25 °C. Anandamide and 2-AG in their protonated (*M*+1) form were identified as peaks with the appropriate *m/z* values, and quantified by the isotope dilution method, whereas palmitoyl ethanolamide was quantified by comparison with its external synthetic standard run under the same conditions. Values are expressed as pmol or nmol per g wet tissue.

We purified *N*-Ar-PE as described⁹ and quantified it by the amount of anandamide released through its digestion with *Streptomyces chromofuscus* PLD, which cleaves quantitatively all *N*-acyl-phosphatidylethanolamines^{9,18}. We quantified the anandamide released by HPLC–APCI–MS, as described above. The consistency of the yield of *N*-Ar-PE purification and hydrolysis was verified by parallel LC–MS measurement of palmitoyl-ethanolamide released from *N*-palmitoyl-phosphatidylethanolamine, which was added to each sample as an external standard before the purification. Values are expressed as pmol per g wet tissue.

Enzyme assays

We measured FAAH activity as described²⁹. Rats were killed by decapitation 30 min after the intravenous injection of vehicle or 250 μg leptin. Membrane fractions from the

hypothalamus were incubated with 100 μM [^{14}C]anandamide in Tris-HCl, pH 9, at 37 °C for 15 min, after which 2 vol of chloroform/methanol 2:1 was added to the incubation mixture. The [^{14}C]ethanolamine produced from the enzymatic reaction was quantified by counting the aqueous phase, and was used as a measure of FAAH activity.

The activity of the N-Ar-PE specific PLD was measured by using [^3H]N-Ar-PE (5 mCi mmol^{-1}) as substrate²⁰. We prepared whole homogenates from the hypothalamus of rats killed within 30 min of an intravenous injection of vehicle or 250 μg leptin. The homogenates were incubated with [^3H]N-Ar-PE (100,000 c.p.m., 20 μM) in 1 ml of 20 mM HEPES-NaOH buffer, pH 7.4, containing 2 mM dithiothreitol, 2 mM CaCl_2 , 2 mM Triton-X100 and 1 mM phenylmethyl sulphonyl fluoride for 30 min at 37 °C, and the reaction terminated by the addition of 2 vol of chloroform/methanol 2:1. The organic phase was fractionated by thin layer chromatography, developed with chloroform/methanol/ NH_4OH (80:20:2, v/v/v), and the band corresponding to the R_f (migration index) value of synthetic anandamide was scraped off the plate and quantified by liquid scintillation spectrometry.

Diacyl glycerol lipase activity was measured in rat hypothalamic homogenates as described²². We quantified *sn*-2-arachidonate-containing DAGs by the arachidonoyl methyl ester (AME) produced from their *trans*-esterification with methanol. We purified hypothalamic lipid extracts by thin layer chromatography on silica plates eluted with diethyl ether/petroleum ether/ammonia (50:50:1, by vol), under conditions allowing the separation of DAGs ($R_f = 0.5$) from triacylglycerols ($R_f = 1$), phospholipids ($R_f = 0.1$) and free fatty acids ($R_f = 0.25$). Bands corresponding to a standard of 1-oleoyl-2-arachidonoyl-glycerol were eluted from silica gel, lyophilized and reacted with 0.5 ml anhydrous methanol in the presence of 3 mg anhydrous sodium carbonate overnight at room temperature. The fatty acid methyl esters produced from the reaction (> 90% yield) were purified by phase extraction, mixed with 1 μg [$^2\text{H}_6$]AME and analysed by gas chromatography-electron impact mass spectrometry (GC-EIMS) as described⁹. Selected fragments were at $m/z = 326$ and 318, corresponding to the molecular ions of [$^2\text{H}_6$]AME and AME, and at $m/z = 255$ and 247, corresponding to the loss of the terminal *n*-pentyl chain. AME was quantified by isotope dilution with [$^2\text{H}_6$]AME, using the GC signal area ratios of the peaks with $m/z = 255$ and 247.

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Induction of the mammalian node requires Arkadia function in the extraembryonic lineages

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The early mammalian embryo is patterned by signals emanating from extraembryonic and embryonic signalling centres, most notably the anterior visceral endoderm (AVE) and the node, respectively¹. The AVE is responsible for anterior development, whereas further axis specification depends on the node, the equivalent of Spemann's organizer^{2,3}. Formation of the node, at the anterior primitive streak, depends on expression of the transcription factor HNF3 β (ref. 4). However, both the source and the nature of the signals responsible for inducing the node have been unknown. Here we describe a recessive lethal mutation, *arkadia*, generated using gene-trap mutagenesis. Mutant embryos establish an AVE but fail to maintain anterior embryonic structures and lack a node. The mutation has disrupted the *Arkadia* gene, which encodes a putative intracellular protein containing a RING domain. *Arkadia* is essential for HNF3 β expression in the anterior primitive streak. Analysis with chimaeras, however, shows that *Arkadia* functions within extraembryonic tissues, revealing that these are required to induce the node. Furthermore, our experiments show that *Arkadia* interacts genetically with the transforming growth factor (TGF) β -like factor Nodal^{5–7}, implying that Nodal mediates the function of *Arkadia* in node induction.

We generated recessive lethal gene-trap insertion mutations in genes expressed in embryonic stem (ES) cells⁸ using the retroviral ROSA β geo gene-trap vector (GT). We describe one such mutation, *arkadia*. The promoterless GT-vector marker, β geo, is expressed widely, indicating that the promoter of the endogenous *Arkadia* gene is ubiquitously active (Fig. 1a). The homozygous *arkadia*

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