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Original Article

Serotonergic Signals Enhanced Hamster Sperm Hyperactivation

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ABSTRACT

In the present study, we investigated the regulatory mechanisms underlying sperm hyperactivation enhanced by 5-hydroxytryptamine (5-HT) in hamsters. First, we examined the types of 5-HT receptors that regulate hyperactivation. Hyperactivation was significantly enhanced by 5-HT_{2A} and 5-HT_4 receptor agonists. Moreover, the results of the motility assay revealed that 5-HT_{2A}, 5-HT_3, and 5-HT_4 receptor agonists significantly decreased the velocity and/or amplitude of sperm. Under 5-HT_2 receptor stimulation, hyperactivation was associated with phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP_3) receptor, soluble adenylate cyclase (sAC), and protein kinase A (PKA). In contrast, under 5-HT_4 receptor stimulation, hyperactivation was associated with transmembrane adenylate cyclase (tmAC), sAC, PKA, and CatSper channels. Accordingly, under the condition that sperm are hyperactivated, 5-HT likely stimulates PLC/IP_3 receptor signals via the 5-HT_{2A} receptor and tmAC/PKA/CatSper channel signals via the 5-HT_4 receptor. After sAC and PKA are activated by these stimulations, sperm hyperactivation is enhanced.

Key words: 5-HT, 5-HT_2 receptor, 5-HT_4 receptor, hyperactivation, sperm

INTRODUCTION

Mammalian sperm are activated after ejaculation and are capacitated in the oviduct. Under *in vitro* capacitation conditions, sperm are reportedly capacitated after activation. During capacitation, sperm motility changes from activated to hyperactivated [1, 2, Supplementary movies 1 and 2]. Activated sperm motility consists of a small-bend amplitude and linear swimming patterns. In contrast, hyperactivated sperm motility consists of a large amplitude and a substantial asymmetric beating pattern [1, 2, Supplementary movie 1]. Notably, hyperactivation allows sperm to move through the oocyte envelope [1, 2]. In addition, capacitated sperm exhibit an acrosome reaction that exposes proteases for digestion of the oocyte envelope [2].

Under *in vitro* capacitation conditions, albumin, Ca^{2+}, and HCO_3^- play important roles [2]. Mammalian sperm are not hyperactivated in the absence of albumin [3, 4]. Albumin removes cholesterol from the sperm cell membrane [5] and induces Ca^{2+} influx via the CatSper channel [6]. Ca^{2+} and HCO_3^- activate soluble adenylate cyclase (sAC) and increase cAMP concentrations [7–10]. Moreover, Ca^{2+} and cAMP control phosphorylation, activating protein kinases and phosphatases [2, 10–12].

5-Hydroxytryptamine (5-HT) is a neurotransmitter formed by hydroxylation and decarboxylation of tryptophan. In several tissues and organs, 5-HT controls numerous functions via specific receptors [13, 14]. Notably, 5-HT receptors are composed of seven types (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7). The 5-HT_1 receptor consists of five subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, and 5-HT_{1F}), and the 5-HT_2 receptor consists of three subtypes (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}). 5-HT_1 and 5-HT_5 receptors inhibit transmembrane

adenylate cyclase (tmAC) through a Gi-protein, decreasing cAMP concentrations; however, 5-HT_4, 5-HT_6, and 5-HT_7 receptors activate tmAC through Gs-protein and increase cAMP concentrations. The 5-HT_2 receptor activates phospholipase C (PLC) through a Gq-protein and increases the inositol 1,4,5-trisphosphate (IP_3) concentration. IP_3 binds to the IP_3 receptor (IP_3R) and releases Ca^{2+} from the Ca^{2+}-store. The 5-HT_3 receptor is a ligand-gated ion channel. As 5-HT and 5-HT receptors can be detected in mammalian reproductive organs such as ovaries, testes, oocytes, cumulus-oocyte complexes (COCs), follicular fluid, and embryos [13, 15–18], some studies have suggested that serotonergic signals are associated with the regulation of steroidogenesis, oocyte maturation, spermatogenesis, and embryonic development. Recently, it has been reported that 5-HT regulates sperm function in mammals. In hamster sperm, 5-HT was found to enhance hyperactivation and induce the acrosome reaction via 5-HT_2 and 5-HT_4 receptors [19, 20]. In human sperm, 5-HT increases straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP) [21]. Furthermore, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_3 receptors have been identified in human and stallion sperm [21, 22]. In mice, 5-HT reportedly increases sperm hyperactivation via the 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors and improves the success rate of *in vitro* fertilization (IVF) [23]. In the present study, we attempted to determine the 5-HT receptor type involved in enhancing hyperactivation and examine how signals regulate hyperactivation in hamster sperm.

Materials and Methods

Chemicals

Sumatriptan succinate (sumatriptan), α-methylserotonin maleate (MS), 1-(3-chlorophenyl) biguanide hydrochloride (mCPBG), 5-methoxytryptamine (MT), WAY208466, LP12, 2',3'-dideoxyadenosine (ddAdo), 2-hydroxyestradiol (2-CE), KH7, 2,4-dithenoyl-1,2,5-oxadiazone n2-oxide (HC-056456, HC), U73122, U73343, D609, ET-18-OCH3, neomycin, spermine, and bisindolylmaleimide 1 (Bis-1) were purchased from Merck KGaA (Darmstadt, Germany). TCB2, BW723C86, and MK212 were purchased from TOCRIS Bioscience (Bristol, UK). H-89, mibefradil (Mib), and NNC 55–0396 (NNC) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti 5-HT_{2A} receptor antibody (SR-2A (A-4); sc-166775) and anti-5-HT_4 receptor antibody (SR-4 (G-3); sc-376158) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). The molecular weight marker set was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). EzWestLumi Plus® was purchased from ATTO Corporation (Tokyo, Japan). Xestospongin C, anti-mouse IgG antibody conjugated peroxidase, bovine serum albumin (BSA), fraction V, and other reagent grade chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals

Syrian hamsters (*Mesocricetus auratus*) were bred at the Research Center for Laboratory Animals, Dokkyo Medical University. The present study was approved by the Animal Care and Use Committee of the University (experimental permission numbers: 0107 and 1248), and all

experiments were performed in accordance with the University's Guidelines for Animal Experimentation.

Preparation of hyperactivated sperm

Sperm were collected from the cauda epididymis of male hamsters (10–20 weeks old). Hyperactivated sperm were prepared as described previously [11]. Modified Tyrode's albumin lactate pyruvate medium [24] was used as the capacitation medium. A drop (~ 5 μl) of cauda epididymis sperm was placed on a culture dish (35 mm diameter; Iwaki, Asahi Glass Co., Ltd., Tokyo, Japan), and 3 ml of medium was added to the dish. The sperm were incubated for 5 min at 37°C for activation. Then, the supernatant containing motile sperm was placed in a new dish containing the vehicle or inhibitors. After incubation for 5 min, the supernatant was transferred to a new dish containing the vehicle or agonist. Sperm were incubated for 4 h at 37°C to induce hyperactivation under 5% CO_2. As stock solutions, sumatriptan (100 μM), MS (100 pM), mCPBG (100 mM), WAY208466 (7.3 μM), LP12 (0.13 μM), TCB2 (0.75 μM), MK212 (0.3 μM), and ddAdo (100 mM) were dissolved in pure water. MT (10 nM), U73122 (1 mM), U73343 (1 mM), D609 (10 mM), ET-18-OCH3 (15 mM), neomycin (65 mM), spermine (1 M), and 2-CE (20 mM) were dissolved in ethanol. BW723C86 (2 mM), Bis-1 (10 μM), H-89 (100 mM), xestospongin C (1 mM), KH7 (10 mM), HC (30 mM), Mib (40 mM), and NNC (20 mM) were dissolved in dimethyl sulfoxide. For all experiments, the maximum concentration of the vehicle was 0.2%.

Measurements of motility and hyperactivation

Motility and hyperactivation were measured as previously described [25]. Motile sperm were recorded on a DVD recorder (RDR-HX50; Sony Corp., Tokyo, Japan) using a CCD camera

(Progressive 3CCD, Sony) attached to a microscope (IX70, Olympus Corp., Tokyo, Japan) with phase-contrast illumination and a small CO_2 incubator (MI-IBC, Olympus). Observations were performed at 37°C for 1 min. Visual analyses of the movies comprised manual counts of the number of total sperm, motile sperm, and hyperactivated sperm in ten different fields. For all experiments, visual analyses were performed in a blinded manner. Motile sperm exhibiting asymmetric and whiplash-like flagellar movements were defined as hyperactivated [1, 2, Supplementary movie 1]. The percentage of motility and hyperactivation were defined as the number of motile sperm/number of total sperm × 100 and the number of hyperactivated sperm/number of total sperm × 100, respectively. Each experiment was repeated four times using four different hamsters. If the proportion of motile sperm was equal to or below 80%, the experiment was repeated. Data were statistically analyzed using a repeated-measures ANOVA post-hoc test in Microsoft Excel (Microsoft Japan, Tokyo, Japan), with ystat2018 (Igakutosho Shuppan, Saitama, Japan) add-on. Statistical significance was set at $p < 0.05$.

Motility assay by the sperm motility analysis system (SMAS)

The motility assay was evaluated using SMAS for animals (Ver. 3.18) with the loaded parameter file mouse_BM10×_ 640 nm _Bright59_150fps-shutter200.ini (Ditect Co. Ltd., Tokyo, Japan) as previously described [23]. The suspension containing motile sperm (20 µl) was transferred to an observation chamber (0.1 mm deep, 18 mm wide, and 18 mm long) made of mending tape attached to the glass slide in two parallel strips, which were then covered with a cover glass. Sperm movement was recorded for 1 s on the hard disk drive of SMAS via a high-speed digital camera (HAS-L2; Ditect) attached to a microscope (ECLIPSE E2000; Nikon Corp., Tokyo, Japan) with phase-contrast illumination, a 650 nm band-pass filter, and a warm plate (MP10DM; Kitazato Corp., Shizuoka, Japan). SMAS analyzed 150 consecutive images

obtained from a single field at 10× magnification in negative phase contrast. SMAS automatically calculated VSL (μm/s), VCL (μm/s), VAP (μm/s), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH; μm), and beat-cross frequency (BCF; Hz), with the wobbler coefficient (WOB; defined as VAP/VCL) manually calculated [26]. SMAS analysis was repeated five times using five different hamsters. In each experiment, ≥ 300 sperm were detected. Only motile sperm judged to be significant were analyzed. The effects of agonists were statistically analyzed by Student's t-test performed using Microsoft Excel or by repeated-measures ANOVA post-hoc test, using Microsoft Excel with ystat2018. Statistical significance was set at $p < 0.05$.

Preparation of sperm protein extracts

Sperm proteins were extracted using the following method. In brief, sperm obtained from the epididymis were washed once with 0.9% (w/v) NaCl and collected by centrifugation at 4°C for 10 min at 15,000 × *g*. Sperm pellets were suspended at 100 mg/ml (w/v) in sodium dodecyl sulfate (SDS) buffer containing 5 M urea, 0.1% SDS, 1% 2-mercaptoethanol, and 75 mM Tris-HCl (pH 6.8) [27]. After pipetting, the suspension was incubated on ice for 10 min. Next, the suspension was centrifuged at 4°C for 20 min at 15,000 × *g* and the supernatant was used as the sperm protein extract.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method described by Laemmli [28] using a separating gel of 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS. After the gel was stained with Coomassie Brilliant Blue (CBB), images of stained gels were scanned using a densitometer (GS-800 densitometer, Bio-Rad Laboratories).

Western blotting

Western blotting was performed according to a previously described method [11, 27] with some modifications. The blotted membrane was blocked with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.15 M NaCl and 20 mM Tris-HCl (pH 7.5) for 1 h at 25°C. After washing three times with TBS, the membrane was incubated with the primary antibody (1:1000 dilution with 5% [w/v] BSA in TBS) for 1 h at 25°C. After washing three times with TBS, the membrane was incubated with secondary antibody conjugated peroxidase (1:5000 dilution with 5% [w/v] BSA in TBS). After the membrane was washed with Tween-TBS containing 0.05% (w/v) Tween-20 and TBS three times, the color reaction was performed using the EzWestLumi Plus® (ATTO). Western blotting was performed using Ez-Capture MG (ATTO).

Results

Effects of 5-HT receptors on hyperactivation and motility assay

Although a previous study [20] has suggested that 5-HT enhanced hyperactivation via 5-HT_2 and 5-HT_4 receptors in hamster sperm, it remains unknown whether 5-HT enhances hyperactivation via other receptors. As shown in Supplementary Fig. 1, the 5-HT receptor types affecting hyperactivation were examined. As shown in Supplementary Fig. 1A-F, 100 fM MS (5-HT_2 receptor agonist) and 10 pM MT (5-HT_4 receptor agonist) [20, 23] significantly increased hyperactivation, although sumatriptan (17 nM, 5-$HT_{1B/1D}$ receptor agonist; 100 nM, 5-HT_{1A} receptor agonist) [23, 29], 100 μM mCPBG (5-HT_3 receptor agonist) [23, 30], 7.3 nM WAY208466 (5-HT_6 receptor agonist) [instruction manual, 23], and 0.13 nM LP12 (5-HT_7 receptor agonist) [instruction manual, 23] did not impact hyperactivation. Moreover, we examined which 5-HT_2 receptor subtypes affected hyperactivation, as the 5-HT_2 receptor consists of three known subtypes [13, 14] (Supplementary Fig. 1G-I). TCB2 (5-HT_{2A} receptor agonist) at 0.75 nM [instruction manual] significantly increased hyperactivation; however, this effect was observed with 2 μM BW723C86 (5-HT_{2B} receptor agonist) [31] and 0.3 nM MK212 (5-HT_{2C} receptor agonist) [instruction manual]. Furthermore, motility was not affected by 5-HT receptor agonists (Supplementary Fig. 1). As shown in Supplementary Fig. 2, the 5-HT_{2A} receptor was detected as an approximately 55-kDa band from sperm protein extracts. Moreover, the 5-HT_4 receptor was detected as an approximately 40-kDa band.

Following treatment with 5-HT receptor agonists, sperm motility was evaluated using SMAS (Supplementary Table 1). MS at 100 fM significantly decreased VSL and VAP. mCPBG at 100 μM significantly decreased VCL. MT at 10 pM significantly reduced ALH. TCB2 (0.75 nM) significantly decreased VSL. Other agonists did not affect these parameters.

Regulatory mechanisms of hyperactivation enhanced by 5-HT_2 receptor stimulation

After 5-HT binds to the 5-HT_2 receptor, it stimulates PLC and produces IP_3 [13, 14]. As shown in Fig. 1, we examined whether PLC was associated with MS-enhanced hyperactivation. As shown in Fig. 1A, 1 μM U73122 (standard PLC inhibitor) [3] significantly suppressed the enhancement observed at 1 h, whereas no such effect was observed with 1 μM U73343 (control of U73122) [3]. Although 10 μM D609 (phosphatidylcholine-PLC inhibitor) [3] did not affect the enhancement, 15 μM ET-18-OCH3 (phosphatidylinositol (PI)-PLC inhibitor) [3] significantly inhibited the enhanced hyperactivation at 1 h (Fig. 1B and 1C). Neomycin at 65 μM (non-specific PLC inhibitor) [3] significantly inhibited the enhancement at 1.5 h; this finding was not observed with 1 mM spermine (PLCα inhibitor) [3] (Fig. 1D and 1E). PLC produces IP_3 and diacylglycerol [13, 14]. IP_3 binds to IP_3R, and diacylglycerol activates protein kinase C (PKC). Xestospongin C at 1 μM (IP_3R inhibitor) [32] significantly inhibited the enhancement at 1 and 1.5 h; these findings were not observed with 10 nM Bis-1 (PKC inhibitor) [32] (Fig. 1F and 1G).

Hyperactivation is regulated by sAC [10], and in addition, it has been reported that tmAC and sAC exist in hamster sperm and produce cAMP [7]. Next, we examined whether adenylate cyclase was associated with MS-enhanced hyperactivation. As shown in Fig. 2A and 2B, 50 and 100 μM ddAdo (tmAC inhibitor) [33] did not affect motility and hyperactivation in the absence or presence of MS. In contrast, 20 μM 2-CE (sAC inhibitor) [33] significantly decreased motility after 2 h in the absence of MS and after 3 h in the presence of MS (Fig. 2C). Moreover, in the absence and presence of MS, 50 μM 2-CE [33] significantly decreased motility at 0, 0.5, and 1 h and did not allow sperm to swim after 1.5 h (Fig. 2C). In terms of hyperactivation (Fig. 2D), 20 μM 2-CE significantly inhibited hyperactivation after 2 h in the absence of MS and

after 2.5 h in the presence of MS. Moreover, 50 μM 2-CE did not allow sperm hyperactivation in the absence and presence of MS (Fig. 2D). KH7, another sAC inhibitor, did not affect motility (Fig. 2E). In the absence and presence of MS, 10 μM KH7 [33] significantly inhibited hyperactivation at 4 h (Fig. 2F). Furthermore, 25 μM KH7 [33] significantly inhibited MS-enhanced hyperactivation at 1, 1.5, and 2 h, significantly suppressing hyperactivation at 3 and 4 h in the absence and presence of MS (Fig. 2F). As sAC produces cAMP and activates PKA [7, 10], we examined the effects of H-89 (a PKA inhibitor) on motility and hyperactivation in the absence and presence of MS (Fig. 3). In the absence of MS, 100 μM H-89 significantly inhibited motility after 1.5 h, whereas 1 and 10 μM H-89 did not affect motility (Fig. 3A). As for hyperactivation, 100 μM H-89 did not enable sperm hyperactivation, whereas 1 and 10 μM H-89 did not affect the ability of the sperm to be hyperactivated(Fig. 3B). In the presence of MS, 100 μM H-89 significantly inhibited motility after 1 h and did not allow sperm hyperactivation (Fig. 3C and 3D). In contrast, 1 and 10 μM H-89 significantly inhibited MS-enhanced hyperactivation without impacting motility (Fig. 3C and 3D).

As shown in Fig. 1, MS-enhanced hyperactivation is associated with Ca^{2+} signals. In addition, sACs are activated by Ca^{2+} [10]. During hyperactivation, Ca^{2+} signals are reportedly associated with a CatSper channel [34]; therefore, we examined whether a CatSper channel was associated with MS-enhanced hyperactivation (Supplementary Fig. 3). As shown in Supplementary Fig. 3A and 3C, 3 and 10 μM HC (a potent CatSper channel blocker) [35] did not affect motility in the absence or presence of MS. Regarding hyperactivation, 3 μM HC did not impact motility in the absence or presence of MS, although 10 μM HC significantly inhibited motility after 1.5 h in the absence of MS and after 2 h in the presence of MS (Supplementary Fig. 3B and 3D). Mib and NNC are typical T-type voltage-activated Ca^{2+} channel blockers [instruction manual] and are proven to be potent CatSper channel blockers in sperm studies [36–38]. As shown in

Supplementary Fig. 3E, in the absence and presence of MS, 30 and 40 μM Mib [38] significantly suppressed motility after 2 h and 1.5 h, respectively, and did not enable sperm hyperactivation (Supplementary Fig. 3F). As shown in Supplementary Fig. 3G, in the absence and presence of MS, 10 and 20 μM NNC [38] significantly inhibited motility after 1.5 and 0.5 h, respectively; both doses did not allow sperm hyperactivation in the absence or presence of MS (Supplementary Fig. 3H).

Regulatory mechanisms of hyperactivation enhanced by stimulation of 5-HT_4 receptor

After 5-HT binds to the 5-HT_4 receptor, it stimulates tmAC and induces cAMP production [13, 14]. Additionally, hyperactivation is regulated by sAC [10]. We examined whether tmAC and sAC were associated with MT-enhanced hyperactivation (Fig. 4). As shown in Fig 4A, 50 and 100 μM ddAdo did not affect motility in the absence or presence of MT and did not affect hyperactivation in the absence of MT. In terms of MT-enhanced hyperactivation, 100 μM ddAdo significantly suppressed this effect; however, 50 μM ddAdo demonstrated no such effect (Fig. 4B). As shown in Fig. 4C, 20 μM 2-CE significantly inhibited motility at 4 h, both in the absence and presence of MT. Moreover, 50 μM 2-CE significantly inhibited motility after 1 h in the absence of MT and after 2.5 h in the presence of MT (Fig. 4C). Moreover, 20 μM 2-CE significantly inhibited hyperactivation after 2.5 h in the absence and presence of MT (Fig. 4D). In addition, 50 μM 2-CE did not enable sperm hyperactivation in the absence or presence of MT (Fig. 4D). As shown in Fig. 4E and 4F, in the absence and presence of MT, 10 and 25 μM KH7 significantly inhibited hyperactivation at 4 h, with no impact on motility.

As tmAC produces cAMP and activates PKA [33], we examined the effects of H-89 on motility and hyperactivation in the presence of MT (Fig. 5). At 100 μM, H-89 significantly suppressed motility after 1 h and did not facilitate sperm hyperactivation. H-89 at 1 μM and 10

μM significantly inhibited MT-enhanced hyperactivation without impacting motility.

In mouse sperm, cAMP-induced Ca^{2+} influx possibly occurs through the CatSper channel [34]. Additionally, a recent mouse study suggested that the CatSper channel is activated by PKA [39]; thus, the present study examined whether the CatSper channel was associated with MT-enhanced hyperactivation (Fig. 6). As shown in Fig. 6A and 6C, 3 and 10 μM HC did not affect motility in the absence or presence of MT. As shown in Fig. 6B, 3 μM HC did not inhibit hyperactivation in the absence or presence of MT. In contrast, 10 μM HC did not affect MT-enhanced hyperactivation (Fig. 6D). Furthermore, 10 μM HC significantly inhibited hyperactivation after 2.5 h in the absence of MT; however, MT canceled the inhibition of hyperactivation (Fig. 6D). As shown in Fig. 6E and 6F, in the absence and presence of MT, 30 and 40 μM Mib significantly inhibited motility after 1.5 h and did not allow sperm hyperactivation. Moreover, in the absence and presence of MT, 10 and 20 μM NNC significantly inhibited motility after 1.5 h and did not enable sperm hyperactivation.

Discussion

A recent human study has suggested that the capacity for sperm hyperactivation can be correlated with IVF success [40]. Some hormones induce sperm hyperactivation [41]; thus, the success of IVF might be controlled by artificial regulation of hyperactivation. Progesterone (P_4) is a popular inducer of hyperactivation [3, 36, 37]. In human sperm, P_4 induces hyperactivation via activation of the CatSper channel [36, 37]. In hamster sperm, P_4 binds to a membrane progesterone receptor and enhances hyperactivation via signals related to PLC, IP_3R, PKA, and PKC [3, 33]. Reportedly, 5-HT and melatonin enhance hyperactivation via specific receptors in hamster sperm [4, 20]. Estrogen suppresses the enhancement of hyperactivation mediated by P_4 and melatonin via membrane estrogen receptors [42–44]. γ-Aminobutyric acid (GABA) suppressed the enhancement of hyperactivation mediated by P_4 and 5-HT via a $GABA_A$ receptor in hamster sperm [25, 45], but it induced hyperactivation via the $GABA_A$ receptor in human sperm [46]. In a human study, the regulation of hyperactivation by P_4 was not correlated with IVF success [40], although 5-HT increased hyperactivation and the success of IVF in rodents [20, 23]. It remains unknown whether melatonin, estrogen, and GABA affect IVF success.

Previous studies [20, 23], as well as the present study, have shown that 5-HT enhanced hyperactivation via several types of 5-HT specific receptors. Typically, 5-HT receptors are comprised of seven major types [13, 14]. The $5\text{-}HT_2$ and $5\text{-}HT_4$ receptor-specific agonists significantly enhanced hamster sperm hyperactivation [20] (Supplementary Fig. 1). Moreover, the enhancement of hyperactivation mediated by 5-HT was suppressed by $5\text{-}HT_2$ and $5\text{-}HT_4$ receptor-specific antagonists [20]. In addition, $5\text{-}HT_2$ receptors consist of three subtypes [13, 14]. The $5\text{-}HT_{2A}$ receptor-specific agonist significantly enhanced the hyperactivation of hamster sperm (Supplementary Fig. 1). As $5\text{-}HT_{2A}$ and $5\text{-}HT_4$ receptors were detected in hamster sperm,

it appears that 5-HT enhanced hamster sperm hyperactivation via 5-HT_{2A} and 5-HT_4 receptors [20] (Supplementary Fig. 1 and 2). A previous study [23] using receptor-specific agonists and antagonists has revealed that 5-HT increased hyperactivation and the success of IVF via 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors [23]. As the 5-HT_{2A} receptor was detected in human sperm [21], the 5-HT_2 receptor is considered a vital receptor for artificially regulating the success of IVF.

As shown in Supplementary Table 1, stimulation with the 5-HT_2 or 5-HT_{2A} receptors decreased VSL. In mouse sperm, stimulation of the 5-HT_2 receptor decreases VSL [23]. As stimulation of the 5-HT_2 receptor enhanced hyperactivation [20, 23] (Supplementary Fig. 1), the decrease in VSL by MS can likely be correlated with MS-enhanced hyperactivation. Stimulation of the 5-HT_3 receptor decreased VCL and ALH (Supplementary Table 1), but the 5-HT_3 receptor was not associated with hyperactivation (Supplementary Fig. 1). In mouse sperm, stimulation of the 5-HT_3 receptor did not impact VCL and ALH, but stimulation increased hyperactivation [23]. These findings indicate that stimulation of the 5-HT_3 receptor can be associated with hyperactivation in a species-specific manner. Moreover, stimulation of the 5-HT_4 receptor decreased ALH levels (Supplementary Table 1). In mouse sperm, stimulation of the 5-HT_4 receptor decreases ALH [23]. Stimulation of the 5-HT_4 receptor enhances hyperactivation [20, 23] (Supplementary Fig. 1), and thus, it is likely that the decrease in ALH by MT can be correlated with MT-enhanced hyperactivation.

When MS enhanced hyperactivation, PI-PLC, IP_3R, sAC, and PKA were associated with enhanced hyperactivation (Fig. 1, 2, and 3). Therefore, it is likely that stimulation of the 5-HT_2 receptor activates sAC and PKA via Ca^{2+} signals, which are known to be related to PLC and IP_3R. Moreover, sAC appears to be associated with hyperactivation regulation, as sAC inhibitors inhibit hyperactivation in the absence of MS (Fig. 2). These findings suggest that sAC

is associated with the basal regulatory mechanism of hyperactivation. The CatSper channel is an important channel for regulating hyperactivation and induces an increase in Ca^{2+} [34, 36, 37]. Although 3 μM HC did not inhibit hyperactivation in the absence and presence of MS, 10 μM HC inhibited hyperactivation in the absence and presence of MS (Supplementary Fig. 3B and 3D). As HC did not inhibit motility (Supplementary Fig. 3A and 3C), the results suggest that the CatSper channel is associated with a basal regulatory mechanism of hyperactivation. When hyperactivation occurs through the basal regulatory mechanism, MS possibly enhances hyperactivation by activating the basal regulatory mechanism without CatSper (Supplementary Fig. 4). Conversely, 100 μM H-89, Mib, and NNC suppressed motility and did not allow sperm hyperactivation in the absence and presence of MS (Fig. 3 and Supplementary Fig. 3). In addition, hyperactivation was suppressed after a decrease in motility induced by the sAC inhibitor (Fig. 2). These observations reveal that motility is an important event when sperm are hyperactivated. HC, which is a CatSper inhibitor, did not affect motility (Supplementary Fig. 3). Mib and NNC are typical inhibitors of T-type Ca^{2+} channels, and it appears that T-type Ca^{2+} channels are associated with the maintenance of motility.

Based on the observed effects of MT, ddAdo, and H-89 on motility and hyperactivation (Fig. 4 and 5), it is likely that stimulation of the 5-HT_4 receptor activates tmAC and PKA. Moreover, the results of MT and sAC inhibition (Fig. 4) suggest that stimulation of the 5-HT_4 receptor is associated with the activation of sAC. sAC is activated by Ca^{2+} [10], and thus, stimulation of the 5-HT_4 receptor is possibly associated with Ca^{2+} signals. In mouse sperm, it has been suggested that cAMP-induced Ca^{2+} influx occurs through the CatSper channel, which is activated by PKA [34, 39]. Inhibition of hyperactivation by HC was abolished by stimulation of the 5-HT_4 receptor (Fig. 6); therefore, it is likely that stimulation of the 5-HT_4 receptor activates PKA via tmAC and activates sAC via the CatSper channel, which is activated by PKA.

Herein, we propose a hypothesis regarding the regulatory mechanisms of 5-HT-enhanced hyperactivation in hamster sperm (Supplementary Fig. 4). In hamster sperm, 5-HT enhances hyperactivation through 5-HT_{2A} and 5-HT_4 receptors. Stimulation of the 5-HT_2 receptor is associated with PI-PLC/IP_3R/Ca^{2+} signals. Stimulation of the 5-HT_4 receptor can be associated with tmAC/cAMP/PKA/CatSper/Ca^{2+} signals. Both stimulations activate sAC/PKA signaling and enhance hyperactivation.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure Legends

Figure 1. Suppression of MS-enhanced hyperactivation by PLC, IP_3R, and PKC inhibitors. Percentages of hyperactivation were detected after sperm were cultured for 4 h with 100 fM MS and inhibitors, including 1 μM U73122 and 1 μM U73343 (A), 10 μM D609 (B), 15 μM ET-18-OCH3 (C), 65 μM neomycin (D), 1 mM spermine (E), 1 μM xestospongin C (F), and 10 nM Bis-1 (G). Data represent the mean ± standard deviation (SD). **(A)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (MS) medium with 100 fM MS and vehicle; (MS + U73122) medium with 100 fM MS, 1 μM U73122, and vehicle; (MS + U73343) medium with 100 fM MS, 1 μM U73343, and vehicle. **(B)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (MS + D609) medium with 100 fM MS, 10 μM D609, and vehicle. **(C)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (MS + ET-18-OCH3) medium with 100 fM MS, 15 μM ET-18-OCH3, and vehicle. **(D)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (MS + Neomycin) medium with 100 fM MS, 65 μM neomycin, and vehicle. **(E)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (MS + Spermine) medium with 100 fM MS, 1 mM spermine, and vehicle. **(F)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide as vehicle; (MS) medium with 100 fM MS and vehicle; (MS + Xestospongin C) medium with 100 fM MS, 1 μM xestospongin C, and vehicle. **(G)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (MS + Bis-1) medium with 100 fM MS, 10 nM Bis-1, and vehicle. * indicates significant differences compared with "Vehicle" ($P < 0.05$). ** indicates significant differences compared with "Vehicle," "MS + U73122" ($P < 0.05$). # indicates significant differences compared with "Vehicle" and "MS + inhibitors" ($P < 0.05$). MS, α-methylserotonin maleate; Bis-1, bisindolylmaleimide 1; PLC, phospholipase C; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate;

Figure 2. Suppression of MS-enhanced hyperactivation by adenylate cyclase inhibitors. Percentages of motility (A, C, and E) and hyperactivation (B, D, and F) were detected after sperm were cultured for 4 h with 100 fM MS and inhibitors, including 50 and 100 μM ddAdo (A and B), 20 and 50 μM 2-CE (C and D), and 10 and 25 μM KH7 (E and F). Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (MS) medium with 100 fM MS and vehicle; (50 μM ddAdo) medium with 50 μM ddAdo and vehicle; (MS + 50 μM ddAdo) medium with 100 fM MS, 50 μM ddAdo, and vehicle; (100 μM ddAdo) medium with 100 μM ddAdo and vehicle; (MS + 100 μM ddAdo) medium with 100 fM MS, 100 μM ddAdo, and vehicle. **(C and D)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (MS) medium with 100 fM MS and vehicle; (20 μM 2-CE) medium with 20 μM 2-CE and vehicle; (MS + 20 μM 2-CE) medium with 100 fM MS, 20 μM 2-CE, and vehicle; (50 μM 2-CE) medium with 50 μM 2-CE and vehicle; (MS + 50 μM 2-CE) medium with 100 fM MS, 250 μM 2-CE, and vehicle. **(E and F)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide as vehicle; (MS) medium with 100 fM MS and vehicle; (10 μM KH7) medium with 10 μM KH7 and vehicle; (MS + 10 μM KH7) medium with 100 fM MS, 10 μM KH7, and vehicle; (25 μM KH7) medium with 25 μM KH7 and vehicle; (MS + 25 μM KH7) medium with 100 fM MS, 25 μM KH7, and vehicle. * indicates significant differences compared with "Vehicle" and "Inhibitors" ($P < 0.05$). ** indicates significant differences compared with "Vehicle," "MS," "Low concentration of inhibitor," and "MS + inhibitors" ($P < 0.05$). # indicates significant differences compared with "Vehicle" and "MS" ($P < 0.05$). ## indicates significant differences compared with "Vehicle," "MS," "Low concentration of inhibitor," and "MS + low concentration of inhibitor" ($P < 0.05$). ¢ indicates significant differences compared with "Vehicle," "MS," "High concentration

of inhibitor," and "MS + High concentration of inhibitor" ($P < 0.05$). ¢¢ indicates significant differences compared with "Vehicle," "Inhibitors," and "MS + Inhibitor" ($P < 0.05$). $ indicates significant differences compared with "Vehicle," "Inhibitors," and "MS + High concentration of inhibitor" ($P < 0.05$). $$ indicates significant differences compared with "Vehicle," "High concentration of inhibitor," and "MS + High concentration of inhibitor" ($P < 0.05$). MS, α-methylserotonin maleate; 2-CE, 2-hydroxyestradiol; ddAdo, 2',3'-dideoxyadenosine.

Figure 3. Suppression of motility and hyperactivation by H-89 (PKA inhibitor). Percentages of motility (A and C) and hyperactivation (B and D) were determined after sperm were cultured with various concentrations of H-89 for 4 h in the absence and the presence of 100 fM MS. Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) dimethyl sulfoxide as vehicle; (1 μM H-89) medium with 1 μM H-89 and vehicle; (10 μM H-89) medium with 10 μM H-89 and vehicle; (100 μM H-89) medium with 100 μM H-89 and vehicle. **(C and D)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide as vehicle; (MS) medium with 100 fM MS and vehicle; (MS + 1 μM H-89) medium with 100 fM MS, 1 μM H-89, and vehicle; (MS + 10 μM H-89) medium with 100 fM MS, 10 μM H-89, and vehicle; (MS + 100 μM H-89) medium with 100 fM MS, 100 μM H-89, and vehicle. * indicates significant differences compared with "Vehicle," "1 μM H-89," and "10 μM H-89" ($P < 0.05$). ** indicates significant differences compared with "Vehicle" and "1 μM H-89" ($P < 0.05$). # indicates significant differences compared with "Vehicle" ($P < 0.05$). ## indicates significant differences compared with "Vehicle," "MS," "MS + 1 μM H-89," and "MS + 10 μM H-89" ($P < 0.05$). $ indicates significant differences compared with "Vehicle" ($P < 0.05$). $$ indicates significant differences compared with "Vehicle" and "MS + 10 μM H-89" ($P < 0.05$). □ indicates significant differences compared with "Vehicle," "MS + 1 μM H-89," and

"MS + 10 μM H-89" (*P < 0.05*). MS, α-methylserotonin maleate.

Figure 4. Suppression of MT-enhanced hyperactivation by adenylate cyclase inhibitors. Percentages of motility (A, C, and E) and hyperactivation (B, D, and F) were determined after sperm were cultured for 4 h with 10 pM MT and inhibitors, including 50 and 100 μM ddAdo (A and B), 20 and 50 μM 2-CE (C and D), and 10 and 25 μM KH7 (E and F). Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (MT) medium with 10 pM MT and vehicle; (50 μM ddAdo) medium with 50 μM ddAdo and vehicle; (MT + 50 μM ddAdo) medium with 10 pM MT, 50 μM ddAdo, and vehicle; (100 μM ddAdo) medium with 100 μM ddAdo and vehicle; (MT + 100 μM ddAdo) medium with 10 pM MT, 100 μM ddAdo, and vehicle. **(C and D)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (MT) medium with 10 pM MT and vehicle; (20 μM 2-CE) medium with 20 μM 2-CE and vehicle; (MT + 20 μM 2-CE) medium with 10 pM MT, 20 μM 2-CE, and vehicle; (50 μM 2-CE) medium with 50 μM 2-CE and vehicle; (MT + 50 μM 2-CE) medium with 10 pM MT, 50 μM 2-CE, and vehicle. **(E and F)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide as vehicle; (MT) medium with 10 pM MT and vehicle; (10 μM KH7) medium with 10 μM KH7 and vehicle; (MT + 10 μM KH7) medium with 10 pM MT, 10 μM KH7, and vehicle; (25 μM KH7) medium with 25 μM KH7 and vehicle; (MT + 25 μM KH7) medium with 10 pM MT, 25 μM KH7, and vehicle. * indicates significant differences compared with "Vehicle," "Inhibitors," and "MT + Inhibitors" (*P < 0.05*). # indicates significant differences compared with "Vehicle" and "MT" (*P < 0.05*). ## indicates significant differences compared with "Vehicle," "Inhibitors," and "MT + High concentration of inhibitor" (*P < 0.05*). $ indicates significant differences compared with "Vehicle," "MT," "Low concentration of inhibitor," and "MT + Inhibitors" (*P < 0.05*).

$$ indicates significant differences compared with “Vehicle,” “MT,” and “High concentration of inhibitor” ($P < 0.05$). ¢ indicates significant differences compared with “Vehicle,” “MT,” “Low concentration of inhibitor,” and “MT + Low concentration of inhibitor” ($P < 0.05$). ¢¢ indicates significant differences compared with “Vehicle,” “MT,” “High concentration of inhibitor,” and “MT + High concentration of inhibitor” ($P < 0.05$). £ indicates significant differences compared with “Vehicle” and “Inhibitors” ($P < 0.05$). MT, 5-methoxytryptamine; 2-CE, 2-hydroxyestradiol; ddAdo, 2’,3’-dideoxyadenosine.

Figure 5. Suppression of MT-enhanced hyperactivation by PKA inhibitors. Percentages of motility (A) and hyperactivation (B) were determined after sperm were cultured for 4 h with 10 pM MT and various concentrations of H-89. Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide as vehicle; (MT) medium with 10 pM MT and vehicle; (MT + 1 μM H-89) medium with 10 pM MT, 1 μM H-89, and vehicle; (MT + 10 μM H-89) medium with 10 pM MT, 10 μM H-89, and vehicle; (MT + 100 μM H-89) medium with 10 pM MT, 100 μM H-89, and vehicle. * indicates significant differences compared with “Vehicle,” “Inhibitors,” and “MT + Inhibitors” ($P < 0.05$). ** indicates significant differences compared with “Vehicle,” “MT,” “MT + 1 μM H-89,” and “MT + 10 μM H-89” ($P < 0.05$). $ indicates significant differences compared with “Vehicle,” “MT + 1 μM H-89,” and “MT + 10 μM H-89” ($P < 0.05$). MT, 5-methoxytryptamine; PKA, protein kinase A.

Figure 6. Suppression of MT-enhanced hyperactivation by CatSper inhibitors. Percentages of motility (A, C, E, and G) and hyperactivation (B, D, F, and H) were determined after sperm were cultured for 4 h with 10 pM MT and inhibitors such as 3 μM HC (A and B), 10 μM HC (C

and D), 30 and 40 μM Mib (E and F), and 10 and 20 μM NNC (G and H). Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) dimethyl sulfoxide as vehicle; (MT) medium with 10 pM MT and vehicle; (3 μM HC) medium with 3 μM HC and vehicle; (MT + 3 μM HC) medium with 10 pM MT, 3 μM HC, and vehicle. **(C and D)** (Vehicle) same as above; (MT) medium with 10 pM MT and vehicle; (10 μM HC) medium with 10 μM HC and vehicle; (MT + 10 μM HC) medium with 10 pM MT, 10 μM HC, and vehicle. **(E and F)** (Vehicle) same as above; (MT) medium with 10 pM MT and vehicle; (30 μM Mib) medium with 30 μM Mib and vehicle; (MT + 30 μM Mib) medium with 10 pM MT, 30 μM Mib, and vehicle; (40 μM Mib) medium with 40 μM Mib and vehicle; (MT + 40 μM Mib) medium with 10 pM MT, 40 μM Mib, and vehicle. **(G and H)** (Vehicle) same as above; (MT) medium with 10 pM MT and vehicle; (10 μM NNC) medium with 10 μM NNC and vehicle; (MT + 10 μM NNC) medium with 10 pM MT, 10 μM NNC, and vehicle; (20 μM NNC) medium with 20 μM NNC and vehicle; (MT + 20 μM NNC) medium with 10 pM MT, 20 μM NNC, and vehicle. * indicates significant differences compared with "Vehicle," "inhibitors," and "MT + inhibitors" ($P < 0.05$). ** indicates significant differences compared with "Vehicle," "MT," and "MT + inhibitors" ($P < 0.05$). # indicates significant differences compared with "Vehicle" and "inhibitors" ($P < 0.05$). ## indicates significant differences compared with "inhibitors" and "MT + inhibitors" ($P < 0.05$). $ indicates significant differences compared with "Vehicle" and "MT" ($P < 0.05$). MT, 5-methoxytryptamine; HC, 2,4-dithenoyl-1,2,5-oxadiazone n2-oxide; Mib, mibefradil.

Supplementary Figure Legends

Supplementary Figure 1. Effects of 5-HT receptor agonists on hamster sperm hyperactivation. Percentages of motility and hyperactivation were determined after 2 h of culture when sperm were cultured for 4 h with 17 nM or 100 nM sumatriptan (A), 100 fM MS (B), 100 μM mCPBG (C), 10 pM MT (D), 7.3 nM WAY (E), 0.13 nM LP12 (F), 0.75 nM TCB2 (G), 2 μM BW723C86 (H), and 0.3 nM MK212 (I). Data represent the mean ± standard deviation (SD). **(A)** (Vehicle) the medium with 0.1% (v/v) pure water as vehicle; (respective concentrations of sumatriptan) the medium with indicated concentrations of sumatriptan and vehicle. **(B)** (Vehicle) same as above; (MS) the medium with 100 fM MS and vehicle. **(C)** (Vehicle) same as above; (mCPBG) the medium with 100 μM mCPBG and vehicle. **(D)** (Vehicle) medium with 0.1% (v/v) ethanol as vehicle; (MT) medium with 10 pM MT and vehicle. **(E)** (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (WAY) medium with 7.3 nM WAY and vehicle. **(F)** (Vehicle) same as above; (LP12) medium with 0.13 nM LP12 and vehicle. **(G)** (Vehicle) same as above; (TCB2) medium with 0.75 nM TCB2 and vehicle. **(H)** (Vehicle) medium with 0.1% (v/v) dimethyl sulfoxide as vehicle; (BW723C86) medium with 2 μM BW723C86 and vehicle. **(I)** (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (MK212) medium with 0.3 nM MK212 and vehicle. * indicates significant differences compared with "Vehicle" ($P < 0.05$). MS, α-methylserotonin maleate; MT, 5-methoxytryptamine; mCPBG, 1-(3-chlorophenyl) biguanide hydrochloride.

Supplementary Figure 2. Detection of 5-HT_{2A} and 5-HT_4 receptors from hamster sperm. Left lane shows CBB stained gel. Middle lane shows western blotting against the anti-5-HT_{2A} receptor antibody. Right lane shows western blotting against anti-5-HT_4 receptor antibody. The

numbers on the left side show molecular weight markers. Sperm protein extracts were applied at 10 μl in each lane. Arrow indicates the antibody reaction.

Supplementary Figure 3. Suppression of MS-enhanced hyperactivation by CatSper inhibitors. Percentages of motility (A, C, E, and G) and hyperactivation (B, D, F, and H) were determined after sperm were cultured for 4 h with 100 fM MS and inhibitors, including 3 μM HC (A and B), 10 μM HC (C and D), 30 and 40 μM Mib (E and F), and 10 and 20 μM NNC (G and H). Data represent the mean ± standard deviation (SD). **(A and B)** (Vehicle) medium with 0.1% (v/v) dimethyl sulfoxide as vehicle; (MS) medium with 100 fM MS and vehicle; (3 μM HC) medium with 3 μM HC and vehicle; (MS + 3 μM HC) medium with 100 fM MS, 3 μM HC, and vehicle. **(C and D)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (10 μM HC) medium with 10 μM HC and vehicle; (MS + 10 μM HC) medium with 100 fM MS, 10 μM HC, and vehicle. **(E and F)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (30 μM Mib) medium with 30 μM Mib and vehicle; (MS + 30 μM Mib) medium with 100 fM MS, 30 μM Mib, and vehicle; (40 μM Mib) medium with 40 μM Mib and vehicle; (MS + 40 μM Mib) medium with 100 fM MS, 40 μM Mib, and vehicle. **(G and H)** (Vehicle) same as above; (MS) medium with 100 fM MS and vehicle; (10 μM NNC) medium with 10 μM NNC and vehicle; (MS + 10 μM NNC) medium with 100 fM MS, 10 μM NNC, and vehicle; (20 μM NNC) medium with 20 μM NNC and vehicle; (MS + 20 μM NNC) medium with 100 fM MS, 20 μM NNC, and vehicle. * indicates significant differences compared with "Vehicle," "inhibitors," and "MS + inhibitors" ($P < 0.05$). # indicates significant differences compared with "Vehicle" and "inhibitors" ($P < 0.05$). ## indicates significant differences compared with "inhibitors" ($P < 0.05$). $ indicates significant differences compared with "Vehicle" and "MS" ($P < 0.05$). MS, α-methylserotonin maleate; HC, 2,4-dithenoyl-1,2,5-oxadiazone n2-oxide; Mib,

mibefradil.

Supplementary Figure 4. Hypothesis regarding regulatory mechanisms of 5-HT-enhanced hyperactivation in hamster sperm. 5-HT, 5-hydroxytryptamine; 5-HT_{2A}R, 5-HT_{2A} receptor; 5-HT_4R, 5-HT_4 receptor; Gq, Gq-protein; Gs, Gs-protein; tmAC, transmembrane adenylate cyclase; sAC, soluble adenylate cyclase; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-phospholipase C; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; PKA, protein kinase A.

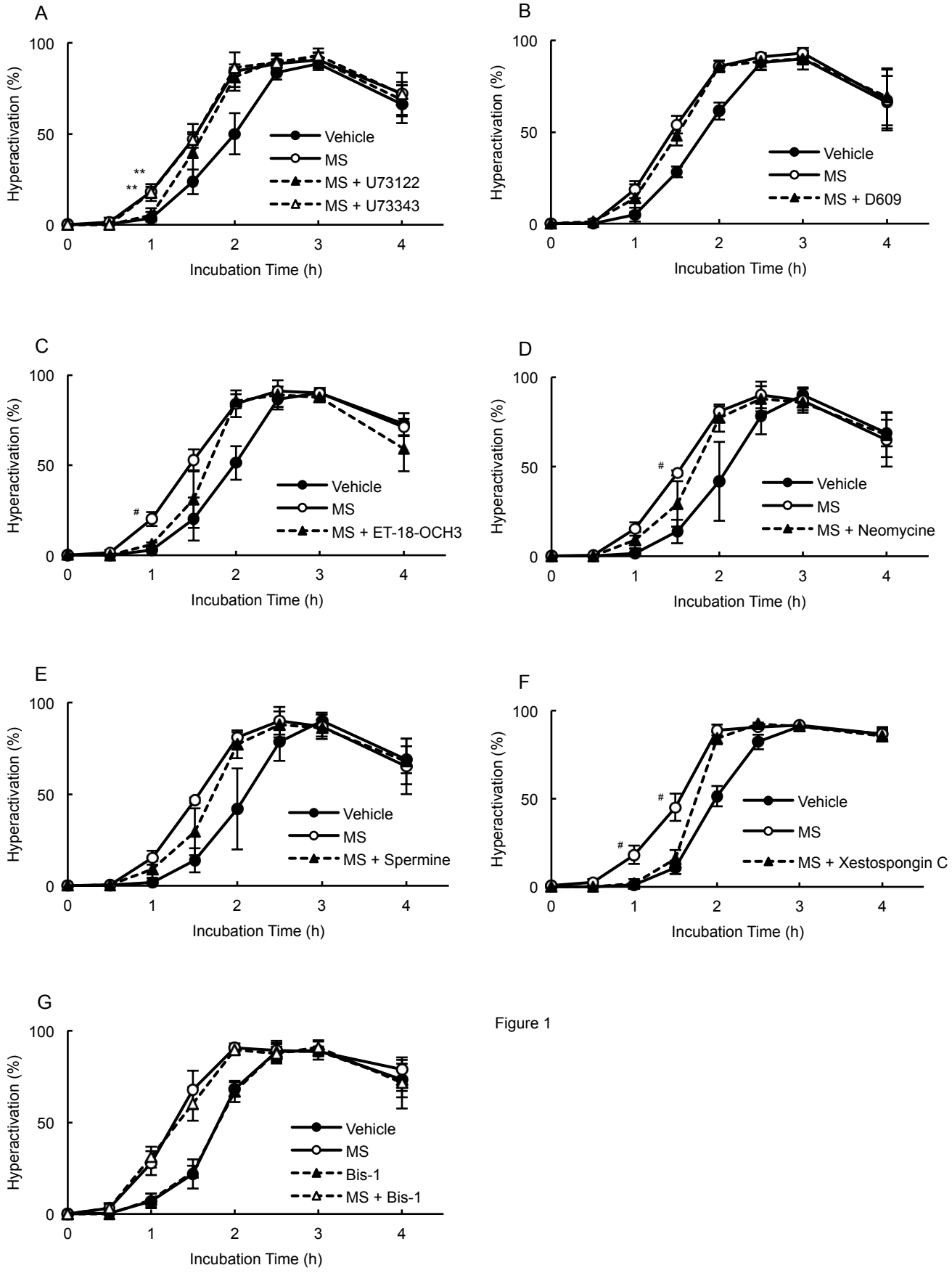

A
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + U73122
MS + U73343
**
**
B
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + D609
C
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + ET-18-OCH3
#
D
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + Neomycine
#
E
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + Spermine
F
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
MS + Xestospongin C
#
#
G
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
Bis-1
MS + Bis-1

Figure 1

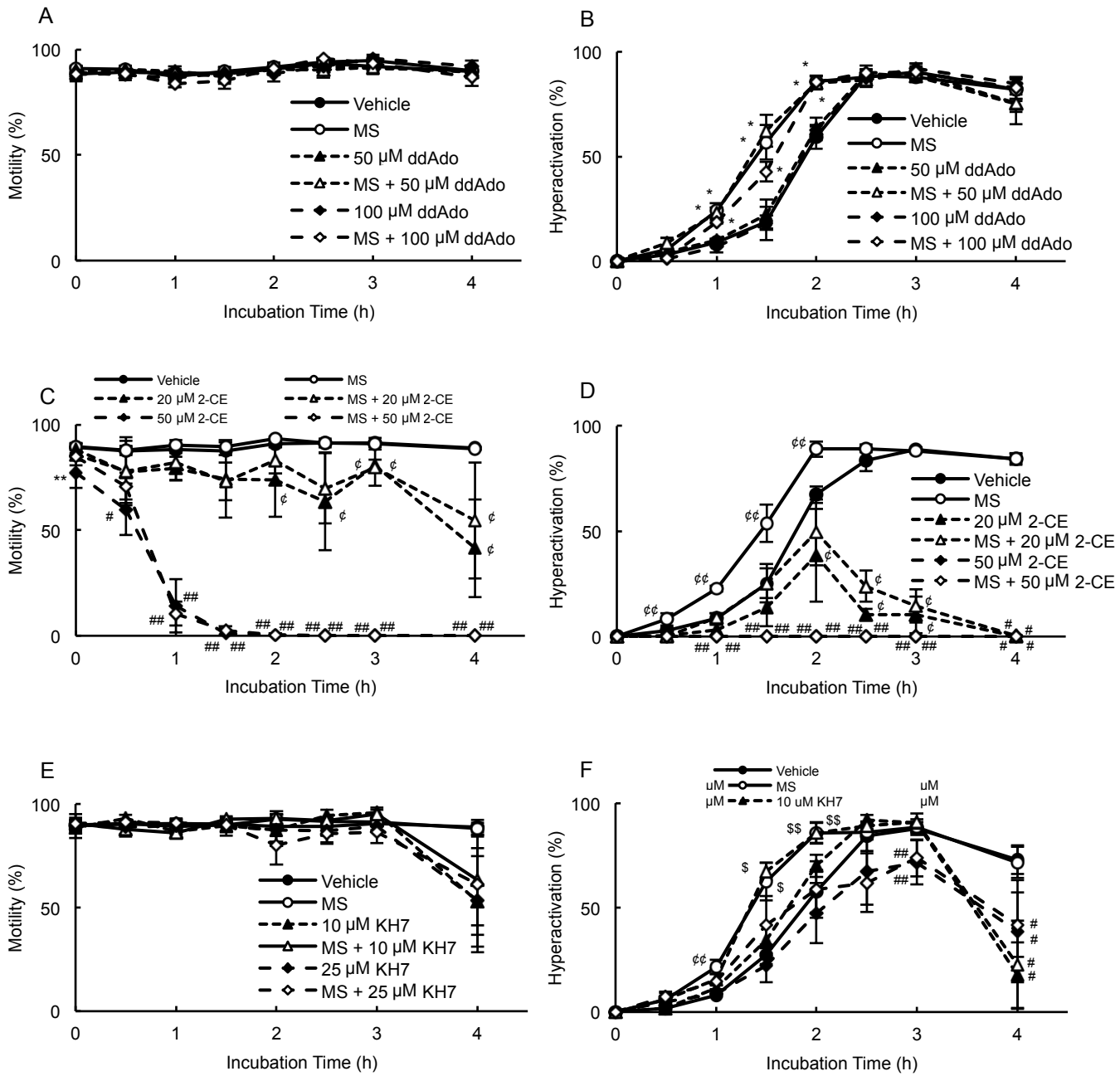

A
Motility (%)
Incubation Time (h)
Vehicle
MS
50 μM ddAdo
MS + 50 μM ddAdo
100 μM ddAdo
MS + 100 μM ddAdo
B
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
50 μM ddAdo
MS + 50 μM ddAdo
100 μM ddAdo
MS + 100 μM ddAdo
C
Motility (%)
Incubation Time (h)
Vehicle
MS
20 μM 2-CE
MS + 20 μM 2-CE
50 μM 2-CE
MS + 50 μM 2-CE
D
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
20 μM 2-CE
MS + 20 μM 2-CE
50 μM 2-CE
MS + 50 μM 2-CE
E
Motility (%)
Incubation Time (h)
Vehicle
MS
10 μM KH7
MS + 10 μM KH7
25 μM KH7
MS + 25 μM KH7
F
Hyperactivation (%)
Incubation Time (h)
Vehicle
MS
10 uM KH7

Figure 2

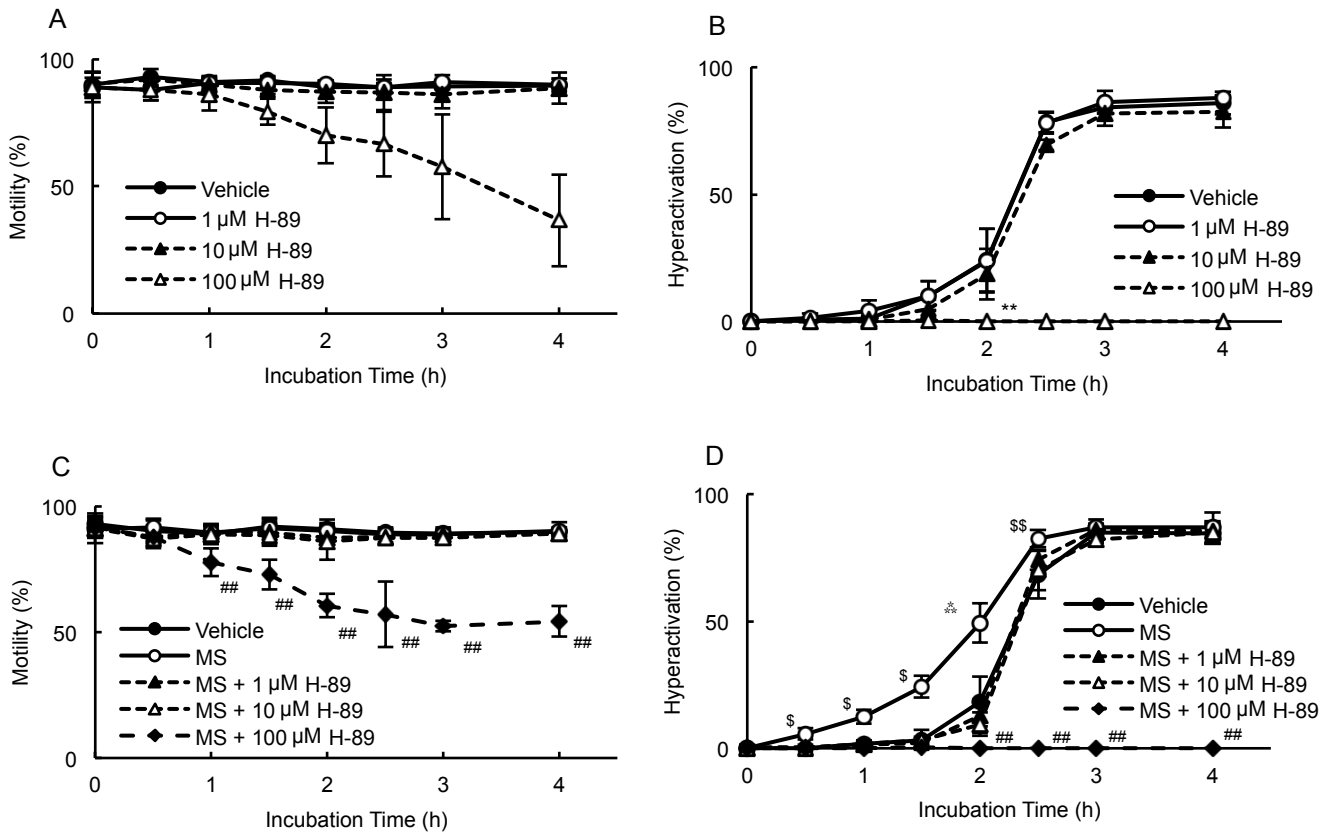

A
Motility (%)
Incubation Time (h)
Vehicle
1 μM H-89
10 μM H-89
100 μM H-89
B
Hyperactivation (%)
Incubation Time (h)
**
C
Motility (%)
Incubation Time (h)
MS
MS + 1 μM H-89
MS + 10 μM H-89
MS + 100 μM H-89
##
D
Hyperactivation (%)
Incubation Time (h)
$
$$
⁂

Figure 3

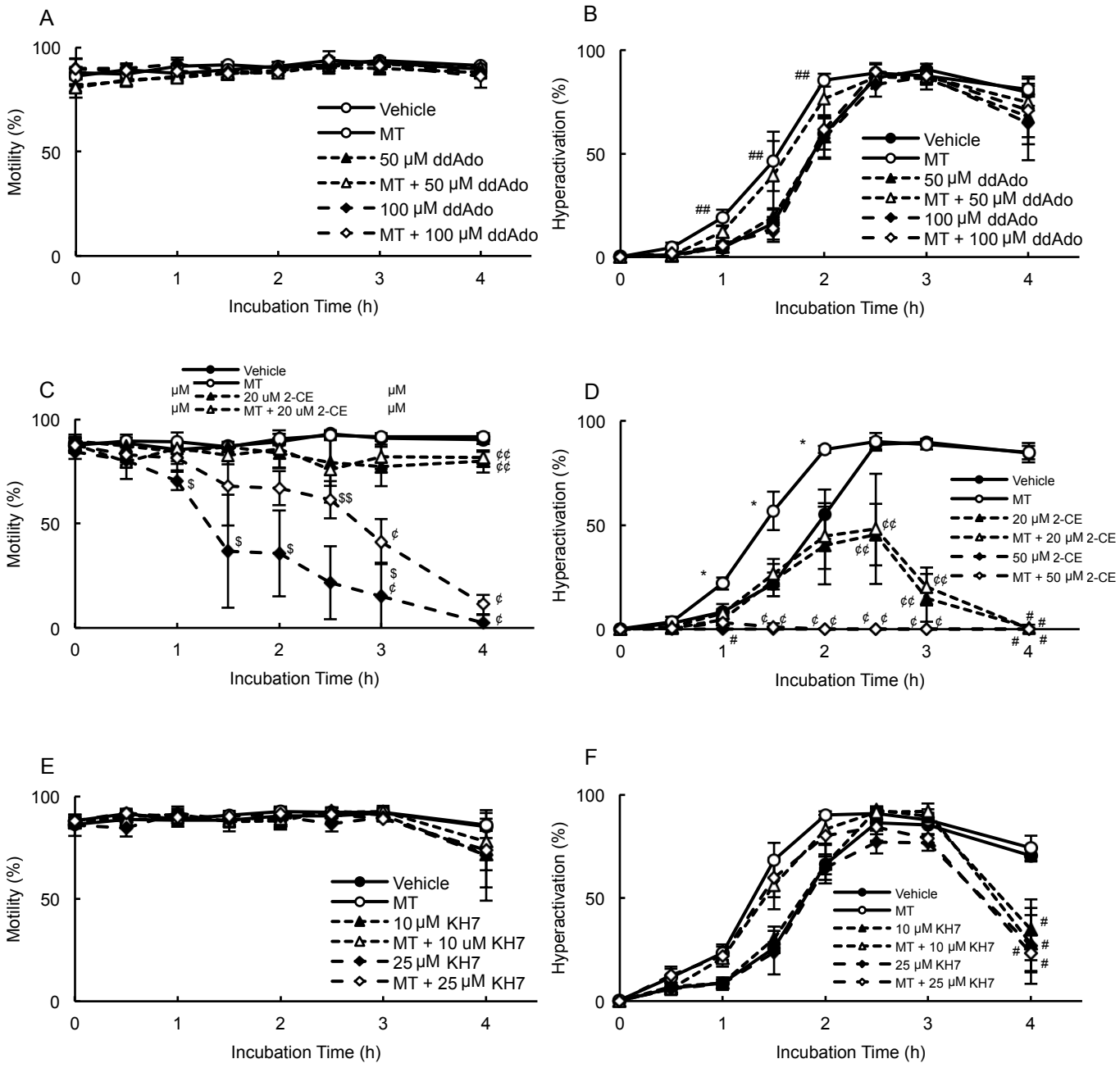

A
Motility (%)
Incubation Time (h)
Vehicle
MT
50 μM ddAdo
MT + 50 μM ddAdo
100 μM ddAdo
MT + 100 μM ddAdo
B
Hyperactivation (%)
Incubation Time (h)
C
Motility (%)
Incubation Time (h)
20 uM 2-CE
MT + 20 uM 2-CE
D
Hyperactivation (%)
Incubation Time (h)
20 μM 2-CE
MT + 20 μM 2-CE
50 μM 2-CE
MT + 50 μM 2-CE
E
Motility (%)
Incubation Time (h)
10 μM KH7
MT + 10 uM KH7
25 μM KH7
MT + 25 μM KH7
F
Hyperactivation (%)
Incubation Time (h)

Figure 4

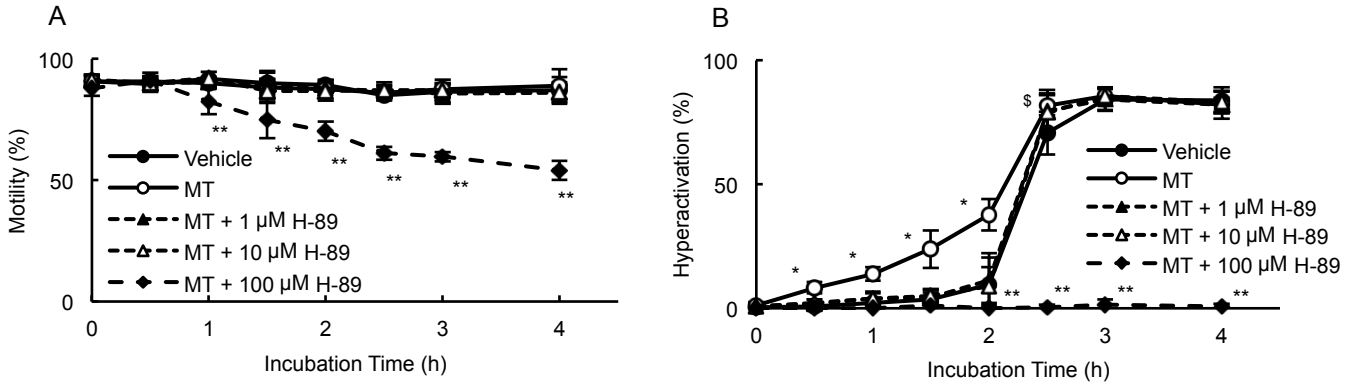

A
100
50
0
Motility (%)
0
1
2
3
4
Incubation Time (h)
Vehicle
MT
MT + 1 μM H-89
MT + 10 μM H-89
MT + 100 μM H-89
**
B
100
50
0
Hyperactivation (%)
0
1
2
3
4
Incubation Time (h)
$
*
**
Vehicle
MT
MT + 1 μM H-89
MT + 10 μM H-89
MT + 100 μM H-89

Figure 5

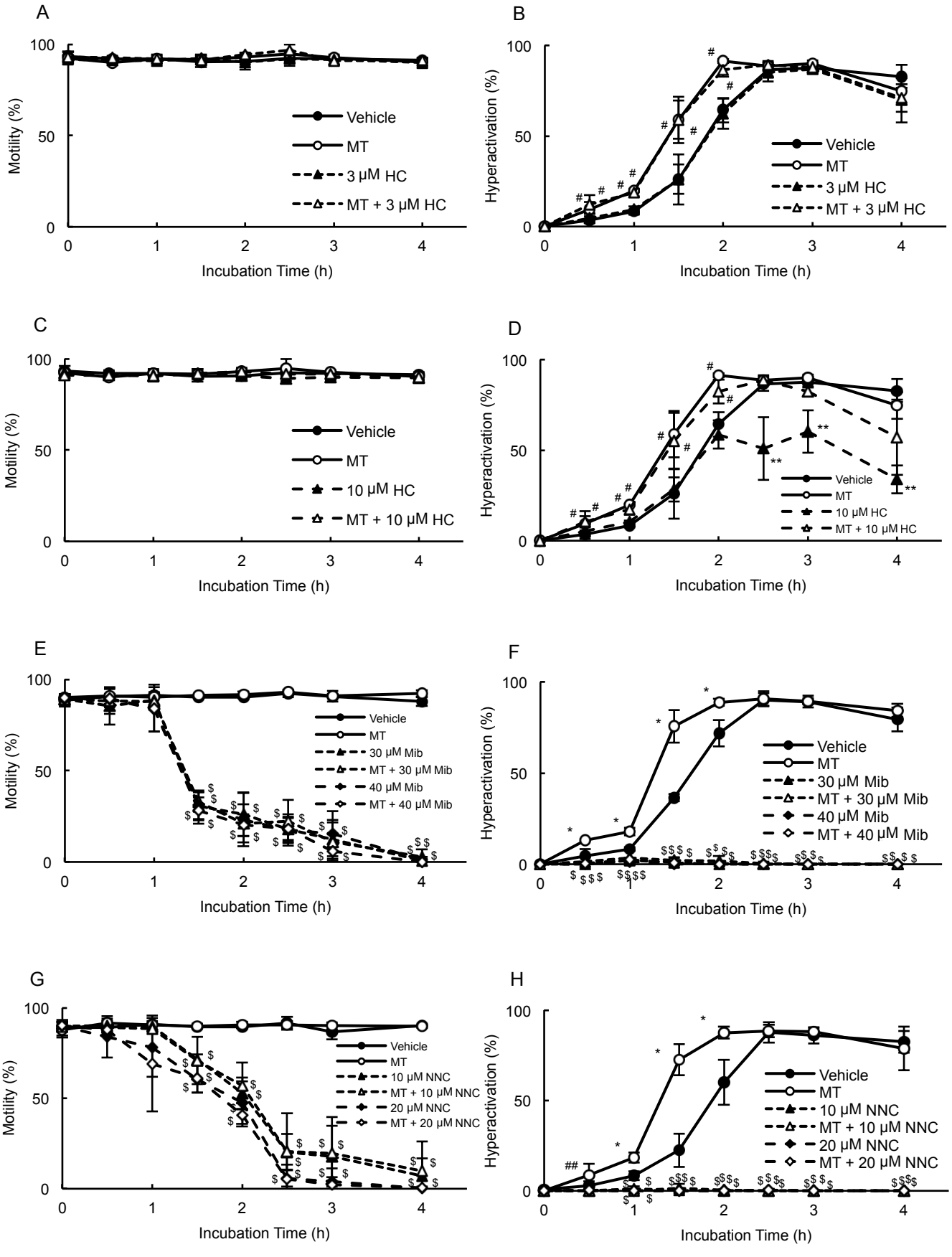
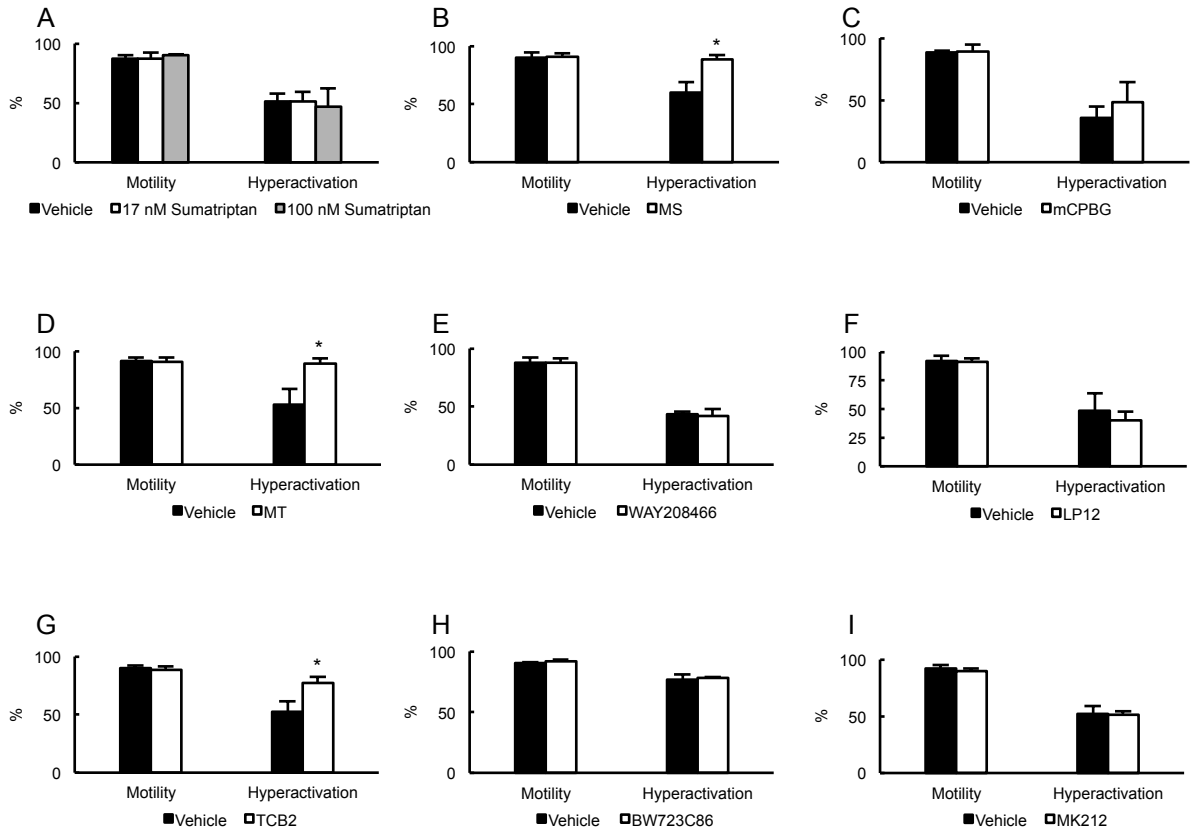
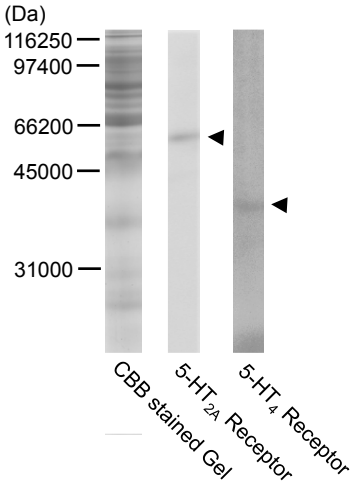

A
Motility (%)
Incubation Time (h)
Vehicle
MT
3 μM HC
MT + 3 μM HC
B
Hyperactivation (%)
Incubation Time (h)
Vehicle
MT
3 μM HC
MT + 3 μM HC
C
Motility (%)
Incubation Time (h)
Vehicle
MT
10 μM HC
MT + 10 μM HC
D
Hyperactivation (%)
Incubation Time (h)
Vehicle
MT
10 μM HC
MT + 10 μM HC
E
Motility (%)
Incubation Time (h)
Vehicle
MT
30 μM Mib
MT + 30 μM Mib
40 μM Mib
MT + 40 μM Mib
F
Hyperactivation (%)
Incubation Time (h)
Vehicle
MT
30 μM Mib
MT + 30 μM Mib
40 μM Mib
MT + 40 μM Mib
G
Motility (%)
Incubation Time (h)
Vehicle
MT
10 μM NNC
MT + 10 μM NNC
20 μM NNC
MT + 20 μM NNC
H
Hyperactivation (%)
Incubation Time (h)
Vehicle
MT
10 μM NNC
MT + 10 μM NNC
20 μM NNC
MT + 20 μM NNC

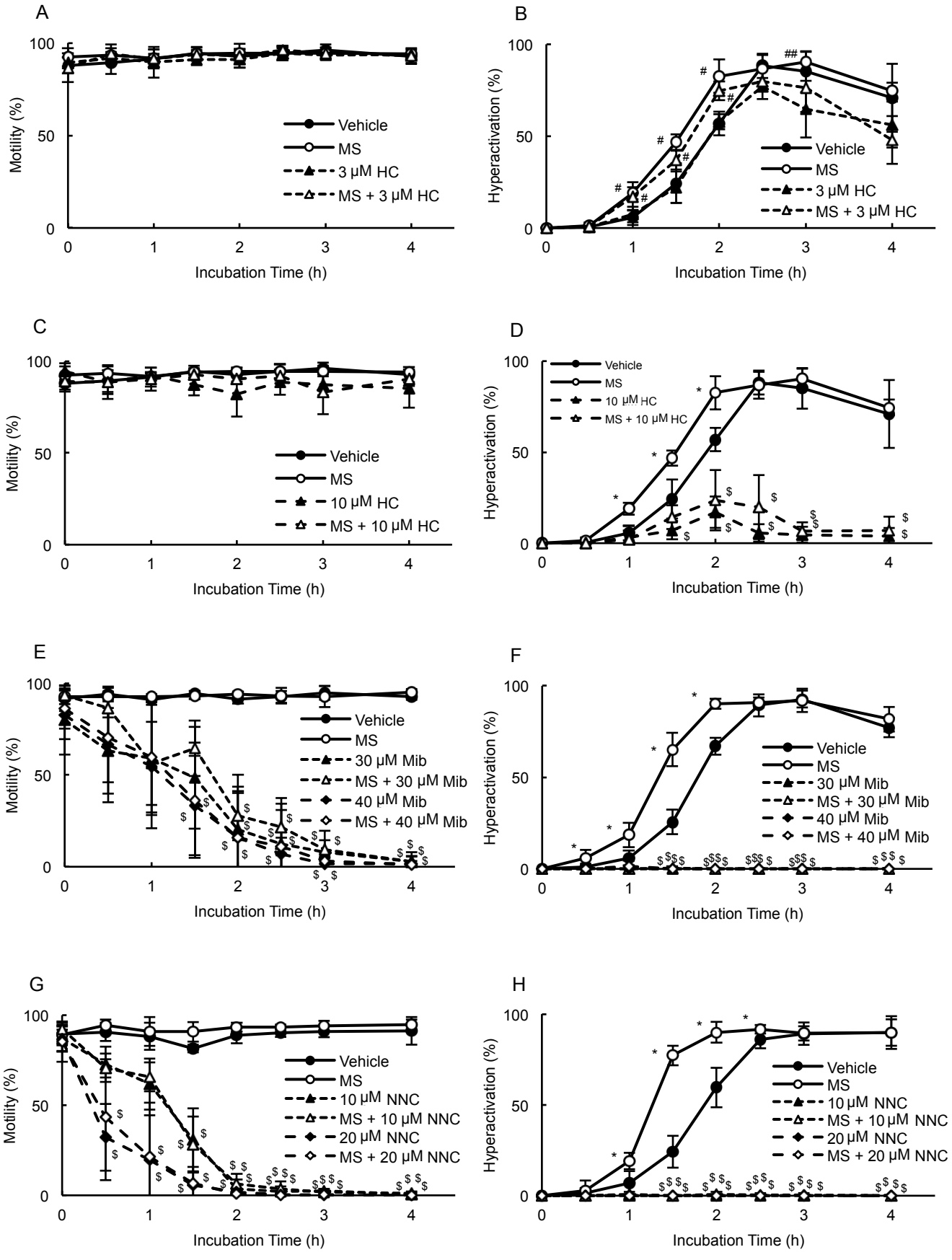
Figure 6



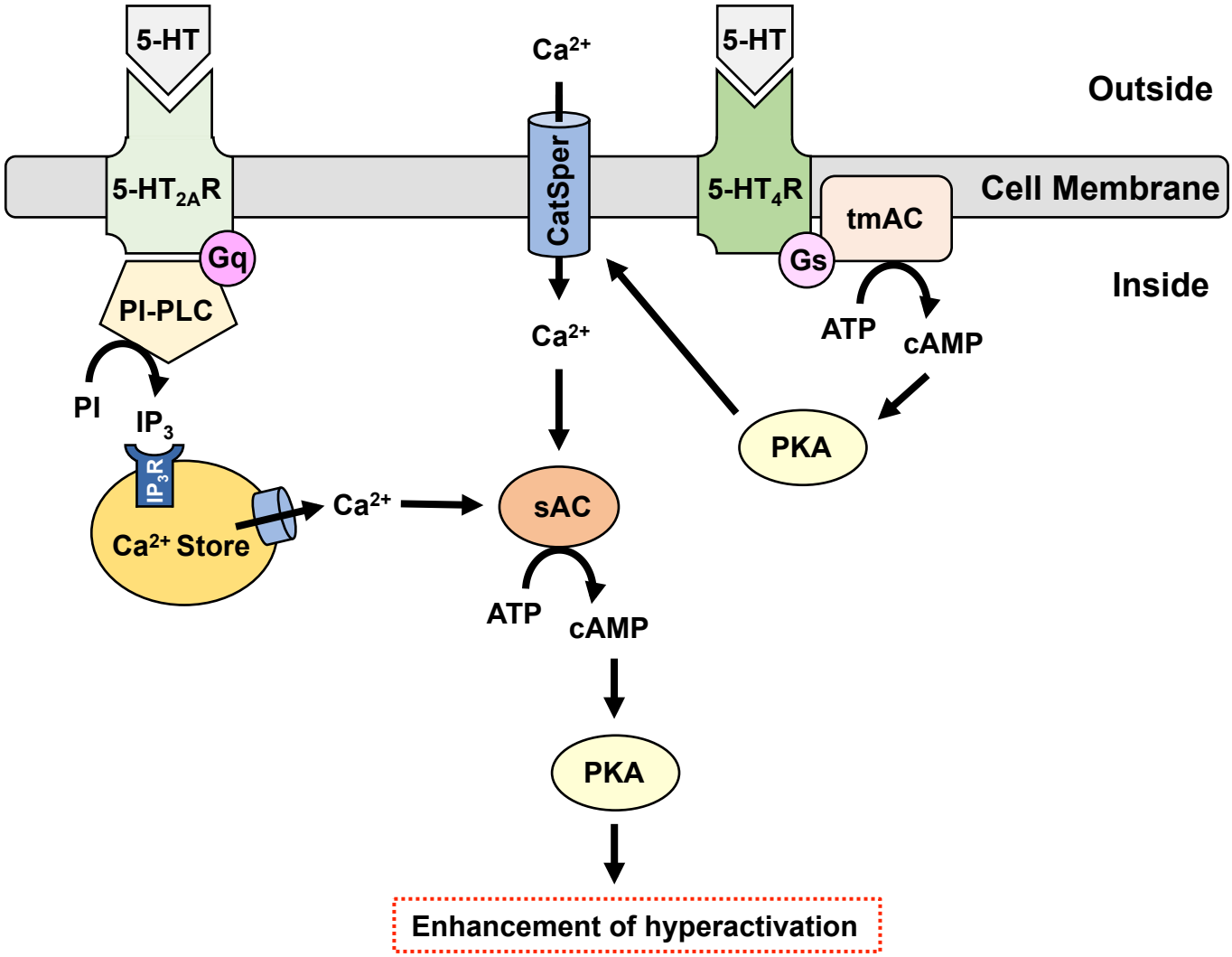
Supplementary Figure. 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

	5-HT$_1$ Receptor			5-HT$_2$ Receptor		5-HT$_3$ Receptor	
	Vehicle	17 nM Sumatriptan	100 nM Sumatriptan	Vehicle	MS	Vehicle	mCPBG
VSL (μm/sec)	110.73 ± 12.46	104.82 ± 16.56	90.28 ± 18.96	104.90 ± 18.57	83.45 ± 6.22*	115.85 ± 39.66	85.16 ± 17.29
VCL (μm/sec)	447.59 ± 104.29	400.38 ± 53.59	549.33 ± 94.50	498.89 ± 89.79	462.56 ± 44.30	512.13 ± 38.24	391.47 ± 62.71*
VAP (μm/sec)	163.11 ± 12.16	176.04 ± 6.14	178.58 ± 23.42	155.91 ± 15.98	132.44 ± 13.65*	176.05 ± 24.89	135.47 ± 34.99
LIN	0.26 ± 0.09	0.30 ± 0.08	0.19 ± 0.08	0.23 ± 0.08	0.19 ± 0.03	0.24 ± 0.09	0.25 ± 0.07
STR	0.60 ± 0.07	0.62 ± 0.09	0.52 ± 0.15	0.67 ± 0.07	0.64 ± 0.07	0.65 ± 0.14	0.65 ± 0.03
ALH (μm)	11.50 ± 1.80	10.33 ± 0.75	11.11 ± 0.78	10.94 ± 1.55	10.03 ± 0.94	12.18 ± 0.76	9.39 ± 0.74*
BCF (Hz)	7.34 ± 3.15	9.91 ± 1.65	7.41 ± 2.62	7.08 ± 3.94	5.98 ± 1.61	7.01 ± 1.75	7.94 ± 1.22
WOB	0.39 ± 0.10	0.47 ± 0.06	0.35 ± 0.06	0.34 ± 0.09	0.31 ± 0.03	0.35 ± 0.07	0.38 ± 0.12

	5-HT$_4$ Receptor		5-HT$_6$ Receptor		5-HT$_7$ Receptor	
	Vehicle	MT	Vehicle	WAY208466	Vehicle	LP12
VSL (μm/sec)	98.06 ± 13.24	94.60 ± 14.92	114.66 ± 28.42	102.94 ± 26.26	80.91 ± 17.19	90.91 ± 26.39
VCL (μm/sec)	437.32 ± 17.68	373.94 ± 92.14	492.85 ± 142.35	464.14 ± 91.87	479.68 ± 170.69	585.77 ± 90.05
VAP (μm/sec)	143.07 ± 15.47	131.59 ± 22.68	164.97 ± 39.79	169.98 ± 39.95	140.03 ± 27.62	145.30 ± 38.68
LIN	0.24 ± 0.05	0.27 ± 0.07	000.26 ± 0.06	0.24 ± 0.05	0.19 ± 0.06	0.16 ± 0.03
STR	0.69 ± 0.06	0.72 ± 0.07	0.70 ± 0.06	0.62 ± 0.09	0.59 ± 0.03	0.64 ± 0.07
ALH (μm)	10.53 ± 0.68	9.49 ± 0.94*	11.21 ± 2.88	11.25 ± 2.06	10.22 ± 3.28	12.35 ± 1.80
BCF (Hz)	7.72 ± 3.00	9.45 ± 3.89	6.62 ± 1.77	6.74 ± 0.41	4.48 ± 1.18	3.49 ± 1.37
WOB	0.34 ± 0.07	0.37 ± 0.07	0.36 ± 0.07	0.38 ± 0.05	0.32 ± 0.08	0.25 ± 0.05

	5-HT$_{2A}$ Receptor		5-HT$_{2B}$ Receptor		5-HT$_{2C}$ Receptor	
	Vehicle	TCB2	Vehicle	BW723C86	Vehicle	MK212
VSL (μm/sec)	120.62 ± 14.38	80.47 ± 20.12*	128.74 ± 37.81	148.62 ± 34.69	121.64 ± 22.16	109.13 ± 21.69
VCL (μm/sec)	350.12 ± 34.71	276.68 ± 70.44	410.98 ± 112.59	421.54 ± 115.61	386.79 ± 68.93	344.52 ± 60.71
VAP (μm/sec)	179.49 ± 20.84	142.07 ± 32.29	215.71 ± 52.05	225.38 ± 52.92	215.23 ± 46.85	179.78 ± 19.41
LIN	0.36 ± 0.06	0.36 ± 0.07	0.32 ± 0.04	0.37 ± 0.07	0.32 ± 0.03	0.33 ± 0.06
STR	0.67 ± 0.08	0.62 ± 0.05	0.61 ± 0.12	0.67 ± 0.06	0.57 ± 0.07	0.63 ± 0.06
ALH (μm)	8.46 ± 1.03	7.79 ± 2.07	9.71 ± 1.97	8.91 ± 2.89	9.20 ± 0.73	8.92 ± 8.92
BCF (Hz)	7.83 ± 1.40	7.91 ± 1.58	8.12 ± 0.92	9.15 ± 1.67	7.98 ± 1.97	8.68 ± 0.98
WOB	0.53 ± 0.05	0.55 ± 0.05	0.54 ± 0.05	0.55 ± 0.07	0.56 ± 0.03	0.53 ± 0.07

Supplementary Table 1

Effects of 5-HT receptor agonists on motility assay of hamster sperm. Each value was indicated at 2 h culture when sperm were cultured for 4 h with 17 nM or 100 nM sumatriptan, 100 fM MS, 100 μM mCPBG, 10 pM MT, 7.3 nM WAY, 0.13 nM LP12, 0.75 nM TCB2, 2 μM BW723C86 and 0.3 nM MK212. Data represent the mean ± SD. (5-HT$_1$ Receptor) (Vehicle) the medium with 0.1% (v/v) pure water as vehicle; (respective concentrations of sumatriptan) the medium with indicated concentrations of sumatriptan and vehicle. (5-HT$_2$ Receptor) (Vehicle) same as above; (MS) the medium with 100 fM MS and vehicle. (5-HT$_3$ Receptor) (Vehicle) same as above; (mCPBG) the medium with 100 μM mCPBG and vehicle. (5-HT$_4$ Receptor) (Vehicle) medium with 0.1% (v/v) ethanol as vehicle; (MT) medium with 10 pM MT and vehicle. (5-HT$_6$ Receptor) (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (WAY) medium with 7.3 nM WAY and vehicle. (5-HT$_7$ Receptor) (Vehicle) same as above; (LP12) medium with 0.13 nM LP12 and vehicle. (5-HT$_{2A}$ Receptor) (Vehicle) same as above; (TCB2) medium with 0.75 nM TCB2 and vehicle. (5-HT$_{2B}$ Receptor) (Vehicle) medium with 0.1% (v/v) dimethyl sulfoxide as vehicle; (BW723C86) medium with 2 μM BW723C86 and vehicle. (5-HT$_{2C}$ Receptor) (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (MK212) medium with 0.3 nM MK212 and vehicle. * indicates significant differences compared with "Vehicle" ($P < 0.05$).