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Genetic deletion of Fatty Acid Amide Hydrolase results in improved long-term outcome in chronic autoimmune encephalitis

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ABSTRACT

The enzyme Fatty Acid Amide Hydrolase (FAAH) is a key regulator of the endogenous levels of a family of biologically active lipid mediators, the fatty acid amides. These include anandamide, oleoyl ethanolamide and palmitoyl ethanolamide, and their effects are mediated by a variety of downstream targets including cannabinoid receptors and *peroxisome proliferator-activated receptors* (PPARs). Activation of both of these may have anti-inflammatory and neuroprotective effects. Levels of all three mediators are low in normal nervous tissue, but substantially elevated in mice lacking FAAH as a result of genetic deletion. There is a long anecdotal history of cannabis use by patients suffering from multiple sclerosis, and preclinical studies have indicated beneficial effects of cannabinoid receptor stimulation on both long-term outcome and acute muscle spasm in rodent models of multiple sclerosis (experimental autoimmune encephalitis; EAE). Thus far no report has appeared on the effect of inhibition of FAAH on the progression of EAE. Using a chronic mouse EAE model, we present data indicating that mice lacking FAAH experience an initial inflammatory phase of EAE similar in severity to wild type controls, but exhibited a more substantial clinical remission compared to wild type mice.

Experimental autoimmune encephalitis (EAE) is a family of animal models in which an inflammatory disease of the CNS is induced by immunization with myelin or myelin components. EAE is thought to mimic some aspects of human multiple sclerosis, including mediation via self reactive T cells, CNS inflammation, demyelination and loss of neuronal fibres with resultant motor signs of paralysis and (in some models) muscle spasm.

Several reports indicate that activation of the endocannabinoid system may have beneficial effects on various aspects of the disease, including amelioration of the accompanying pain and muscle spasm, and also potential disease modification [2,22,19,5,21,13]. These studies have employed various pharmacological reagents that might be expected to activate the endocannabinoid system, including both the Fatty Acid Amide Hydrolase (FAAH) inhibitor AM347 [2], and inhibitors of the putative anandamide transporter (some of which may also inhibit FAAH). Several mechanisms by which beneficial effects are achieved have been suggested, including reduction in inflammatory processes [21,19], direct control of muscle spasm [2], and neuroprotective mechanisms which were evoked by CB1 activation [22]. We now present direct evidence that FAAH knockout mice, which have elevated levels of several potentially relevant FAAH substrates including anandamide (AEA) palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA), exhibit a more substantial clinical remission compared with wild type mice after experiencing an initial phase of similar magnitude.

C57BL/6 mice were obtained from the Jackson laboratory (Bar Harbor, ME). Knockout mice with a genetic deletion of FAAH on a C57BL/6 background were supplied by Dr. B. Cravatt's laboratory at The Scripps Research Institute, San Diego. Active EAE was induced on day 1 by immunizing 6–9-week-old female C57BL/6 or FAAH knockout mice on a C57BL/6 background, with 300 μg of $MOG_{aa35–55}$ (American Peptide Co., Sunnyvale, CA) emulsified in CFA (total volume 100 ul) (BD Diagnostic Systems, Franklin lakes, NJ) supplemented with 5 mg/ml nonviable, desiccated *Mycobacterium tuberculosis* (H37Ra; Difco/BD Diagnostic Systems) s.c. On day 1 and 3 they also received an intraperitoneal injection of 250 ng of *Bordetella pertussis* toxin (List Biological Labs, Campbell, USA).

In accordance with the protocol approved by the local Institutional Animal Care and Use Committee (IACUC), mice were examined twice daily, and were weighed and clinical scores recorded either daily or at two daily intervals using a clinical scale as follows: 0 = healthy mouse, 1 = flaccid tail, 2 = hind limb weakness, 3 = full paralysis of one or both hind limbs, 4 = hind limb and fore limb paralysis, and 5 = death. Animals were euthanased if they reached a score of 4. All procedures were approved by the local IACUC Committee.

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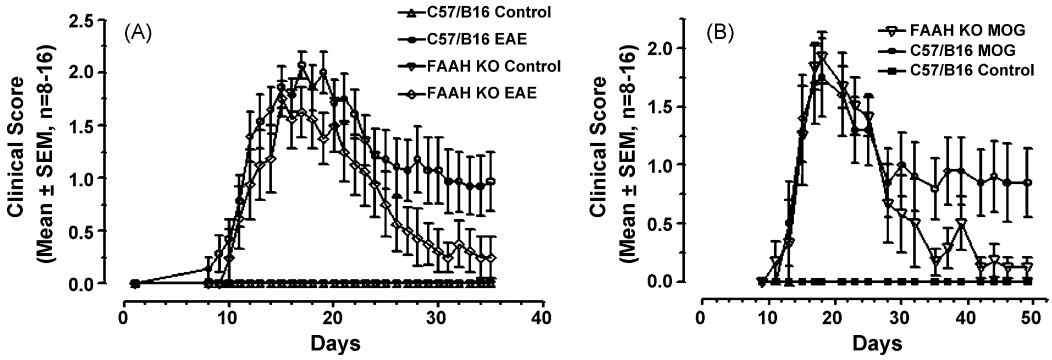

Fig. 1. Progression of EAE in wild type C57BL/6 and FAAH knockout mice of the same genetic background in two independent experiments. Control mice of either genotype received a sham inoculation of Freund's Complete Adjuvant without the encephalitogenic MOG peptide ($FAAH^{+/+}$ $n = 16$ experiment 1 and 2; $FAAH^{-/-}$ $n = 9$ experiment 1, $n = 12$ experiment 2).

Mice at the peak of the early severe phase of EAE (day 14–16) were perfused intracardially with 10% formalin and post fixed in 10% formalin for 48 h. Spinal cord segments corresponding to lumbar regions 2 and 3 were removed from the lumbar vertebra and processed in a tissue processor, embedded in paraffin and ten coronal series sections were cut. Sections were stained with haematoxylin and eosin (HE) to identify infiltrating leucocytes and Luxol Fast Blue (LFB) to identify myelin.

Standard procedures were employed for immunohistochemical staining. Goat anti-CD3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Cat. No. sc-1127) was used at a dilution of 1:100. Donkey anti-goat biotinylated IgG (Chemicon International Inc., Temecula CA, Cat. No. AP180B) was used as secondary antibody. The immunoreactivity was visualized by ABC reagents (Vector, Burlingame, Cat. No. PK-6100) and diaminobenzidine (Dako, Carpinteria, CA) followed by counterstaining with haematoxylin.

Demyelination was quantified on LFB stained sections at a magnification of 40× using Image-Pro Plus 4.0 software, and the percentage of demyelination in the white matter was averaged from all mice in the sample group. T lymphocytes were quantitated by manually counting at 400×.

Time course data are expressed as mean ± standard error of mean of clinical scores (S.E.M., $n = 9$–16 individual animals). For comparisons between wild type and knockout EAE data, a Mann–Whitney non-parametric test was used. Unpaired *T*-test was used on the objectively quantified measurement of demyelination by the Image-Pro Plus 4.0 software.

Two independent studies yielded similar results (Fig. 1A and B). In both experiments, a synchronized response occurred in both $FAAH^{+/+}$ and $FAAH^{-/-}$ groups, with onset on day 8 or 9 after induction. The majority (87–88%) of immunized animals developed clinical signs in both wild type and knockout groups in both experiments (experiment 1, $FAAH^{+/+}$, $n = 16$; $FAAH^{-/-}$, $n = 9$; experiment 2, $FAAH^{+/+}$, $n = 16$; $FAAH^{-/-}$, $n = 12$). A well-defined first phase was seen with a maximal mean clinical score of approaching 2 at day 16–17 (mean scores; experiment 1: $FAAH^{+/+}$, 1.75 ± 0.68; $FAAH^{-/-}$ 1.9 ± 0.4; experiment 2: $FAAH^{+/+}$, 1.8 ± 0.4; $FAAH^{-/-}$ 1.5 ± 0.5). Some individual mice in the control groups achieved scores of 3, while a few mice in the knockout group achieved a maximal score of 2.5. The initial peak of response resolved slowly and incompletely in the control group in both experiments. A plateau of clinical score of about 1 was reached between day 28 and 32, and as expected for this model, this was maintained without sharp further remission or relapse until termination of the experiments at day 36 or 50. The knockout groups in both experiments continued to improve in mean clinical score, reaching 0.25 or less at about day 30, and then maintaining at this level until the end of the experiment.

Fig. 2 presents the data as area under the curve for both experiments as first phase (day 1–28 in both experiments) and "late phase", defined as the remaining time course until the end of the experiment. In both cases, the late phase shows a significant ($p < 0.0001$) decrease in clinical score in the knockout group compared with the wild type controls.

Since the pattern of response in these two experiments suggested that any beneficial effect of FAAH absence was not realised during the early phase of the disease, we hypothesized that during the early phase of EAE, wild type and knockout mice spinal cords should show similar levels of leucocyte infiltration and myelin loss. To test this, a third experiment was initiated to provide tissue for early phase spinal cord histology. By day 14–16 the mean score in both knockout and control groups was approximately 2. Animals from wild type and knockout groups having the same clinical scores were taken at this stage for histological examination of the spinal cords.

Demyelination was observed at day 14–16 in both wild type and knockout mice (Figs. 3A and 4A and B.) Myelinated fibres in the ventral, lateral and occasionally in the dorsal columns of the white matter of spinal cords degenerated and disappeared. Similar numbers of infiltrating leucocytes (revealed by HE staining, Fig. 4C

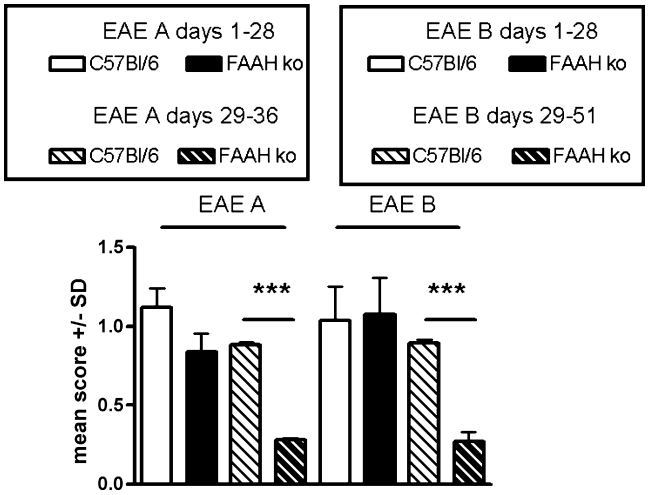

Fig. 2. Data from the two experiments shown in Fig. 1 are expressed as area under the curve for the two phases of the disease, day 1–28, and day 29–termination of the experiments. No statistically significant difference was seen in the first phase of active EAE, but the knockouts showed a highly statistically significant improvement over controls in the later phases of the disease (Mann–Whitney non-parametric test: $^{***}p < 0.001$). $FAAH^{+/+}$ $n = 16$ experiment 1 and 2; $FAAH^{-/-}$ $n = 9$ experiment 1, $n = 12$ experiment 2.