Metabotropic Glutamate Receptor 5 Antagonism with Fenobam

Examination of Analgesic Tolerance and Side Effect Profile in Mice

Michael C. Montana, B.A.,* Beth A. Conrardy, M.D.,† Laura F. Cavallone, M.D.,‡ Benedict J. Kolber, Ph.D.,§ Lesley K. Rao, M.D.,† Suellen C. Greco, D.V.M.,‖ Robert W. Gereau IV, Ph.D.#

ABSTRACT

Background: The metabotropic glutamate receptor 5 noncompetitive antagonist fenobam is analgesic in rodents. Future development of fenobam as an analgesic in humans will require a favorable long-term treatment profile and a lack of significant deleterious side effects. This study aimed to determine whether tolerance to fenobam's analgesic effects developed over 14 days and to assess for side effects in mice.

Methods: Mouse models of pain, locomotor behavior, and coordination were used. Fenobam or vehicle (n = 8 or 11 per group) was administered for 14 days, and analgesic tolerance to fenobam was assessed using the formalin test. Histopathologic examination, hematology, and clinical chemistry analysis after 14-day fenobam administration were also assessed (n = 12 or 9). The effects of fenobam on locomotor activity were assessed in the open field and elevated zero maze (n = 8 or 7). Coordination was assessed using ledge crossing and vertical pole descent tasks (n = 11 or 10).

Results: Tolerance to fenobam's analgesic effect did not develop after 14 days. Chronic fenobam administration resulted in statistically significantly less weight gain compared with vehicle control subjects, but did not cause any physiologically or statistically significant hematologic abnormalities, altered organ function, or abnormal histopathology of the liver, brain, or testes. Fenobam administration resulted in a metabotropic glutamate receptor 5-dependent increase in exploratory behavior but does not impair motor coordination at analgesic doses.

Conclusions: Analgesic tolerance to repeat fenobam dosing does not develop. Chronic dosing of up to 14 days is well tolerated. Fenobam represents a promising candidate for the treatment of human pain conditions.

What We Already Know about This Topic

- The metabotropic glutamate receptor 5 antagonist fenobam produces acute analgesia in rodents, and may be developed for clinical use
- Whether analgesic tolerance develops with chronic fenobam treatment has not been studied

What This Article Tells Us That Is New

- Two-week daily dosing, a duration that produces extreme tolerance to opioids, did not result in tolerance to fenobam's analgesic effect in mice

* M.D./Ph.D. Candidate, Medical Scientist Training Program, Department of Anesthesiology, Washington University Pain Center, Program in Neuroscience, Washington University School of Medicine, St. Louis, Missouri. † Clinical Fellow, Department of Anesthesiology, Washington University Pain Center. ‡ Assistant Professor, Department of Anesthesiology, Washington University Pain Center. § Postdoctoral Fellow, Department of Anesthesiology, Washington University Pain Center. ‖ Assistant Director, Diagnostics, Division of Comparative Medicine, Washington University School of Medicine. # Professor, Department of Anesthesiology and Chief of the Basic Research Division, Washington University Pain Center, Program in Neuroscience, Washington University School of Medicine.

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Address correspondence to Dr. Gereau: Washington University Pain Center, Department of Anesthesiology, 660 S. Euclid Avenue, St. Louis, Missouri 63110. gereaur@wustl.edu. Anesthesiology 2011; 115:1239–50

GLUTAMATE is the primary excitatory neurotransmitter in the mammalian nervous system. Abnormal glutamatergic signaling may play a prominent role in several disease processes, including chronic pain.[1,2] The G-protein coupled glutamate receptor metabotropic glutamate receptor 5 (mGlu5) is expressed at synapses throughout the nervous system, in particular the pain neuraxis,[3] and agents that modulate mGlu5 may have therapeutic potential in the treatment of pain. Significant inroads into the use of pharmacologic agents acting at mGluRs in human patients have been made recently,[4,5] and findings that activation of mGlu5 is pronociceptive[6–8] whereas its inhibition is antinociceptive[9–11] have led to the suggestion that antagonism of mGlu5 may have analgesic efficacy in humans. However, new pharmacologic agents that target mGlu5 will also have to possess acceptable safety profiles along with clinical efficacy. This is illustrated by the recent discontinuation of clinical trials involving the Addex Pharmaceuticals (Plan-les-Ouates, Switzerland) mGlu5 antagonist ADX-10059, which, despite fourfold improvement *versus* placebo in inducing a pain-free state in migraineurs, resulted in unacceptable liver enzyme increases.[12]

The recent finding that the clinically validated compound fenobam is a potent and selective mGlu5 negative allosteric modulator[13] has led to a resurgence in interest in testing the efficacy of fenobam as a treatment for various neurologic conditions. Originally developed in the 1970s as an anxiolytic with a then-unknown mechanism of action, fenobam was found to have a favorable safety profile;[14,15] however, initial results regarding its anxiolytic efficacy were mixed.[16,17] Further human clinical testing was discontinued in the early 1980s. Fenobam has subsequently been demonstrated to be analgesic in rodents[10,11] and moderately effective in managing some of the symptoms of fragile X syndrome in humans.[4] However, in these studies fenobam was only administered as a one-time dose, and future treatment strategies involving fenobam or other mGlu5 antagonists in pain conditions will likely require longer-term treatment. It is currently unknown whether tolerance develops to fenobam's analgesic effects. In addition, whereas the elevations in liver enzymes seen with ADX-10059 may be compound-specific, the effects of chronic mGlu5 antagonist administration on liver function and other vital systems are not well characterized in the literature.

Here, we performed a series of experiments to determine whether tolerance develops to the analgesic effects of fenobam and to assess for deleterious side effects. We test whether tolerance to the analgesic effects of fenobam develops after repeated dosing of up to 14 days. In addition, we assessed for alterations in liver function, gross liver histopathology, and hematologic abnormalities after chronic fenobam treatment. Sedation and impairment of motor coordination are common dose-limiting factors for some classes of analgesic drugs. Fenobam may act as a stimulant,[10,16] but it is unknown whether this stimulant effect is due to specific activity at mGlu5. Therefore, we also assessed the locomotor activity of mGlu5 knockout mice treated with fenobam. Finally, mGlu5 has been shown to play a role in weight gain and energy balance.[18,19] We sought to expand these results by determining if fenobam mediates any effects on appetitive behavior.

Materials and Methods

Materials

Subjects. Experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of Washington University School of Medicine (St. Louis, Missouri). Male Swiss Webster mice (5 weeks old) were purchased from Taconic (Germantown, New York) and group-housed five animals to a cage for 1 week before any behavioral experiments were started. For experiments involving mice lacking mGlu5 (mGlu5 knockout; 6–9 weeks old), animals were bred in-house on a C57BL/6 background and compared with wild type (WT) littermates.[20] All C57BL/6 WT mice used in behavioral experiments were also derived from this colony. For experiments involving knockout animals the experimenter was blinded to genotype. Genotyping of mice bred in-house was performed using standard polymerase chain reaction techniques. All mice were group-housed on a 12/12-light/dark schedule with *ad libitum* access to food and water, except as described in the next paragraphs during food deprivation experiments.

Chemicals and Reagents. Fenobam was purchased from Tocris (Ellisville, MO) or was custom-synthesized for our use by Scynexis (Research Triangle Park, NC) and dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich Chemical Company, St. Louis, MO) on the day of the experiment. All intraperitoneal injection volumes were 20 μl. Throughout all experiments the investigator was blinded to pharmacologic treatment. All other reagents were high-performance liquid chromatography grade and purchased from Sigma–Aldrich Chemical Company.

Behavioral Assays

Open-Field Locomotor Test. Locomotor activity was measured in an open-field chamber (42 l × 42W × 30H cm) attached to the VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH). For experiments involving drug-naive C57BL/6 WT and mGlu5 knockout mice littermates, animals were brought to the testing room in their home cage and acclimated for at least 2 h before testing. Locomotor activity was assessed individually by recording photobeam breaks for 60 min. Total distance traveled, time spent moving, and the number of beam breaks (horizontal activity) were calculated for the entire chamber. For experiments designed to assess the effects of fenobam on locomotor behavior in mGlu5 knockout mice, animals were habituated in their home cages for at least 2 h, and then injected intraperitoneally with either 30 mg/kg fenobam or

vehicle (DMSO) immediately before placement in the chamber. Animals were allowed to explore the chamber for 90 min, locomotor activity was measured individually as described above, and the total distance traveled was calculated for the entire chamber.

Elevated Zero Maze. Locomotor activity was measured in low light conditions using a zero maze (Stoelting Co., Wood Dale, IL) placed 70 cm off of the ground and consisting of two closed sections (wall height, 30 cm) and two open sections (wall height, 1.3 cm) on a circular track (diameter of track, 60 cm). C57BL/6 WT mice were habituated to testing room for 1 h before injections, and then injected intraperitoneally with fenobam (30 mg/kg) or vehicle (DMSO). Fifteen minutes after injection, mice were placed individually in the closed area of the zero maze for a 600-s trial. Movement during the trial was recorded using two digital video cameras (Logitech 9000 Pro webcam, Romanel-sur-Morges, Switzerland). Total distance traveled, number of entries into open sections, and time spent in the open sections was scored off-line using AnyMaze video tracking software (Stoelting Co.).

Motor Sensory Assessment. A series of three tests was performed on drug-naive C57BL/6 WT mice to assess the effects of fenobam on gross motor behavior and coordination.[21,22] All tests were performed two times.

Ledge Crossing Task. Each mouse was tested to see how long it could maintain its balance on a 0.75 cm-wide Plexiglass ledge without falling (60 s maximum). A score of 60 s was also assigned if the mouse traversed the entire 51-cm length of the ledge and returned to the starting point in less than 60 s without falling.

Vertical Pole Descent Task. Mice were placed head upward at the top of a vertical metal rod (8 mm diameter, 55 cm height). The rod was finely textured with a file to provide a gripping surface. Mice were given a maximum of 120 s to turn 180 degrees and climb down to reach the bottom of the pole and place all four paws on the tabletop. Mice were required to reverse direction and actively climb down. If a mouse slid down the pole without reversing direction or fell down the pole it was given a score of 120 s.

Inverted Screen Hang. Mice were placed on a wire mesh screen (16 squares per 10 cm^2, 47 cm high × 18 cm wide) oriented at 60 degrees. The screen was then inverted to 180 degrees so that mice were upside down. The time mice spent hanging on the screen was measured for 2 min. A maximum score of 120 s was given to an animal that did not fall.

Spontaneous Formalin-induced Nocifensive Behavior after Chronic Fenobam Treatment. Male Swiss Webster mice received chronic intraperitoneal injection with 30 mg/kg fenobam or vehicle (DMSO) once per day for 5 days. On the sixth day, mice were placed in transparent Plexiglas boxes (10 l × 10W × 15H cm, assembled by the Washington University School of Medicine Instrument Machine Shop) on a glass surface and acclimated for 2 h before any drug injection. Animals were then pretreated by intraperitoneal injection with vehicle or fenobam (30 mg/kg). Ten microliters of dilute formalin solution (5% dissolve in normal saline, Sigma-Aldrich Chemical Company) was injected subcutaneously into the plantar surface of the right hind paw. Some mice from the chronic-vehicle group were injected with fenobam, whereas others received vehicle, followed 5 min later by intraplantar formalin injection. Thus, three separate groups were analyzed: chronic vehicle/acute vehicle; chronic vehicle, acute fenobam; and chronic fenobam, acute fenobam, with chronic administration defined as 5 days. Spontaneous behavior was recorded using a digital video camera (Logitech 9000 Pro webcam, Romanel-sur-Morges, Switzerland) placed underneath the glass platform. Video recordings were scored after the collection of all data by an observer blinded to treatment (both chronic and acute) and the time spent in nocifensive behavior defined as licking, lifting, or flicking of the injected paw, and was scored in 5-min intervals for 45 min after paw injection. A separate set of experiments was performed exactly as just described, except animals were injected with fenobam (30 mg/kg) or vehicle for 14 days instead of 5 days, with formalin testing performed on the 15th day. Animals from this longer time period of injections were killed immediately after the cessation of the formalin test, and tissue obtained from them was used in the serum chemistry analysis and tissue histology described in the following paragraphs. All animals were weighed daily immediately before intraperitoneal injection.

Effects of Fenobam on Food Intake and Weight Gain. Food deprivation studies were performed in a manner similar to that previously described [18]. For experiments designed to assess the acute effects of fenobam on food intake, mice were weighed and housed individually in their home cages 3 days before food deprivation. Cage bedding was replaced with a raised wire mesh bottom. On the third day mice were weighed again and food deprived for 24 h starting 1 h after lights on until 1 h after lights on the next day. Mice were then weighed again and injected intraperitoneally with either 30 mg/kg fenobam or vehicle, and preweighed food was placed onto the cage bottom. Food intake was measured for each mouse at 15, 30, 60, 90, and 180 min, as well as 3 h before lights off (*i.e.*, 8 h later). To compensate for variation of body weight, mouse food intake was normalized to body weight. Mice were weighed to the nearest 0.1 g and food was weighed to the nearest 0.01 g.

Serum Chemistry Analysis and Complete Blood Count with Differential

Plasma and Tissue Collection. All mice from the 14-day chronic fenobam injection study (see previous paragraphs) were administered an overdose of sodium pentobarbital (75 mg/kg) after cessation of the formalin test. Whole blood was obtained by transcutaneous cardiac puncture and placed into plasma separator tubes with lithium heparin for blood chemistries or EDTA tubes for complete blood counts (BD Microtainer, Franklin Lakes, NJ). Serum chemistry analysis and necropsy were performed immediately after tissue collection.

General Instrumentation. Complete blood counts were analyzed using the Hemavet 1700 (Drew Scientific, Waterbury, CT). Leukocyte differentials were determined by direct examination of blood smears. Serum chemistries were analyzed using the Vitros DT60 system dry reagent chemistry analyzer (Ortho Clinical Diagnostics, Rochester, NY).

Histologic Analysis. Tissues were formalin-fixed, paraffin-embedded, and sectioned at 5 μm. Sections were prepared according to standard histologic techniques and stained with hematoxylin and eosin.

Statistical Analysis

Behavioral Data Analysis. Statistical analysis of behavioral data was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). All data collected over multiple time points from the open-field assay, the spontaneous formalin test, and the food deprivation studies were statistically analyzed using a Bonferroni correction multiple comparison test after a nonrepeated measures two-way ANOVA. The factors used for these analyses were genotype/treatment and time. Summed data from these tests were analyzed using a two-tailed Student *t* test when comparisons were made between two groups or a Bonferroni multiple comparisons tests after a one-way ANOVA when comparisons were made between more than two groups. In all studies, the accepted level of significance was $P < 0.05$. In all figures, data are reported as mean ± SEM. Also listed in the text are 95% CIs of the difference between means.

Serum Chemistry and Cell Count Data Analysis. Statistical analysis of data were performed using Prism 5.0 (GraphPad Software, Inc.). All data collected from chronically injected fenobam *versus* vehicle-injected animals were statistically analyzed using an unpaired Student *t* test. In all studies, the accepted level of significance was $P < 0.05$. In all figures data are reported as mean ± SEM.

Results

Effects of Genetic and Pharmacologic Disruption of mGlu5 in the Open-Field Task

Previous work from our laboratory suggests that mGlu5 inhibition *via* fenobam may affect locomotor activity.[10] We

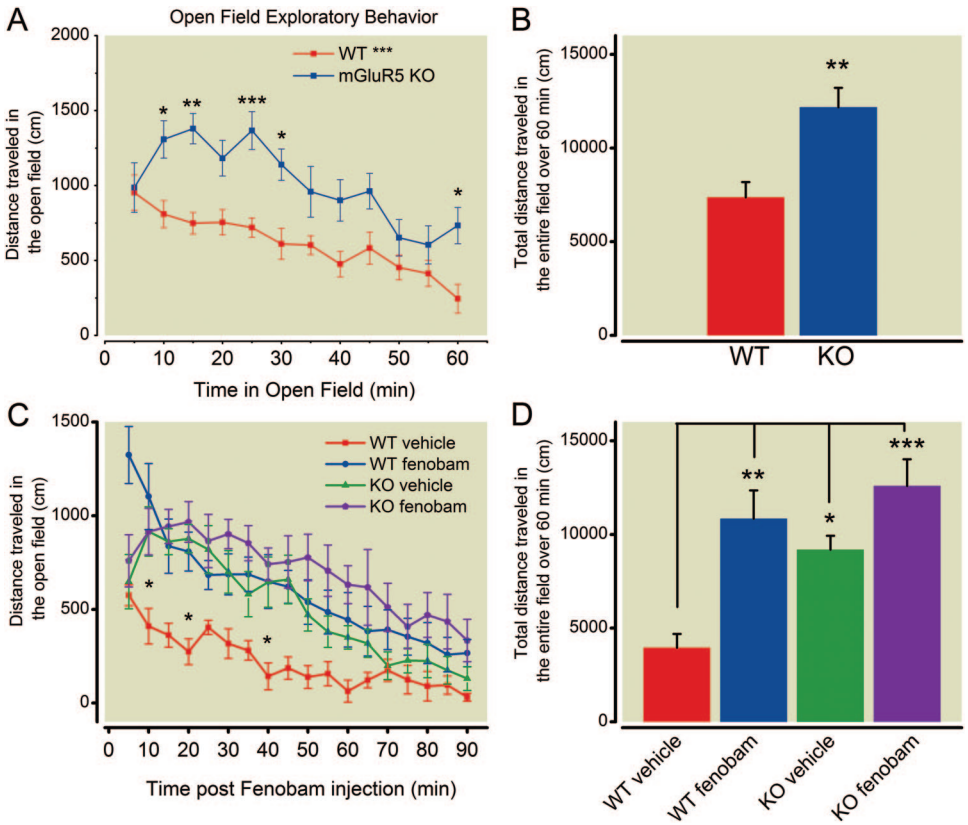

Fig. 1. The effects of fenobam and mGlu5 disruption on open field locomotor behavior. (*A*) Drug-naïve mGlu5 knockout mice traveled significantly farther compared with their wild type littermates at multiple time points (two-way ANOVA main effect $P <$ 0.0001; Bonferroni correction posttest *, **, *** $P < 0.05$, 0.01, and 0.001, respectively) and (*B*) as a sum total of distance traveled in 60 min (unpaired Student *t* test $P = 0.0044$) n = 8 per group. (*C* and *D*) WT mice traveled significantly less than all other groups at multiple time points (*C*, two-way ANOVA main effect $P < 0.0001$; Bonferroni correction posttest, * $P < 0.05$) and as a sum total of distance traveled in 90 min (*D*, one-way ANOVA $P = 0.0001$; Bonferroni correction posttest *, **, ***, $P < 0.05$, 0.01, and 0.001, respectively). Fenobam did not affect the total distance traveled in mGlu5 knockout mice compared with vehicle-treated mGlu5 knockouts (Bonferroni correction posttest $P > 0.05$) (n = 7 per group in WT-vehicle and WT-fenobam and n = 8 per group in KO-vehicle and KO-fenobam groups, respectively). cm = centimeter; KO = knockout; WT = wild type.

expanded upon previous findings to determine whether altered locomotor behavior after fenobam administration is mediated *via* inhibition of mGlu5 or through an off-target effect of the drug. The open-field task was used to assess the effects of mGlu5 deletion and inhibition on locomotor activity. When the effects of genetic deletion of mGlu5 were examined in the open field, we found that drug-naïve mGlu5 knockout mice traveled a statistically significant farther distance in the open field over a 60-min time period in comparison with their drug-naïve WT littermates (7,365 ± 876.7 cm *vs.* 12,171 ± 1111 cm; 95% CI −7843 to −1770 cm; $P < 0.001$; fig. 1, A and B). To assess whether the increased locomotor activity after fenobam administration was due to inhibition of mGlu5, knockout mice and WT littermates were injected with fenobam (30 mg/kg) or vehicle (DMSO) and then immediately placed in the open field for 90 min. There was a statistically significant increase in the total distance traveled in the fenobam-treated WT mice and both the vehicle- and fenobam-treated mGlu5 knockout mice compared with vehicle-treated WT mice (95% CI between vehicle-treated WT mice and fenobam-treated WT, vehicle-treated knockout mice and fenobam-treated knockout mice −1,1735 to −2,044 cm, −9,924 to −541.8 cm, and −13,329 to −3,946 cm, respectively; $P < 0.001$; fig. 1, C and D). No differences were noted between vehicle- or fenobam-treated mGlu5 knockout animals.

Effects of Fenobam on Performance in an Elevated Zero Maze

Mice injected with fenobam (30 mg/kg) traveled a statistically significant farther distance during a 10-min period in an elevated zero maze when compared with vehicle-injected mice (41.44 ± 3.099 m *vs.* 15.59 ± 2.651; 95% CI −35.30 to −16.40 m; $P = 0.0002$). In addition, fenobam-injected mice more frequently entered the open sections of the zero maze ($P = 0.001$; fig. 2A), spent a statistically significant longer amount of time in the open section of the zero maze ($P < 0.01$, fig. 2B), and traveled a statistically significant farther distance as a percentage of total distance traveled in the open sections of the zero maze (*i.e.*, distance in open arms divided by total distance) ($P < 0.01$; fig. 2C) compared with vehicle-injected control mice.

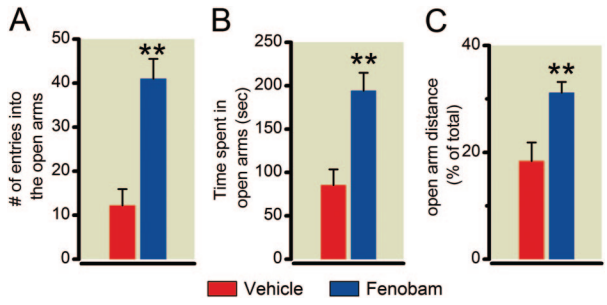

Fig. 2. Fenobam increases the time spent in the open sections of an elevated zero maze. Drug-naïve C57 WT mice were injected with either vehicle or fenobam (30 mg/kg). Fenobam injected mice (*A*) entered the open sections significantly more than their vehicle-injected littermates, (*B*) spent significantly more time in the open sections, and (*C*) traveled significantly farther as a percentage of total distance traveled in the open sections (unpaired Student *t* test $^{**}P < 0.01$) over a 10-min period. (n = 5 in the vehicle group and 6 in the fenobam per group). WT = wild type.

When knockout mice were compared with WT control mice, no differences were noted in the total distance traveled (WT n = 8, 19.04 ± 1.633 meters *vs.* knockout n = 7, 21.49 ± 2.770 meters; 95% CI −9.344–4.452 m; $P > 0.05$), the number of entries into the open sections of the zero maze (WT 13.75 ± 1.916 *vs.* knockout 21.14 ± 4.512; 95% CI −15.70–4.450 entries; $P > 0.05$), the distance traveled as a percentage of total distance traveled in the open arms (WT 21.41 ± 3.300% *vs.* knockout 18.99 ± 4.310%; 95% CI −9.223–14.06%; $P > 0.05$), knockout mice exhibited reduced time in the open sections of the maze (WT 86.19 ± 13.83 s *vs.* knockout 156.2 ± 24.43; 95% CI −126.6 to −11.36 s; $P = 0.0229$).

Effects of Fenobam on Motor Coordination

In comparison with vehicle, fenobam injection resulted in no physiologically or statistically significant differences in performance on three separate tasks designed to assess motor coordination. Mice injected with fenobam (30 mg/kg) performed equivalent to vehicle-injected mice when required to hang from an inverted screen for 120 s (95% CI for trial 1, 52.87–6.106; for trial 2, 33.56–25.41; fig. 3A), descend from a thin (8 mm diameter, 55 cm height) vertically-oriented metal pole (95% CI for trial 1, 48.85–49.06; for trial 2, 46.52–51.39; fig. 3B), and cross a thin (0.75 cm wide, 51 cm long) horizontally oriented ledge (fig. 3C). No physiologically or statistically significant difference between vehicle-injected and fenobam-injected C57 WT mice was observed over two consecutive trials.

The Effects of Repeat Dosing of Fenobam on Analgesic Efficacy

To test whether tolerance to the analgesic effects of fenobam develops over time, Swiss Webster WT mice were injected with either vehicle or fenobam (30 mg/kg) for 5 days. On the sixth day, mice from the chronic-vehicle group were injected with either fenobam or vehicle, and mice from the chronic-fenobam group were injected with fenobam 5 min before performing a formalin test. Regardless of whether they had undergone chronic injection of fenobam or vehicle, mice receiving acute injections of fenobam demonstrated statistically significantly less spontaneous nocifensive behavior postformalin injection when compared with mice that had undergone chronic injection of vehicle mice and that received acute injections of vehicle ($P < 0.001$; fig. 4A). Acute pretreatment with fenobam (30 mg/kg intraperitoneal) statistically significantly reduced the time mice spent licking or lifting the formalin-injected paw during the first phase (95% CI of the mice treated with chronic vehicle/acute vehicle

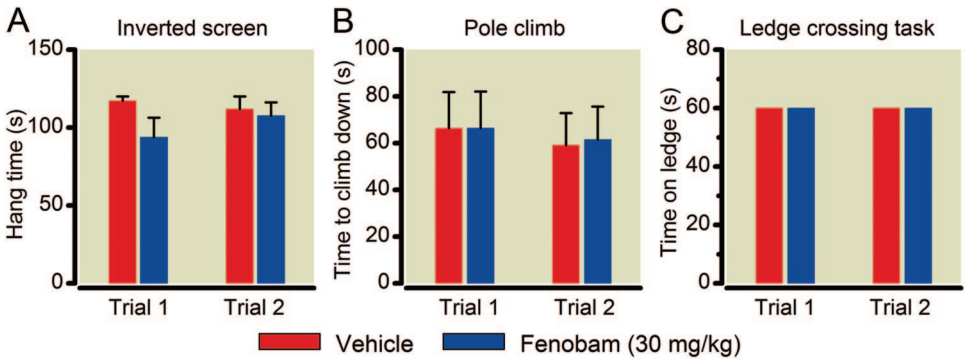

Fig. 3. Fenobam does not impair motor coordination. Drug-naïve C57 WT mice were injected with either vehicle or fenobam (30 mg/kg). Within 20 min their performance was assayed twice on three separate tasks designed to assess motor coordination: (*A*) the inverted screen hang time, (*B*) the vertical pole descent task, and (*C*) the ledge-crossing task. No differences were noted between fenobam- and vehicle-injected mice in any task. (n = 10 in the vehicle group and 11 in the fenobam group). Veh = vehicle; WT = wild type.

compared with chronic vehicle/acute fenobam and chronic fenobam/acute fenobam 14.57–179.0 and 30.16–200.8 s, respectively; $P = 0.0097$; fig. 4B) and the second phase compared with 5-day vehicle/vehicle mice, regardless of fenobam pretreatment (95% CI of the mice treated with chronic vehicle/acute vehicle compared with chronic vehicle/acute fenobam and chronic fenobam/acute fenobam 124.2–740.5 and 81.56–721.1 s, respectively; $P = 0.0069$; fig. 4C).

This experiment was then repeated with a second group of Swiss Webster WT mice, with the one alteration that fenobam or vehicle was injected for 14 days before performing a formalin test on the 15th day. Similar to the 5-day experiment previously discussed, mice undergoing acute treatment with fenobam demonstrated statistically significantly less spontaneous nocifensive behaviors postformalin injection when compared with 14-day vehicle/vehicle mice ($P < 0.001$; fig. 4D). Mice that were chronically treated with vehicle and then pretreated with fenobam exhibited statistically significantly reduced time spent licking or lifting in the first phase compared with 14-day vehicle/vehicle mice (95% CI 16.02–156.7 s; $P = 0.0258$; fig. 4E). Acute pretreatment with fenobam (30 mg/kg intraperitoneal) also statistically significantly reduced the time mice spent licking or lifting the formalin injected paw during the second phase compared with 5-day vehicle/vehicle mice, regardless of fenobam pretreatment (95% CI of the chronic and acutely vehicle-treated mice compared with chronic vehicle/acute fenobam and chronic fenobam/acute fenobam 34.97–575.3 and 65.50–583.9 s, respectively; $P = 0.0154$; fig. 4F).

The Effects of Chronic Fenobam Injection on Histopathology, Serum Chemistries, and Complete Blood Count

Serum was collected from mice who received chronic injections of either vehicle or fenobam for 14 days. Serum chemistry values for cholesterol, total bilirubin, triglycerides, creatinine, total protein, alanine-aminotransferase, aspartate-aminotransferase, lactate dehydrogenase, and amylase were measured. No differences in any values measured were seen between fenobam- (30 mg/kg intraperitoneal for 14 days) and vehicle-injected (vehicle intraperitoneal for 14 days) mice (fig. 5A–I; 95% CIs: cholesterol: −23.87–10.17 mg/dl, total bilirubin: −0.07–0.10 mg/dl, triglycerides: −37.00–9.46 mg/dl, creatine: −0.043–0.021 mg/dl, total protein: −0.42–0.13 g/dl, alanine-aminotransferase: −83.38–289.5 units, aspartate-aminotransferase: −32.61–116.4 units, lactate dehydrogenase: −1,310–3,167 units, and amylase: −1,361–2,788 units; $P > 0.05$ for all comparisons).

Whole blood was also obtained from mice who received chronic injections of either vehicle or fenobam for 14 days and assayed for counts of leukocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, or platelets. No differences were found between mice injected daily with fenobam (30 mg/kg intraperitoneal for 14 days) or vehicle (vehicle intraperitoneal for 14 days). (fig. 6A–H; 95% CIs: leukocytes, −0.8969–1.052°K/cu mm, erythrocytes −3.187–0.8982 M/cu mm, hemoglobin −4.845–1.005 g/dl, hematocrit −18.84–4.038%, mean corpuscular volume −7.191 to 2.258 cu microns, mean corpuscular hemoglobin −2.358–0.4581 pg, mean corpuscular hemoglobin concentration −9.907–4.767%, and platelets −342.6–330.8°K/cu mm; $P > 0.05$ for all comparisons.)

In addition, a 100-count leukocyte differential was performed on all mice from which whole blood was obtained. No differences were noted in the number of segmented neutrophils (all values presented as mean number of cells per cubic mm ± SD) (vehicle = 506.9 ± 140.6; fenobam = 651.8 ± 428.1, $P > 0.05$), lymphocytes (vehicle = 1,194 ± 725.2; fenobam = 945.3 ± 632.5, $P > 0.05$), or monocytes (vehicle = 2.5 ± 6.1; fenobam = 5.1 ± 10.8, $P > 0.05$). No band forms, eosinophils, or basophils were observed from any mice in either injection group.

Several animals from each group were examined by necropsy. In addition, tissue histopathology was performed on brain, liver, and testes on all animals examined by necropsy.

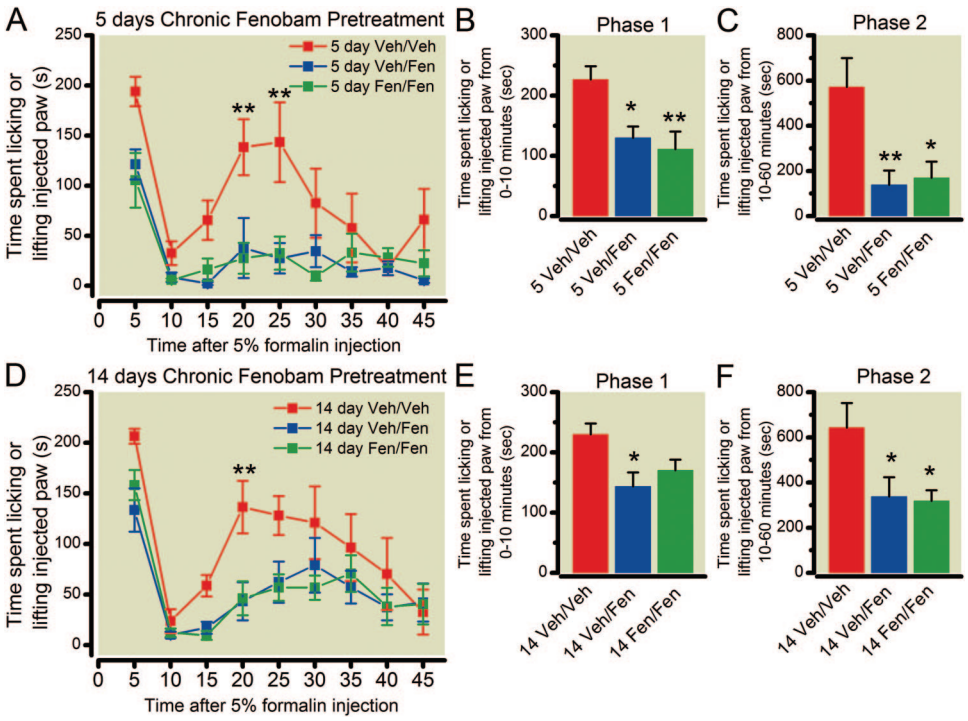

Fig. 4. The effects of chronic fenobam injection on spontaneous formalin behavior. Mice that were administered either fenobam or vehicle for 5 days (*A–C*) before the formalin test demonstrated significantly decreased time spent licking or lifting the injected paw when administered fenobam (30 mg/kg) 5 min before intraplantar formalin injection compared with mice treated with vehicle for 5 days and vehicle on the day of the experiment (two-way ANOVA main effect of treatment $P < 0.0001$; Bonferroni correction posttest ** $P < 0.01$ compared with 5-day Veh/Veh). Both the first phase (*B*) and the second phase (*C*) were reduced (one-way ANOVA main effect of treatment $P = 0.0097$, $P = 0.0069$, respectively; Dunnett posttest *, ** $P < 0.05$ and 0.01, respectively compared with 5-day Veh/Veh mice, n = 6 in Veh/Veh per group and n = 7 in Veh/Fen and Fen/Fen groups, respectively). Mice that were administered either fenobam or vehicle for 14 days (*D–F*) before the formalin test demonstrated significantly decreased time spent licking or lifting the injected paw when administered fenobam (30 mg/kg) 5 min before intraplantar formalin injection compared with mice treated with vehicle for 14 days and vehicle on the day of the experiment (two-way ANOVA main effect of treatment $P < 0.0001$; Bonferroni correction posttest ** $P < 0.01$ compared with 14-day Veh/Veh). The second phase (*F*) was reduced in both groups compared to Veh/Veh mice (one-way ANOVA main effect of treatment $P = 0.0154$; Dunnett posttest * $P < 0.05$) as compared to 14-day Veh/Veh mice. The first phase (*E*) was significantly reduced in vehicle/fenobam mice when compared with Veh/Veh mice (one-way ANOVA main effect of treatment $P = 0.0258$; Dunnett posttest * $P < 0.05$ compared with 14-day Veh/Veh mice). (n = 8 in the Veh/Veh and n = 11 in the Veh/Fen and Fen/Fen groups, respectively). Fen = fenobam; Veh = vehicle.

Additional organs that were noted to be abnormal by gross necropsy also underwent tissue histopathology on a case-by-case basis. In all animals, the hair, coat, and skin were normal, skeletal palpation revealed no evidence of malformation or trauma, there was no nasal or ocular discharge or diarrhea, and hydration and body fat were normal. Whole body and individual organ weights are detailed in table 1. No physiologically or statistically significant differences were found in total body weight or any organ weight between fenobam- and vehicle-treated mice. The major thoracic and abdominal organs and brain were examined in all animals. No abnormalities were noted in the respiratory system, digestive system, musculoskeletal system, urinary system, genital system, brain, thymus, spleen, lymph nodes, adrenal, thyroid, pituitary, or eye. Gross abnormalities and histopathologic examinations of the brain, liver, testes, and any other organs found to exhibit abnormalities on gross examination are noted in table 2. No physiologically or statistically significant differences were found between the fenobam- and vehicle-treated animals with regard to the type or prevalence of any abnormalities.

The Effects of Fenobam on Weight Gain and Postfood Deprivation Food Intake

Animals injected with fenobam or vehicle for the 14-day tolerance study described previously were weighed daily before injection. When the percentage change in body weight was calculated as a function of starting weight for each animal, daily fenobam injection was found to have a statistically significant effect on percent change in body weight over a 14-day period, compared with vehicle (fig. 7A, $P < 0.0001$). No differences were found in the average starting weights of the animals (vehicle = 29.34 ± 0.77g, fenobam = 29.42 ± 0.65 g, 95% CI −2.112–1.946 g; fig. 7A, inset, $P > 0.05$).

The effects of fenobam on food intake after a 24-h food deprivation were assessed in WT Swiss Webster mice (fig.

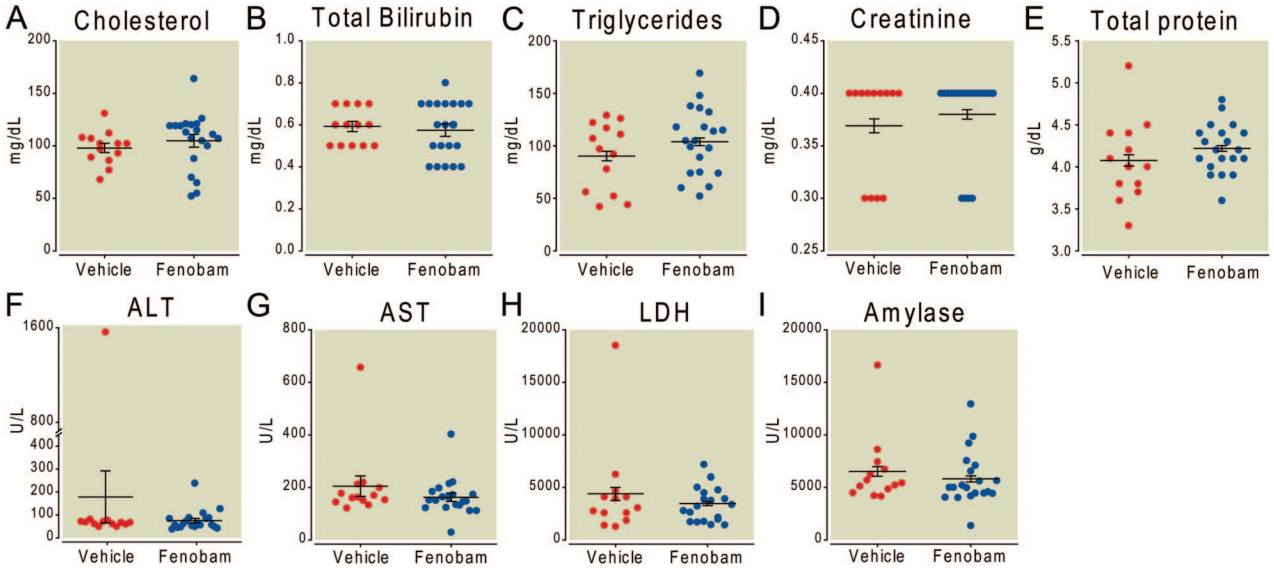

Fig. 5. The effects of chronic fenobam injection on serum chemistries. No differences were found between mice injected daily with fenobam (30 mg/kg intraperitoneal) or vehicle in (*A*) serum cholesterol, (*B*) total bilirubin, (*C*) triglycerides, (*D*) creatinine, (*E*) total protein, (*F*) alanine-aminotransferase (ALT), (*G*) aspartate-aminotransferase (AST), (*H*) lactate dehydrogenase (LDH), or (*I*) or amylase (unpaired Student *t* test p more than 0.05 vehicle compared with fenobam). (n = 13 for vehicle and 20 for fenobam). U = units.

7B). Fenobam (30 mg/kg) administered at the time of refeeding statistically significantly decreased the amount of food consumed, compared with vehicle-treated animals (fig. 7B, $P < 0.001$). Fenobam-treated mice consumed more food 60 min (95% CI −36.42 to −11.72 mg/g body weight), 90 min (95% CI −36.97 to −12.28 mg/g body weight) and 180 min (95% CI −26.17 to −1.470 mg/g body weight) after refeeding compared with vehicle-treated

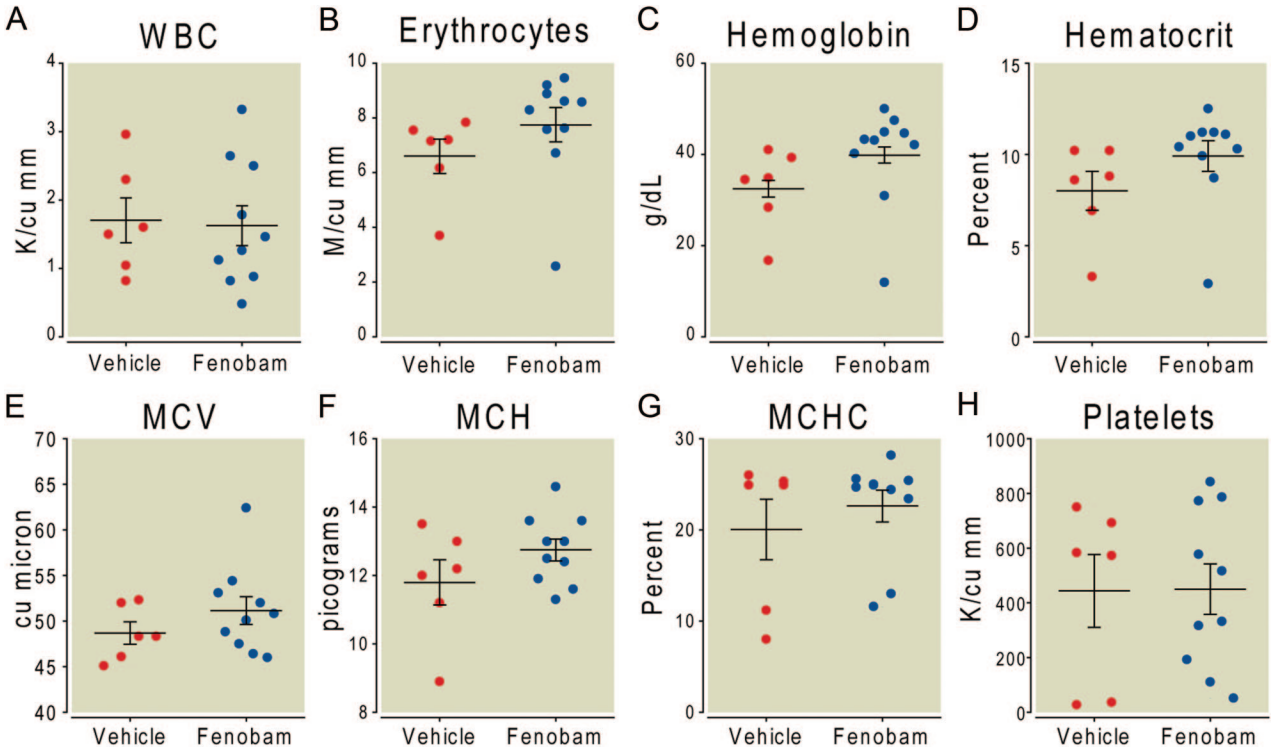

Fig. 6. The effects of chronic fenobam injection on complete blood count. No differences were found between mice injected daily with fenobam (30 mg/kg intraperitoneal) or vehicle in serum (*A*) leukocyte count (WBC), (*B*) erythrocyte cell count, (*C*) hemoglobin, (*D*) hematocrit, (*E*) mean corpuscular volume (MCV), (*F*) mean corpuscular hemoglobin (MCH), (*G*) mean corpuscular hemoglobin concentration (MCHC), or (*H*) platelets. (Unpaired Student *t* test $P > 0.05$ vehicle compared with fenobam). (n = 6 for vehicle and 10 for fenobam). cu = cubic; K = thousand; M = million.

Table 1. The Effects of Chronic Fenobam Injection on Visceral Organ Weights

—	Total Animal	SD	Heart	SD	Lung	SD	Liver	SD
Vehicle	30.11	2.32	0.16	0.01	0.20	0.02	1.65	0.16
Fenobam	29.83	2.04	0.16	0.05	0.20	0.02	1.59	0.17

—	Spleen	SD	Thymus	SD	Right Kidney	SD	Right Testicle	SD
Vehicle	0.09	0.01	0.08	0.01	0.26	0.03	0.12	0.01
Fenobam	0.10	0.02	0.06	0.02	0.25	0.04	0.11	0.01

All weights in grams, n = 9 for vehicle, n = 12 for fenobam. No significant differences were noted for any organs (unpaired Student *t* test, $P > 0.05$).

mice. However, by 8 h after the start of refeeding food intake of the fenobam-treated mice was not different from vehicle-treated animals (vehicle = 97.3 ± 8.8 mg/g; fenobam = 87.9 ± 7.8 mg/g; 95% CI −16.77–35.55 mg/g body weight; unpaired *T*-test $P = 0.44$). No differences in body weight were noted in animals before refeeding (Veh = 32 ± 0.82 g, Fenobam = 31.73 ± 1.25 g; 95% CI −3.052–3.585; unpaired Student *t* test $P = 0.86$).

Discussion

Activation of mGlu5 with the group I agonist (R,S)-3,5-dihydroxyphenylglycine is proalgesic,[6] whereas antagonism with fenobam results in decreased nociceptive behaviors.[10,11] These data provide evidence that mGlu5 may represent a viable therapeutic target for the treatment of pain. However, any future development of mGlu5 antagonists as analgesics in human patients will require both drugs that are selective for mGlu5 and for mGlu5 antagonism at sites outside the pain neuraxis to be devoid of clinically significant deleterious effects. Here we report that drug-naïve mGlu5 knockout mice exhibited increased locomotor activity at multiple time points when compared with WT littermates, and the level of locomotor activity of mGlu5 knockout mice was unchanged after either vehicle or fenobam injection. In contrast, when fenobam was injected into WT mice before placement in the open field, increased locomotor activity was exhibited at multiple time points compared with vehicle-treated mice. This suggests that the increased locomotor activity is due to antagonism of mGlu5. The fact that only a single difference (increased open arm time in knockout mice) was seen in drug-naive mGlu5 knockout mice compared with WT control mice may be due to a compensatory effect caused by genetic deletion of mGlu5 since birth in knockout animals.

Table 2. The Effects of Chronic Fenobam Injection on Gross Organ Appearance and Histopathology

	Vehicle	Fenobam
Gross necropsy examination		
No gross abnormalities	5/9	4/12
Heart enlarged due to left ventricle dilation	0/9	1/12
Lesions related to repeated intraperitoneal injection		
Focal adhesions between abdominal fat and viscera	3/9	5/12
Seminal vesicles dark in color	1/9	2/12
Liver capsule minimally thickened by inflammatory infiltrate	1/9	1/12
Seminal vesicles showed mild fibrosis and inflammatory infiltrate	0/9	2/12
Histopathologic examination		
No significant brain lesions	9/9	12/12
No significant liver lesions	8/9	11/12
No significant testicular lesions	8/9	11/12
Cardiomyopathy	0/9	1/12
Testes showed moderate seminiferous tubule degeneration affecting a limited number of tubules	0/9	1/12
Testes showed advanced seminiferous tubule degeneration affecting 5 − 40% of tubules	1/9	0/12

Fenobam was originally developed as an anxiolytic,[15] and when administered to rodents[13] and humans[15] it is anxiolytic. Increased locomotion in an open-field enclosure could represent an anxiolytic effect of fenobam. To test this possibility, we assessed the effects of fenobam on drug-naive mice in an elevated zero maze. Fenobam-injected mice entered the open sections of the zero maze significantly more and spent significantly more time in the open sections when compared with vehicle-injected mice. They also traveled significantly farther in the zero maze as a whole and traveled significantly more of this distance in the open sections of the maze when compared with vehicle-injected mice. These findings suggest that the effects of fenobam are both stimulatory and anxiolytic. The fact that these findings were not seen in drug-naive mGlu5 knockout mice compared with WT control mice may be due to a compensatory effect due to genetic deletion of mGlu5 since birth in knockout animals.

Increased locomotor activity after mGlu5 antagonism with fenobam is in opposition to results seen with 2-methyl-6-(phenylethynyl)pyridine[9,23] and the related mGlu5 antagonist 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine,[9] which have been shown to reduce locomotor activity at analgesic doses. 2-Methyl-6-(phenylethynyl)pyridine has also been reported to impair motor coordination at analgesic doses.[9] Although our previous findings[10] did not show im-

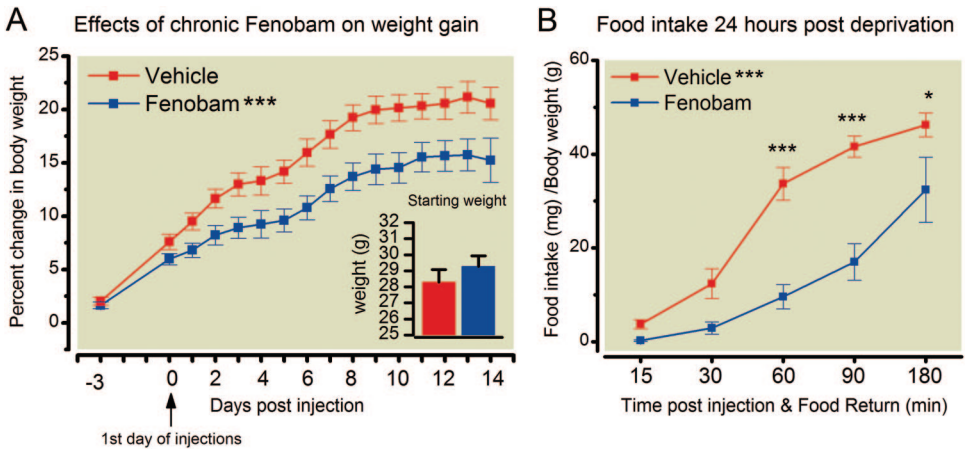

Fig. 7. The effects of mGlu5 inhibition on weight gain and postfasting food intake. (*A*) The percentage change in weight of fenobam injected animals was significantly less than that of vehicle injected animals over a 2-week period (two-way ANOVA main effect of fenobam *** $P < 0.0001$). (*A*, *inset*) The average starting weights of the animals in the two groups did not differ (unpaired Student *t* test $P > 0.05$). (n = 13 for the vehicle and 16 for the fenobam group). (*B*) Fenobam (30 mg/kg) significantly decreased food intake after a 24-h fast compared with vehicle (two-way ANOVA main effect of fenobam *** $P < 0.0001$, Bonferroni correction posttest, *, *** $P < 0.05$, 0.001). n = 6 per group. WT = wild type.

paired motor coordination after fenobam injection, the discrepant findings between 2-methyl-6-(phenylethynyl)pyridine and fenobam compelled us to perform further assessment of the effects of fenobam on motor coordination. No differences were seen in fenobam- *versus* vehicle-injected mice in the ledge crossing test or the vertical pole descent task. Both of these tasks require a mouse to navigate with limited foot purchase and execute complex turning behaviors without falling. A test of strength where mice hung upside down for 2 min also yielded no differences, further suggesting that although mGlu5 antagonism may result in increased locomotive behaviors, motor coordination remains intact. Thus, although fenobam may increase locomotor activity, the potentially more deleterious side effect of altered motor coordination appears to be absent at the tested analgesic dose. Furthermore, the stimulant side effects of fenobam could be beneficially exploited in patients who are also suffering from concurrent depression, assuming that any psychostimulant effects are not aversive. No major adverse reactions were reported by three healthy volunteers administered fenobam at doses of 150 mg.[4] Although these findings are encouraging they represent results from a limited number of subjects. Further human clinical testing will be necessary to confirm these data.

A major limitation of certain analgesics, such as opiates, is that tolerance to their analgesic effects occurs over time. For mGlu5 antagonists to be useful in humans they would have to avoid this limitation. Seven days of repeated dosing of the mGlu5 antagonist 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine has been demonstrated to result in analgesic tolerance in the formalin test.[24] Whether tolerance develops to the analgesic effects of fenobam is not currently known. Therefore, we injected groups of mice daily for either 5 or 14 days with an analgesic dose of fenobam (30 mg/kg) or vehicle. On the 6th or 15th day, respectively, mice were injected with either fenobam or vehicle, followed by a formalin test. Mice receiving chronic injections of fenobam did not display tolerance to the analgesic effects of acute fenobam on the test day. Fenobam was analgesic in both the first and second phase of the formalin test. Although this is not conclusive evidence that tolerance to fenobam would not develop if the drug was administered over a longer time course, it is nonetheless encouraging evidence that tolerance may not be a limiting factor in the use of fenobam as an analgesic.

A fascinating effect of mGlu5 deletion and pharmacologic inhibition is a significant effect on weight gain and postfast feeding behavior. mGlu5 knockout mice have been previously reported to weigh less than their WT littermates.[18,19] Differences in *ad libitum* food intake were not found,[18] so this is not believed to account for the differences. However, both mGlu5 knockout mice and mice injected with 2-methyl-6-(phenylethynyl)pyridine exhibit significantly less refeeding behavior after a 14-h fast, suggesting that mGlu5 does play a role in appetite and feeding behavior.[18] Here we report that fenobam administration significantly decreases postfast refeeding. Fenobam's psychostimulant properties and appetite-suppressive effects should certainly be viewed as separate from its analgesic effects. However, rather than being dose limiting, it might be possible to exploit these off-target effects to beneficial clinical use in certain diseases that have pain as a major symptom. Osteoarthritis is a prime candidate as weight loss has been associated with both a decrease in risk of developing osteoarthritis[25] and weight loss and increased activity result in an improvement of existing osteoarthritis symptoms.[26] Again, further testing in human patients will be necessary to know for certain.

Future endeavors to bring mGlu5 antagonists to human patients will require that pharmacologic agents have acceptable safety profiles. This is underscored by the cessation of clinical trials involving the mGlu5 antagonist ADX-10059

due to unacceptable increases in liver enzymes.[12] Although these changes were thought to be compound-specific,[27] mGlu5 is expressed in liver tissue,[28] and thus mGlu5 antagonists as a class could theoretically cause damage to that tissue. Therefore, we sought to determine whether chronic administration of fenobam would also result in unacceptable changes to the liver, as well as other organs. Fenobam administration for up to 14 days resulted in no significant gross lesions in multiple organs and systems, including hepatobiliary, respiratory, digestive, urinary, nervous, or endocrine, when compared with vehicle-injected control subjects, or changes seen by histopathology in the brain, the liver, or the testes. In addition, no major changes were seen in standard blood chemistry measurements or in complete blood counts. Although some animals did demonstrate cardiomyopathy and testicular degeneration, these lesions can occur spontaneously as animals age.[29] Given that the animal with the significant testicular lesions was treated with vehicle, this is likely to be an incidental finding. An absence of brain lesions and a lack of altered motor coordination suggests an absence of adverse effects in the spinal cord; however, we did not directly verify this finding histologically. Many other lesions seen, including focal adhesions, liver capsule inflammatory infiltrates, and the darkened color of the seminal vesicles, were seen in both vehicle- and fenobam-injected mice and may simply be due to repeated intraperitoneal injections.[30]

Finally, although these experiments were designed to test the analgesic tolerance of fenobam and better understand its toxicity profile, it is critical to note that only one dose was tested. This dose was chosen based on previous dose-response studies indicating that 30 mg/kg was an effective analgesic dose in mice.[10] Furthermore, this dose corresponds to the highest single dose of fenobam so far tested in human trials with different clinical indications.[4,14,15] Possible toxicity of fenobam at higher doses or administered for longer time periods have not been ruled out. In addition, as mGlu5 has been implicated in learning and memory,[11] future studies should test the effects of fenobam on these processes.

In conclusion, we report that fenobam, a clinically validated mGlu5 antagonist with analgesic and anxiolytic properties, has an acceptable safety profile in rodents after chronic daily dosing of 30 mg/kg for up to 14 days. In addition, we report that tolerance to the analgesic effects of fenobam does not develop during this time period. Furthermore, although this study and previous studies suggest that fenobam and other mGlu5 antagonists may have a psychostimulant effect, we continue to find no evidence that fenobam impairs motor coordination. Finally, we suggest that although fenobam may have effects outside of the pain neuraxis, at least some of its nonanalgesic effects could be used for beneficial clinical effect. Fenobam, and mGlu5 antagonists in general, represent promising candidates for the treatment of human pain conditions.

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