Published in final edited form as:

Pain. 2017 December ; 158(12): 2376–2385. doi:10.1097/j.pain.0000000000001042.

Monomethyl fumarate (MMF) inhibits pain behaviors and amygdala activity in a rat arthritis model

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Abstract

Neuroplasticity in the amygdala, a brain center for emotions, leads to increased neuronal activity and output that can generate emotional-affective behaviors and modulate nocifensive responses. Mechanisms of increased activity in the amygdala output region (central nucleus, CeA) include increased reactive oxygen species, and so we explored beneficial effects of monomethyl fumarate (MMF), which can have neuroprotective effects through the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) anti-oxidant response pathway. Systemic (intraperitoneal) MMF dose-dependently inhibited vocalizations and mechano-sensitivity (hindlimb withdrawal reflexes) of rats in an arthritis pain model (kaolin/carrageenan-induced mono-arthritis in the knee). Stereotaxic administration of MMF into the CeA by microdialysis also inhibited vocalizations but had a limited effect on mechano-sensitivity, suggesting a differential contribution to emotional-affective versus sensory pain aspects. Extracellular single-unit recordings of CeA neurons in anesthetized rats showed that stereotaxic administration of MMF into the CeA by microdialysis inhibited background activity and responses of CeA neurons to knee joint stimulation in the arthritis pain model. MMF had no effect on behaviors and neuronal activity under normal conditions. The results suggest that MMF can inhibit emotional-affective responses in an arthritis pain model through an action that involves the amygdala (CeA).

Keywords

Amygdala; pain; fumarate; MMF; electrophysiology

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Conflict of Interest: The authors declare no competing financial interest.

Introduction

Evidence suggests neuroprotective effects of fumarate-containing compounds in oxidative stress in central nervous system cells, possibly by stimulating the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway [4;17;49]. While dimethyl fumarate (DMF) has been used clinically to treat relapsing forms of multiple sclerosis and psoriasis [10;37], the beneficial cytoprotective and antioxidant effects of DMF and its primary metabolite monomethyl fumarate (MMF) in pain management remain to be determined. Despite the fact that DMF is rapidly hydrolyzed to MMF in the body, the two fumarates seem to have distinct pharmacological properties and biological activities but both activate Nrf2 to increase antioxidant and cytoprotective gene expression [17], resulting for example in increased levels of the antioxidant glutathione [1;23].

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, play important roles in cytotoxicity and oxidative stress [6;41;50] and are also involved in pain pathophysiology [8;48]. ROS contribute to peripheral [28;55;60] and spinal [15;22;29;30;33;34;52;54;61] mechanisms of inflammatory and neuropathic pain. Relatively little is known about their role in brain mechanisms of pain, but recent studies have linked ROS in the rostral ventromedial medulla [39] and amygdala [25;36;40] to pain facilitation in different pain models.

The amygdala plays a key role in emotional-affective aspects of pain [43]. Neuroplastic changes have been shown to increase amygdala output and behavioral responses in different pain models. The central nucleus (CeA) serves major amygdala output functions and receives nociceptive information through the spino-parabrachio-amygdaloid pathway and highly processed affect-related information from the lateral-basolateral (LA-BLA) network [43]. CeA activity correlates positively with emotional-affective behaviors and can also modulate pain sensitivity. Therefore, interventions that control CeA activity may be useful therapeutic strategies. Our previous studies showed that inhibition of ROS production inhibits CeA neuronal excitability and responsiveness [25;36] and supraspinally organized vocalizations, anxiety-like behavior and spontaneous behaviors, but not spinal reflexes [25;40].

Therefore, this study tested the hypothesis that MMF can inhibit pain behaviors through an action in the amygdala (CeA) using a rat model of arthritis pain (kaolin-carrageenan-induced monoarthritis in the knee).

Materials and Methods

Adult male Sprague–Dawley rats (200–300 g; Envigo, Indianapolis, IN) were housed in a temperature-controlled room and maintained on a 12-h day/night cycle with unrestricted access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas Tech University Health Sciences Center (TTUHSC).

Behavioral tests

Experimental protocol—Behavioral tests (see “Pain-related behaviors”) were done before and >5–6 h after arthritis induction (see “Arthritis pain model”). To determine the effects of systemic (intraperitoneal, i.p.) application of MMF, pain behaviors were measured every 15 min for 1 h post-injection. For effects of MMF in the amygdala, pain behaviors were measured 15 min after the onset of stereotaxic administration of MMF by microdialysis (see “Drug application”). Rats were randomly assigned to receive a given intervention. Experimenters were blinded to the treatment (drug or vehicle) but not the pain model. Different investigators performed the tests and every effort was made to have equivalent testing parameters between rats and treatment groups.

Arthritis pain model—Rats were briefly anesthetized with isoflurane (2–3%) and a mono-arthritis was induced by separate injections of kaolin (4% in sterile saline, 100 µl) and carrageenan (2% in sterile saline, 100 µl) into the left knee joint cavity through the patellar ligament followed by repetitive flexions and extensions of the leg for 5 minutes after each injection. This well established protocol [44] produces an aseptic use-dependent monoarthritis with damage to the cartilage, inflammation of the synovia and synovial fluid exudate within hours and persists for weeks. Naive animals were used as controls instead of sham-injected animals because of concerns about possible tissue irritation by needle insertion and saline injection into the joint cavity. Our previous studies showed that naive and sham-injected animals are not different >2 h postinjection and do not develop neuroplasticity and pain behaviors observed in the arthritis model [19,45].

Pain-related behaviors

Vocalizations: Vocalizations in the audible (20 Hz to 16 kHz) and ultrasonic (25 ± 4 kHz) ranges were measured using a condenser microphone connected to a preamplifier, and bat detector connected to a filter and amplifier (UltraVox 4-channel system; Noldus Information Technology, Leesburg, VA), respectively [44]. Rats were briefly anesthetized with isoflurane (2–3%) and positioned comfortably in a custom-designed recording chamber (U.S. Patent 7,213,538) at a fixed distance from the sound detectors [20]. After habituation to the chamber, brief (15 s) innocuous (500 g/30 mm²) and noxious (1500 g/30mm²) stimuli were applied to the knee with a calibrated forceps equipped with a force transducer whose output signal was displayed in grams on an LCD screen. Vocalizations were recorded for 1 min starting with the onset of the mechanical stimulus and analyzed using Ultravox 2.0 software (Noldus Information Technology).

Mechanical reflex thresholds: Hindlimb withdrawal thresholds were determined after the vocalization measurements while the rat was lightly restrained in the recording chamber. A calibrated forceps with force transducer was used to compress the left knee joint with continuously increasing intensity until a reflex was evoked. The average value from 2 – 3 trials was used as the withdrawal threshold, which was defined as the force required for a reflex response [44].

Drug applications—MMF was purchased from Sigma-Aldrich and dissolved in 0.9 % isotonic saline, which served as the vehicle control for systemic (i.p.) drug injection (0.5 –

10 mg/kg MMF). For drug application by microdialysis, rats were anesthetized with isoflurane (2–3%) and a guide cannula (CMA/Microdialysis, Solna, Sweden) was implanted stereotactically (David Kopf Instruments) into the right CeA using the following coordinates: 2.1 mm caudal to bregma, 4 mm lateral to midline, and 7 mm deep. The cannula was fixed to the skull with dental acrylic (Plastic One, Roanoke, VA). Bacitracin ointment was applied to the exposed tissue to prevent infection. On the day of the experiment (next day), a microdialysis probe (CMA/Microdialysis 11) extending 1 mm beyond the cannula was inserted and connected to an infusion pump (Harvard Apparatus, Holliston, MA) with polyethylene tubing. MMF or artificial cerebrospinal fluid (ACSF, control; containing in mM: 117 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, and 11 glucose) was applied for 15 min at 5 µl/min before the behavioral tests to establish tissue equilibrium. MMF stock solution was diluted in ACSF to the final concentration in the microdialysis probe, which was 100 times higher than the target concentration in the tissue because of the concentration gradient across the dialysis membrane and diffusion in the tissue. This approach has been described in our previous publications [see for example 19;25;46;59]. Brains were removed after the experiment and stored in 30% sucrose before they were frozen-sectioned to verify the position of the microdialysis probes.

Concentrations for microdialysis administration were based on the literature that reported target concentrations of MMF in the range of 10–30 µM, with 15 µM being the most commonly tested concentration although 5 µM was used occasionally [1;3;49;58]. Due to the concentration gradient across the microdialysis membrane a 100 fold higher concentration is needed inside the probe [12;31;36;46], and so we used 1 mM. For systemic application, DMF and MMF have been shown to be effective at doses of 15, 25, 30 and 50 mg/kg whereas 5 mg/kg was not effective consistently [1;7;49;51;56]. We tested doses of 0.5, 1.0 and 10 mg/kg and found maximum effects with 10 mg/kg (Fig. 1).

Systems electrophysiology

Experimental protocol—Extracellular single-unit recordings were made from neurons in the laterocapsular division of the CeA in anesthetized rats as described before [25–27]. Recordings were made in normal animals and in animals with arthritis (>5–6 h after induction; see “Behavioral tests, Arthritis pain model”). MMF was administered stereotactically into the amygdala (see “Drug application”), and neuronal activity was measured every 5 min.

Animal preparation and anesthesia: Rats were anesthetized with pentobarbital (induction, 50 mg/kg, i.p.; maintenance, 15 mg/kg/h, i.v.), paralyzed with vecuronium (induction, 0.3–0.5 mg, iv; maintenance, 0.3 mg/h, iv), and artificially ventilated (3–3.5 ml; 55–65 strokes/min). End-tidal CO₂ levels (kept at 4.0 ± 0.2%) and heart rate (using ECG) were continuously monitored. Core body temperature was maintained at 37°C by means of a homeothermic blanket system. The animal was mounted in a stereotaxic frame (David Kopf Instr., Tujunga, CA) and a craniotomy was performed at the sutura fronto-parietalis level to allow the insertion of the recording electrode and microdialysis probe for drug application.

Single-unit recording and identification of CeA neurons: Individual neurons were recorded with glass insulated carbon filament electrodes (4 – 6 M Ω) using the following coordinates: 2.1–2.8 mm caudal to bregma, 3.8–4.2 mm lateral to midline, depth 7–8.5 mm. The recorded signals were amplified, bandpass filtered (300 Hz to 3 kHz), processed by an interface (CED 1401 Plus), and displayed on a computer monitor, using Spike2 software (CED, version 4) for spike sorting, data storage and analysis of single-unit activity. Spike size and configuration were continuously monitored. Only those neurons were included in the study whose spike configuration matched a preset template and could be clearly discriminated from background noise throughout the experiment. A template was created for the spikes of an individual neuron during the initial 5 min recording period, capturing the waveform within set limits of variability for parameters such as amplitude, duration, and rise time using Spike 2 software. Subsequent spikes were matched to that template (spike sorting) and only spikes within the set limits of variability were counted as signals of that particular neuron. CeA neurons were identified by monitoring background activity and responses to mechanical compression of the tissue (knee joint) at innocuous (500 g/30 mm²) and noxious (1500 g/30 mm²) intensities with a calibrated forceps. Noxious compression was used sparingly as a search stimulus. One neuron was recorded per animal.

Drug application—For drug application into the amygdala, a microdialysis probe (CMA/Microdialysis 11) was inserted stereotaxically (David Kopf Instruments) into the right CeA while the animal was anesthetized with pentobarbital (see “Animal preparation and anesthesia”). The following coordinates were used: 2.1 mm caudal to bregma, 4 mm lateral to midline, and 7 mm deep. The microdialysis probe was connected to an infusion pump (Harvard Apparatus, Holliston, MA) with polyethylene tubing. MMF or ACSF (as control) was applied at a rate of 5 μ l/min for 15–20 min while measurements were made. MMF stock solution was diluted in ACSF as described in “Behavioral tests, Drug applications”. For drug concentrations, see description in “Behavioral tests”.

Verification of recording site—At the end of each experiment the recording site in the CeA was marked by injecting DC (250 μ A for 3 min) through the carbon filament recording electrode. The brain was removed and submerged in 10% formalin and potassium ferrocyanide. Tissues were stored in 30% sucrose before they were frozen-sectioned at 50 μ m. Sections were stained with Hematoxylin and Eosin (H&E), mounted on gel-coated slides, and coverslipped. The boundaries of the different amygdala nuclei were easily identified under the microscope. Lesion/recording sites and positions of the microdialysis probes were verified.

Statistical analysis

All averaged values are presented as means $\pm$ SEM. Statistical significance was accepted at the level $P < 0.05$. GraphPad Prism 5.0 software (Graph-Pad Software, San Diego, CA) was used for analysis. Statistical analysis was performed on the raw data. Repeated measures ANOVA with Bonferroni posttests and t-tests were used where appropriate. For dose-response analysis sigmoid curves were fitted to the data by nonlinear regression using the formula $y = A + (B - A)/[1 + (10C/10X)^D]$, where A = bottom plateau, B = top plateau, C =

log(IC50), D = slope coefficient (GraphPad Prism 5.0). Two-way ANOVA was used to compare dose-response relationships.

Results

The goal of this study was to test the hypothesis that MMF, which can have Nrf2-mediated antioxidant effects (see Introduction), inhibits arthritis pain-related behaviors through an action in the amygdala output region (CeA). The right amygdala was targeted because of evidence for right-hemispheric lateralization in several pain models [5;18;26;47].

Effects of systemic and intra-amygdalar MMF on vocalizations

In arthritic rats, 5–6 hours after induction of arthritis, systemic (i.p.) MMF inhibited audible and ultrasonic vocalizations to brief (15 s) noxious (1500 g/30 mm²) mechanical stimuli (compression of the left knee joint; Fig. 1). The effect of MMF (10 mg/kg; n = 7 rats) was significant compared to vehicle (n = 7 rats; $P < 0.0001$, $F_{1,72} = 20.54$, audible, $F_{1,72} = 41.57$, ultrasonic; two-way ANOVA with Bonferroni posttests). Inhibition reached a maximum at 60 min after injection of MMF (Fig. 1A and B) and was dose-dependent (0.5 – 10 mg/kg MMF; Fig. 1C). There was no significant difference between effects of MMF on audible and ultrasonic vocalizations ($P > 0.05$, $F_{1,28} = 0.32$, two-way ANOVA), suggesting inhibitory effects on nocifensive and aversive affective responses, respectively.

Based on the time course analysis (Fig. 1), the 1 h time point was used to determine the effect of systemic (i.p.) MMF (10 mg/kg), compared to vehicle, on audible (Fig. 2A–C) and ultrasonic (Fig. 2D–F) vocalizations under normal conditions and in the arthritis pain state. In normal rats, systemic (i.p.) MMF (10 mg/kg; n = 7 rats) had no significant effect on audible (Fig. 2A) and ultrasonic (Fig. 2D) vocalizations to brief (15 s) noxious (1500 g/30 mm²) stimuli compared to vehicle (n = 7 rats; $P > 0.05$, unpaired t-test). Vocalizations were measured 1 h after injection of MMF (10 mg/kg, i.p.) or vehicle. In two separate groups of rats, vocalizations were measured before (“normal”) and after induction of arthritis; and then vehicle or MMF (10 mg/kg, i.p.) was injected. Vocalizations increased significantly in both the vehicle group (n = 8 rats; $P < 0.01$, repeated measures ANOVA with Bonferroni posttest; Fig. 2B and 2E) and the MMF groups (n = 5 rats; $P < 0.01$ and 0.001, audible and ultrasonic vocalizations, respectively; repeated measures ANOVA with Bonferroni posttest; Fig. 2C and 2F). Systemic MMF (10 mg/kg, i.p.; n = 5 rats; Fig. 2C and 2F), but not vehicle (n = 8 rats; Fig. 2B and 2D), inhibited vocalizations of arthritic rats significantly ($P < 0.05$ and 0.01, audible and ultrasonic vocalizations, respectively; repeated measures ANOVA with Bonferroni posttest).

Stereotaxic administration of MMF (1 mM, concentration in microdialysis probe; 15 min) into the central nucleus of the amygdala (CeA, n = 6 rats), but not basolateral amygdala (BLA, n = 5 rats), inhibited audible (Fig. 3A) and ultrasonic (Fig. 3B) vocalizations to brief noxious mechanical stimuli significantly compared to stereotaxic administration of ACSF in the same rats before MMF administration ($P < 0.01$, paired t-tests). Histologically verified sites of drug injection are shown in Figure 4A.

Effects of systemic and intra-amygdalar MMF on mechanical thresholds

In arthritic rats, 5–6 hours after induction of arthritis, systemic (i.p.) MMF increased mechanical thresholds for hindlimb withdrawal reflexes evoked by compression of the arthritic knee joint (Fig. 5A). The effect of MMF ($n = 5$ rats) was significant compared to vehicle ($n = 5$ rats; $P < 0.0001$, $F_{1,48} = 22.65$; two-way ANOVA with Bonferroni posttests). Inhibition of spinal reflexes reached a maximum at 60 min after injection of MMF (Fig. 5A) and was dose-dependent (Fig. 5B).

In normal rats, systemic (i.p.) MMF ($n = 5$ rats) had no significant effect on mechanical withdrawal thresholds compared to vehicle ($n = 5$ rats; $P > 0.05$, unpaired t-test; Fig. 6A). Thresholds were measured at the left knee joint 1 h after application of MMF (10 mg/kg, i.p.) or vehicle. In two separate groups of rats, mechanical reflex thresholds were measured before and 5–6 h after arthritis induction (Fig. 6B and C); and then vehicle (6B) or MMF (10 mg/kg, i.p.; 6C) was injected. Thresholds decreased significantly 5–6 h postinduction of arthritis in the vehicle ($n = 5$ rats) and MMF groups ($n = 5$ rats; $P < 0.001$, repeated measures ANOVA with Bonferroni posttest). Systemic MMF (10 mg/kg, i.p.; $n = 5$ rats; 6C), but not vehicle ($n = 5$ rats; 6B), increased thresholds significantly ($P < 0.05$, repeated measures ANOVA with Bonferroni posttest) 1 h after drug application.

Stereotaxic administration of MMF (1 mM, concentration in microdialysis probe; 15 min) into the CeA ($n = 6$ rats), but not BLA ($n = 5$ rats), had a small but significant inhibitory effect on withdrawal reflexes (Fig. 6D), increasing the mechanical threshold at the arthritic knee ($P < 0.01$, paired t-test, compared to ACSF control in the same rats). Figure 4A shows the histologically verified sites of drug injection. The direct comparison of normalized drug effects (% of predrug control; Fig. 6E) showed that systemically administered MMF (10 mg/kg, i.p.; $n = 5$ rats) had a significantly greater effect on mechanical sensitivity than MMF administered into the CeA (1 mM, concentration in microdialysis probe, 15 min, $n = 6$ rats; $P < 0.05$, unpaired t-test).

Effects of MMF on CeA neurons

The contribution of a direct action of MMF on neuronal activity in the amygdala to its behavioral effects was determined using extracellular single unit recordings of neurons in the latero-capsular CeA, which has been shown to drive pain-related behaviors [43]. Recordings were made in normal rats and in arthritic rats 5–6 h after arthritis induction. So-called “multireceptive” neurons were selected that responded more strongly to brief noxious than innocuous test stimuli [25–27]. Mechanical test stimuli were applied to the knee joint using a calibrated forceps as in the behavioral experiments. ACSF was administered through the microdialysis probe into the CeA before and after MMF. Figure 7 shows the results (A, B, individual examples; C, D, summary).

In normal animals without arthritis, stereotaxic administration of MMF (1mM, concentration in microdialysis probe; 15 min) into the CeA by microdialysis had no effect on background activity of CeA neurons ($n = 7$ neurons) and on their responses to innocuous and noxious stimuli (Fig. 7A and 7C). In the arthritis pain model (5–6 h postinduction), MMF

significantly inhibited background and evoked activity of CeA neurons ($n = 8$ neurons; $P < 0.05$, paired t-test comparing MMF to ACSF in the same neuron; Fig. 7B and 7D).

As a control for drug diffusion, MMF (1 mM, concentration in the microdialysis probe) was administered stereotactically into the adjacent striatum (1.0–1.5 mm away from the laterocapsular CeA) in a different set of experiments. The striatum was selected for off-site controls as in our previous studies [21;24;36] because it is close by but does not project directly to the CeA. MMF administration into the striatum of arthritic rats had no effect on background or evoked activity of CeA neurons ($n = 6$; $P > 0.05$, paired t-test comparing MMF to ACSF in the same neuron; Fig. 8). Recording sites in the CeA (Fig. 4C) and drug application sites into the CeA and striatum (Fig. 4B) were verified histologically.

Discussion

This study addressed beneficial effects and site of action of MMF in the amygdala in an arthritis pain model. MMF applied systemically or into the CeA inhibited emotional-affective responses (vocalizations). Systemic MMF also decreased mechano-sensitivity (hindlimb withdrawal reflexes), whereas intra-CeA had a much smaller albeit statistically significant effect (Fig. 6E), which may perhaps suggest a preferential contribution of MMF in the amygdala to emotional-affective aspects of pain. MMF also inhibited neuronal activity of amygdala neurons when administered into the CeA. The data suggest that MMF can inhibit pain-related changes in the amygdala to affect certain types of pain behaviors while actions outside the amygdala can produce additional effects.

The data were obtained with well-established behavioral and electrophysiological assays. Vocalizations evoked by noxious stimuli reflect supraspinally organized nocifensive (audible vocalizations) and aversive-affective (ultrasonic vocalizations) responses [20;21]. Spinally organized withdrawal reflexes assess mechano-sensitivity. These tests have been used successfully before to determine behavioral changes in the arthritis pain model [44]. The arthritis model used here is a monoarthritis in the knee induced by intra-articular injections of kaolin and carrageenan to produce a combination of mechanical damage to the cartilage and localized inflammation [44]. In this model, behavioral changes occur within hours and last for several days. They are causally linked to increased neuronal activity and synaptic plasticity in the CeA based on pharmacological studies showing that decreasing CeA activity inhibit pain behaviors [reviewed in 43] whereas increasing CeA output can generate pain behaviors in normal animals [24;36].

Importantly, mechanisms of pain-related amygdala plasticity include increased ROS, possibly as a consequence of metabotropic glutamate receptor (mGluR5) activation [36]. Accordingly, ROS scavengers in the amygdala can inhibit increased pain behaviors [25;36;40]. These findings are consistent with an important role of G protein-coupled mGluR5 in amygdala plasticity in pain [9;21;32;35;45] and with an emerging role of ROS in peripheral [28;55;60], spinal [15;22;29;30;33;34;52;54;61], and supraspinal [25;36;39;40;48] mechanisms of inflammatory and neuropathic pain.

MMF, the primary and bioactive metabolite of DMF, has been shown to be cytoprotective against oxidative challenge via transcription factor Nrf2-dependent antioxidant response element pathway [4;16;17;49]. For example, Nrf2 protects against hydrogen peroxide-induced oxidative injury in the brain via MMF-dependent oxidative stress-induced growth inhibitor 1 (OSGIN1) induction [4]. MMF and DMF also increase the transcription of genes involved in the synthesis of glutathione [1;2;23], a major cellular antioxidant against ROS levels [42]. DMF/MMF-mediated Nrf2 induction has been found in neurons and astrocytes [38;49], but the present study was not designed to distinguish neuronal and non-neuronal cellular targets.

The mechanisms of MMF effects, which occurred quite rapidly (after 15 min) with stereotaxic administration into the amygdala, remain to be determined. ROS scavengers also inhibited neuronal activity and pain behaviors with a similar time course in our previous studies [25;36;40]. In addition, glutathione reacts directly with various radical molecules and can interact directly with glutamate receptors to inhibit signaling [42]. In contrast, DMF, but not MMF, inhibit NF κ B signaling to reduce pro-inflammatory cytokines [17], and so this mechanism seems unlikely to be involved in the effect of MMF. MMF is also a potent agonist for the cell-surface G-protein-coupled receptor GPR109A [57], and activation of this receptor blocks inflammation in colon [14;53], retina [13] and brain [11]. As such, the antinociceptive effects of MMF might involve multiple pathways that suppress oxidative stress as well as inflammation.

Drug doses and concentrations were selected based on information in the literature (see Methods). Target concentrations of MMF were reported to be in the range of 10–30 μ M, with 15 μ M being tested most commonly although 5 μ M was used occasionally [1;3;49;58]. For microdialysis drug application we used 1 mM because a 100 fold higher concentration is needed inside the probe due to the concentration gradient across the microdialysis membrane as indicated by our own work comparing drug effects in brain slices with microdialysis drug application for a variety of compounds [12;31;36;46]. For systemic application, DMF and MMF have been shown to be effective at doses of 15, 25, 30 and 50 mg/kg whereas 5 mg/kg was not effective consistently [1;7;49;51;56]. We tested doses of 0.5, 1.0 and 10 mg/kg and found maximum effects with 10 mg/kg (Fig. 1).

A number of tests and observations argue against non-specific actions of MMF. MMF had no behavioral or electrophysiological effects under normal conditions but only in the pain model, which is consistent with our previous study using a ROS scavenger [25]. Stereotaxic administration into the amygdala (CeA) inhibited vocalizations but not spinal reflexes, which is also consistent with the pattern of effects of a ROS scavenger, NADPH oxidase inhibitor or lipoxygenase inhibitor in the amygdala [40]. Lack of effects of intra-BLA or striatal injections of MMF also argues in favor of focal effects of CeA injections and strongly suggests that the CeA is indeed the site of action of MMF. It should be noted that the CeA is distinct from BLA in that it receives a dedicated line of nociceptive input from the parabrachial area [43]. The focus of our systems electrophysiology on a well-defined subpopulation of amygdala/CeA neurons that are closely linked to pain modulation [43] may explain the quite consistent effect of MMF.

In conclusion, the results suggest that MMF, the primary metabolite of clinically available DMF, can inhibit pain behaviors through an inhibitory action in the amygdala, an important node for emotional-affective aspects of pain. Mechanisms of actions remain to be determined. The significance of this study extends beyond the field of pain research. MMF has emerged as a therapeutic target for multiple sclerosis, psoriasis, and other disorders associated with oxidative stress, inflammation or immune activation [16].

Acknowledgments

This work was supported by National Institute of Neurological Disorders and Stroke (NIH/NINDS) grants NS081121 and NS038261.

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Figure 1. Effects of systemic MMF on vocalizations

Audible (A) and ultrasonic (B) vocalizations were evoked by brief (15 s) noxious stimuli (1500 g/30 mm²) in arthritic rats (5–6 hours after induction). Measurements were made before and after systemic MMF (10 mg/kg, i.p.; n = 7 rats) or vehicle (n = 7 rats). *, **, *** P < 0.05, 0.01, 0.001, two-way ANOVA with Bonferroni posttests (see Results for details). (C) Effects of different doses (n = 7 rats each) of MMF (i.p.) on vocalizations of arthritic rats compared to vehicle (n = 8 rats). Symbols show means ± SEM.

Audible vocalizations

Ultrasonic vocalizations

Figure 2. Effects of systemic MMF on vocalizations in normal and arthritic rats

Audible (**A**) and ultrasonic (**D**) vocalizations to brief (15 s) noxious stimuli (1000–1500 g/30 mm²) were measured in normal rats 1 h after application of MMF (10 mg/kg, i.p.; n = 7 rats) or vehicle (n = 7 rats). ns, $P > 0.05$, unpaired t-test. In two separate groups of rats, audible (**B**, **C**) and ultrasonic (**E**, **F**) vocalizations were measured before (“Normal”) and 5–6 h after induction of arthritis, and 1 h after application of vehicle (n=8 rats; **B** and **E**) or MMF (10 mg/kg, i.p.; n=5 rats; **C** and **F**). ns, $P > 0.05$, *,** $P < 0.05$, 0.01, repeated measures one-way ANOVA with Bonferroni posttests (see Results for details). Bar histograms show means $\pm$ SEM.

A Audible vocalizations **B Ultrasonic vocalizations** **Figure 3. Effects of stereotaxic MMF on vocalizations**

Audible (**A**) and ultrasonic (**B**) vocalizations were evoked by brief (15 s) noxious stimuli (1500 g/30 mm²) in arthritic rats (5–6 hours after induction). MMF (1 mM, concentration in microdialysis probe; 15 min) and ACSF (predrug control) were administered stereotaxically into the central nucleus of the amygdala (CeA, n = 6 rats) or basolateral amygdala (BLA, n = 5 rats). ACSF and MMF were tested in the same rats. ns, $P > 0.05$; ** $P < 0.01$, paired t-test. Bar histograms show means $\pm$ SEM.

Figure 4. Histologically verified sites of drug injection and recording sites

Diagrams show coronal brain slices. Numbers indicate distance from the bregma. Symbols show the positions of the tips of the microdialysis fibers for drug application in the behavioral (**A**, $n = 11$) and electrophysiological (**B**, filled symbols, CeA, $n = 15$ CeA; circles, striatum, $n = 6$) experiments. (**C**) Symbols show the positions of the tips of recording electrodes ($n = 21$) in the laterocapsular division of the CeA based on electrolytic lesions (see Methods). Scale bar, 500 μm .

Figure 5. Effects of systemic MMF on mechanical reflex thresholds

(A) Thresholds for hindlimb withdrawal reflexes were measured by compressing the arthritic knee joint (5–6 h postinduction). Measurements were made before and after systemic MMF (10 mg/kg, i.p.; $n = 5$ rats) or vehicle ($n = 5$ rats). *,** $P < 0.05, 0.01$, two-way ANOVA with Bonferroni posttests (see Results for details). (B) Effects of different doses ($n = 5$ rats each) of MMF (i.p.) on spinal reflexes compared to vehicle ($n = 8$ rats). Symbols show means $\pm$ SEM.

Figure 6. Effects of systemic and stereotaxic MMF on mechanical reflex thresholds in normal and arthritic rats

(A) In normal rats (bar histograms on the left), systemic MMF (10 mg/kg, i.p.; $n = 5$ rats) had no significant effect on mechanical withdrawal thresholds compared to vehicle ($n = 5$ rats). ns, $P > 0.05$, unpaired t-test. (B, C) Thresholds were also measured before (“Normal”) and 5–6 h after induction of arthritis, and 1 h after application of vehicle ($n = 8$ rats; B) or MMF (10 mg/kg, i.p.; $n = 5$ rats; C). ns, $P > 0.05$; *,*** $P < 0.05$, 0.001, repeated measures one-way ANOVA with Bonferroni posttests (see Results for details). Thresholds were measured at the left knee joint. (D) Stereotaxic administration of MMF (1 mM, concentration in microdialysis probe; 15 min) and ACSF (predrug control) into CeA ($n = 6$ rats) or BLA ($n = 5$ rats). ACSF and MMF were tested in the same rats. ns, $P > 0.05$; ** $P < 0.01$, paired t-test comparing MMF to predrug ACSF in the same rat. Bar histograms show means $\pm$ SEM. (E) Normalized drug effects (% of predrug ACSF control) of systemically administered MMF (10 mg/kg, i.p.; $n = 5$ rats) and intra-CeA administration of MMF (1 mM, concentration in microdialysis probe, 15 min, $n = 6$ rats). * $P < 0.05$, unpaired t-test. Bar histograms show means $\pm$ SEM.

Figure 7. Effects of stereotaxic MMF on CeA neurons

Extracellular single unit recordings of a neuron in the latero-capsular CeA in a normal rat (**A**) and another neuron in a rat with knee joint arthritis (5–6 h postinduction, **B**). Peristimulus time histograms (PSTHs) show number of action potentials (spikes) per second. Responses to innocuous and noxious mechanical test stimuli (compression of the knee joint) were measured. ACSF was administered through the microdialysis probe into the CeA before and after stereotaxic administration of MMF (1 mM, concentration in microdialysis probe; 15 min) into the CeA by microdialysis. (**C**, **D**) Summary of the effects of stereotaxically administered MMF (1 mM, concentration in microdialysis probe; 15 min) compared to ACSF (vehicle control) on CeA neurons recorded in normal animals ($n = 7$ neurons; **C**) and in the arthritis pain model (5–6 h postinduction; $n = 8$ neurons; **D**). ns, $P > 0.05$; * $P < 0.05$, paired t-test comparing MMF to predrug ACSF in the same neuron. Bar histograms show means $\pm$ SEM.

Figure 8. Offsite controls

Extracellular single unit recordings of a neuron in the latero-capsular CeA in an arthritic rat (5–6 h postinduction) during administration of ACSF (A, predrug) and MMF (B, 1 mM, concentration in the microdialysis probe) into the striatum adjacent to CeA. Same display as Fig. 6. (C) Summary of the effects of stereotactically administered MMF (1 mM, concentration in microdialysis probe; 15 min) compared to ACSF (vehicle control) on CeA neurons recorded in arthritic rats (5–6 h postinduction; $n = 6$ neurons). ns, $P > 0.05$, paired t -test comparing MMF to predrug ACSF in the same neuron. Bar histograms show means $\pm$ SEM.