



Inc. (St. Louis, MO); AEA and AEA- $d_4$  from Cayman Chemical Company (Ann Arbor, MI) and Abcam (Cambridge, MA), respectively.

**In-Gel Analysis of Enzyme Activity.** Mouse testis (obtained from ICR male mouse) was homogenized in 50 mM Tris-HCl buffer (pH 8.0) containing 320 mM sucrose, and the homogenate was centrifuged at  $1,000 \times g$  and  $4^\circ\text{C}$  for 10 min, and the supernatant was then centrifuged at  $20,000 \times g$  for 20 min. The  $20,000 \times g$  pellet was finally reconstituted in the Tris buffer (in the absence of sucrose). *In vitro* screening of target molecule(s) for FNT oxon in the mouse testicular membrane proteome was performed by ABPP<sup>12</sup> with a FP-TAMRA fluorescent probe.<sup>13</sup> In brief, a 40 mg testicular membrane protein was reacted with FNT oxon (0.1–1000  $\mu\text{M}$ ) in competition with the FP-TAMRA serine hydrolase probe (1  $\mu\text{M}$ ) for 30 min at  $25^\circ\text{C}$ , and afterward the sample was subjected to SDS–PAGE separation for analyzing the fluorescence activity by a flatbed scanner FLA-3000 (FUJIFILM, Tokyo, Japan). Identically, the testicular membrane proteome obtained from FNT-treated mouse was reacted with the FP-TAMRA probe for ABPP gel-based analysis.

**Enzyme Assays.** FAAH or monoacylglycerol lipase (MAGL) activity in mice testis or epididymis cauda was assayed by hydrolysis of the corresponding substrate [ $^{14}\text{C}$ ]AEA or [ $^{14}\text{C}$ ]mono-oleoylglycerol, respectively (55 mCi/mmol for both substrates), according to Quistad et al.<sup>14,15</sup> Concisely, the aliquot of testicular membrane preparation was incubated with 1  $\mu\text{M}$  [ $^{14}\text{C}$ ]AEA or [ $^{14}\text{C}$ ]mono-oleoylglycerol for 30 min at  $37^\circ\text{C}$ , and the enzymatic reaction was terminated by addition of organic solvent (chloroform/methanol/hexane, 1.25:1.4:1.0) and 200 mM  $\text{K}_2\text{CO}_3$ . Subsequently, the radioactivity in the aqueous upper phase, as the amount of the [ $^{14}\text{C}$ ]arachidonic acid or [ $^{14}\text{C}$ ]oleic acid produced from the enzymatic reaction, was determined by liquid scintillation counter. A half maximal inhibitory concentration ( $\text{IC}_{50}$ ) value was calculated by iterative least-squares regression using Sigmaplot software ver. 8.0 (SPSS Inc., Chicago, IL).

**Animal Studies.** Throughout the animal experiments, the study was carried out in accordance with the Guide to Animal Experimentation of Nagoya City University (approval no. H23M-17). Nineteen male ICR mice aged 9 weeks were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). The animals were housed in cages in the animal room under controlled environmental conditions: temperature  $23\text{--}25^\circ\text{C}$ , relative humidity  $57\text{--}60\%$ , and a 12-h light-dark cycle (lighting 9:00–21:00). Food and water were provided *ad libitum*.

After 1 week of acclimation, the animals were randomly divided into 3 groups each containing 6–7 mice. The 3 groups were orally administered 50 or 100 mg/kg/day FNT dissolved in corn oil or the vehicle for 10 consecutive days. The mice were observed for about 30 min after oral route administration. On the day following the final administration the animals were sacrificed by decapitation. Testes and epididymides cauda were obtained, weighed, and frozen at  $-80^\circ\text{C}$  until analyzed.

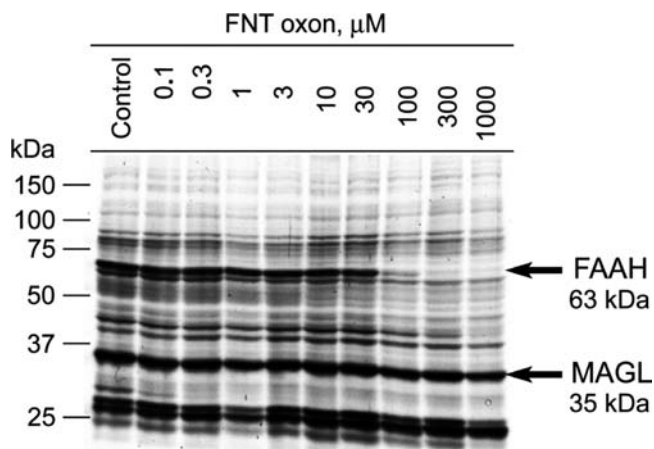
**LC–MS.** Analysis of testicular AEA levels was performed on a LC–MS/MS-8030 system (SHIMADZU, Kyoto, Japan) consisting of a solvent delivery device (LC30AD), an autosampler (SIL-30AC), a system controller (CBM-20A), and a column thermostat CTO-20A) according to the method reported by Zoerner et al.<sup>16</sup> with a slight modification. In short, 50 mg of testis in 150  $\mu\text{L}$  of 250 mM sucrose-phosphate buffer (pH 7.4) was homogenized, and 4 ng of the internal standard, AEA- $d_4$ , was added to 50  $\mu\text{L}$  of the resultant testicular preparations before solvent extraction with toluene. After toluene evaporation under a stream of nitrogen, the samples were reconstituted in water/methanol (1:3), and aliquots of these solutions were analyzed by LC–MS/MS. Separation of analytes was carried out on a Kinetex 1.7  $\mu\text{m}$  XB-C18 100A column (100 mm  $\times$  2.1 mm) (Phenomenex, Torrance, CA).

**Statistics.** Dunnett's multiple comparisons were carried out for data between the FNT-treated and vehicle groups following one-way analysis of variance. When the values were not distributed normally, they underwent a square-root or logarithmic conversion to attain a normal distribution. For variables where the normal distribution was still unattainable even after the conversion, the Kruskal–Wallis test

followed by the Steel's test was employed to detect differences among the groups.

## RESULTS

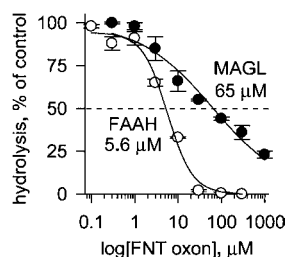
**Potential OP Target in Mouse Testis.** The FP-TAMRA chemical probe broadly labels diverse serine hydrolases. The ABPP approach with the FP-TAMRA probe revealed that FNT oxon (active metabolite of FNT) selectively inhibits the testicular fatty acid amide hydrolase (FAAH, 63 kDa) (Figure 2), known as an endocannabinoid agonist AEA hydrolyzing



enzyme, whereas even at concentrations up to 1000  $\mu\text{M}$ , FNT oxon failed to block FP-TAMRA labeling of the 35 kDa testis protein band assigned as the other endocannabinoid 2-arachidonoylglycerol metabolizing enzyme, MAGL.<sup>12</sup> Several other minor OP-sensitive targets were also detected similar to those in the mouse brain membrane proteome.<sup>17</sup>

**Potency of FNT Oxon As Inhibitor of Testicular FAAH or MAGL.** FNT oxon was markedly more potent against FAAH than MAGL as indicated by their  $\text{IC}_{50}$  values:  $5.6 \pm 0.3$  or  $65 \pm 13 \mu\text{M}$  against FAAH or MAGL, respectively (Figure 3).

**Effects of Subacute FNT Exposure.** At the end of the treatment, no significant difference was observed in body weights or the absolute and relative weights of testis, epididymis cauda, and other organs between the FNT-treated and control groups (data not shown). The activities of FAAH and MAGL in mouse testis and epididymis cauda are shown in Table 1. The FAAH activities in the low- and high-dose groups were  $76 \pm 16$  (50% of relative activity) and  $67 \pm 32$  (44%) pmol/mg/min, respectively, compared with  $150 \pm 18$  pmol/mg/min



**Figure 3.** Potency of FNT oxon as inhibitors of testicular FAAH and MAGL. FAAH or MAGL activity was assayed by hydrolysis of the corresponding substrate, [ $^{14}\text{C}$ ]AEA or [ $^{14}\text{C}$ ]mono-oleoylglycerol, respectively.<sup>14,15</sup>  $\text{IC}_{50}$  values (molar concentration of a test chemical necessary for 50% inhibition of specific enzyme activity) are determined by iterative least-squares regression using Sigmaplot software. The  $\text{IC}_{50}$  value ( $\pm\text{SD}$ ,  $n = 3$ ) of FNT oxon is  $5.6 \pm 0.3$  or  $65 \pm 13 \mu\text{M}$  against FAAH or MAGL, respectively.

**Table 1. Effects of Subacute FNT Exposure (10 Days via Oral Route) on Endocannabinoid Hydrolyzing Enzymes FAAH and MAGL in Mouse Testis and Epididymis Cauda<sup>a</sup>**

treatment	activity, pmol/mg/min ( $\pm\text{SD}$ , $n = 6-7$ )			
	FAAH		MAGL	
	testis	epididymis cauda	testis	epididymis cauda
vehicle	150 $\pm$ 18 (100%)	46 $\pm$ 10 (100%)	710 $\pm$ 26 (100%)	400 $\pm$ 20 (100%)
low	76 $\pm$ 16** (50%)	36 $\pm$ 6.9** (78%)	670 $\pm$ 27 (94%)	390 $\pm$ 36 (98%)
high	67 $\pm$ 32** (44%)	29 $\pm$ 11** (63%)	680 $\pm$ 25 (96%)	380 $\pm$ 15 (95%)

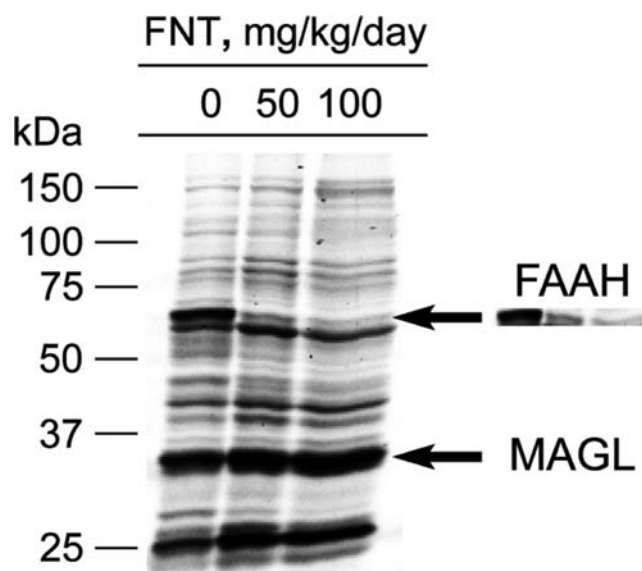
<sup>a</sup>Asterisks indicate significant difference (\*\* $P < 0.01$  based on Dunnett's multiple post hoc test) between the vehicle (corn oil) and treatment (50 or 100 mg/kg/day). FAAH and MAGL activities were evaluated by [ $^{14}\text{C}$ ]AEA and [ $^{14}\text{C}$ ]mono-oleoylglycerol hydrolysis assays. The relative activity (%) is also given in parentheses. AChE activities (nmol/mg/min  $\pm$  SD) in the mouse brain homogenate were compared between the vehicle and treated groups, i.e., vehicle  $66 \pm 4$  (100%); low  $31 \pm 3^{**}$  (47%); high  $28 \pm 7^{**}$  (43%), respectively (\*\* $P < 0.01$ ). No toxic sign was observed for the FNT-treated groups during the entire period of exposure.

(100%) for the vehicle control. Significant decline in the FAAH activities was also observed in the epididymis cauda depending on the FNT exposure. No significant difference in the MAGL activities was discernible between the vehicle and the FNT-exposed groups in either testis or epididymis cauda. Interestingly, no toxic sign was observed for the FNT-administered groups during the whole exposure period, although brain AChE activity after the final administration was reduced (43–47% relative to vehicle control) (see Table 1 footnote). Consistently, selective FAAH inhibition in the FNT-treated testicular proteome was unambiguously evident on the basis of the ABPP analysis (Figure 4).

**AEA Levels.** Testicular AEA levels (pg/mg testis  $\pm$  SD,  $n = 6-7$ ), which may be modulated by FAAH inhibition, did not show any significant difference between the control and the FNT-exposed groups: vehicle  $17 \pm 3.3$ , low  $14 \pm 2.5$ , high  $15 \pm 1.4$ , respectively.

## DISCUSSION

This study describes that the major OP insecticide FNT acts on the FAAH in murine testis and epididymis cauda. The



**Figure 4.** Effects of FNT treatment (10 days via oral route) on the testicular serine hydrolase proteome. A testicular membrane preparation was reacted with the FP-TAMRA probe for ABPP gel-based analysis. A representative ABPP gel image for serine hydrolase proteome is shown in emphasizing selective FAAH inhibition (right).

endocannabinoid system (ECS), regulating a diverse array of physiological functions from energy homeostasis to pain and memory,<sup>18–21</sup> is also known to play important roles on spermatogenesis and sperm motility acquisition.<sup>22–25</sup> The ECS consists of three major players: the cannabinoid type-1 receptor (CB1R); AEA (an endogenous CB1R agonist); and the AEA hydrolyzing enzyme FAAH. OP compounds also act on the mouse brain FAAH and MAGL, consequently resulting in hypomotility or catalepsy.<sup>12,14,15,17</sup> The presence of ECS components in the male reproductive system has been identified in seminal plasma, male reproductive tissues, and Leydig and Sertoli cells, as well as in male germ cells from spermatogonia to mature spermatozoa, and AEA elicits inhibitory effects on male reproduction.<sup>23,25–28</sup>

Down-regulation or inhibition of FAAH, elevating AEA levels, therefore overstimulates the cannabinoid signal, leading to apoptosis of testicular cells such as Sertoli and Leydig. AEA, well characterized in the Sertoli cells, which influence spermatogenesis by providing nutrients and hormonal signals needed for the development of germ cells, has been shown to induce apoptosis of the Sertoli cells and indicated even to be a possible modulator of cell survival and death.<sup>26,28</sup> In the epididymis cauda and sperm cells, AEA also regulates sperm motility through CB1R activation and viability by reducing mitochondrial activity.<sup>22–25</sup> In mice, after spermiation, spermatozoa leave the testis and appear in the epididymis, where they acquire motility traveling from the caput to the cauda. A recent study using wild-type and CB1R knockout mice has shown that AEA inhibits sperm motility in the epididymis in a CB1R-dependent manner.<sup>27</sup> In contrast, CB1R antagonist rimonabant appreciably increases sperm motility and viability.<sup>29</sup>

The present investigation provides two substantial points of evidence that FNT oxon (bioactivated form of FNT) selectively inhibits the mouse testicular FAAH and that repeated FNT exposure leads to diminishing FAAH activity in testis and epididymis cauda. However, unexpectedly, a significant change in testicular AEA levels by the FNT treatment was undetectable



in the present conditions, and accordingly further sustained exposure studies (~6 weeks, a sufficient period for the complete spermatogenesis, or a much longer period) in low FNT doses would be warranted to show the possible relationship between testicular (and possibly epididymal) AEA elevation and spermatotoxicity. This unexpected phenomenon is also observed in the mouse brain FAAH activities and AEA levels after transient OP exposure.<sup>14,17</sup> Indeed, AEA appears to be physiologically labile.<sup>30</sup> The present FNT exposure, in relatively high doses (inducing no neurotoxic sign) and a short period, reflects our goal of exploring a possible OP target in the murine male reproductive system.

Therefore, the present investigation ushers in a new insight that inhibition of FAAH by the OP insecticide in male reproductive tissues appears to trigger spermatotoxicity such as broken sperm, cytoplasmic droplets, and reduced motility and viability.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

ABPP, activity-based protein profiling; AChE, acetylcholinesterase; AEA, anandamide; CB1R, cannabinoid type-1 receptor; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; FNT, fenitrothion; FP-TAMRA, phosphonofluoridate fluorescent probe; MAGL, monoacylglycerol lipase; OP, organophosphate

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