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Impact of lipid raft integrity on 5-HT₃ receptor function and its modulation by antidepressants

Running title: lipid rafts and serotonin type 3 receptor function

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Abstract

Because of the biochemical colocalization of the 5-HT₃ receptor and antidepressants within raft-like domains and their antagonistic effects at this ligand-gated ion channel, we investigated the impact of lipid raft integrity for 5-HT₃ receptor function and its modulation by antidepressants. Treatment with methyl- β -cyclodextrin (M β CD) markedly reduced membrane cholesterol levels and caused a more diffuse membrane distribution of the lipid raft marker protein flotillin-1 indicating lipid raft impairment. Both amplitude and charge of serotonin evoked cation currents were diminished following cholesterol depletion by either M β CD or simvastatin (Sim), whereas the functional antagonistic properties of the antidepressants desipramine (DMI) and fluoxetine (Fluox) at the 5-HT₃ receptor were retained. Although both the 5-HT₃ receptor and flotillin-1 were predominantly found in raft-like domains in Western blots following sucrose density gradient centrifugation, immunocytochemistry revealed only a coincidental degree of colocalization of these two proteins. These findings and the persistence of the antagonistic effects of DMI and Fluox against 5-HT₃ receptors after lipid raft impairment indicate that their modulatory effects are likely mediated through non-raft 5-HT₃ receptors, which are not sufficiently detected by means of sucrose density gradient centrifugation. In conclusion, lipid raft integrity appears to be important for 5-HT₃ receptor function in general, whereas it is not a prerequisite for the antagonistic properties of antidepressants such as DMI and Fluox at this ligand-gated ion channel.

Key words: lipid rafts, antidepressants, 5-HT₃ receptor, ligand-gated ion channel, cholesterol, immunocytochemistry

Introduction

There is increasing evidence that the membrane localization of distinct neurotransmitter receptors within lipid rafts can influence their function by affecting neurotransmitter binding, receptor trafficking and clustering (Allen, *et al* 2007; Tsui-Pierchala, *et al* 2002). Lipid rafts are specific microdomains within the cell membrane that are enriched in cholesterol and glycosphingolipids (Pike, *et a* 2004). They are supposed to be heterogeneous and highly dynamic structures that float within the membrane bilayer (Kusumi and Suzuki, 2005). Lipids and proteins can move in and out of these domains thereby forming distinct clusters which determine their biophysical properties. In a biochemical sense, lipid rafts are commonly characterized as insoluble by non-ionic detergents upon sucrose density gradient centrifugation at 4°C (Brown and Rose, 1992). However, an extraction of these microdomains without the use of detergents seems to more closely reflect the physiological situation (Smart, *et al* 1995; Song, *et al* 1996). Thus, different compositions and properties of lipid rafts have been described depending on the preparation procedure (Foster, *et al* 2003; Garner, *et al* 2008; Kim, *et al* 2004; Pike, 2008; Sprenger and Horrevoets, 2007).

Lipid rafts have also been suggested to play a role for the action of antidepressants. It has recently been shown that the G-protein subunit G α is more likely shifted to lipid rafts, where it is less likely to couple to adenylyl cyclase, in post mortem brain tissue of patients who had suffered from major depression (Donati, *et al* 2008). Chronic treatment with antidepressants may enhance G α signalling through preventing the accumulation of G α in lipid rafts (Donati and Rasenick, 2005). Moreover, a variety of antidepressants and antipsychotics accumulate in raft-like domains together with the serotonin type 3 (5-HT₃) receptor as detected by sucrose density gradient centrifugation (Eisensamer, *et al* 2005). Indeed, various classes of antidepressants (Breitinger, *et al* 2001; Eisensamer, *et al* 2003; Fan, 1994) and antipsychotics (Rammes, *et al* 2004) have been shown to act as non-competitive antagonists at this neurotransmitter receptor.

The 5-HT₃ receptor is a pentameric ligand-gated non-specific cation channel, which functions either as homomeric 5-HT_{3A} or as heteromeric 5-HT_{3AB} receptor (Hannon and Hoyer, 2008; van Hooft and Vijverberg, 2000). However, electrophysiological and immunohistochemical evidence

indicates that the majority of native 5-HT₃ receptor complexes do not contain the 5-HT_{3B} subunit (Hussy, *et al* 1994; Reeves and Lummis, 2006; for review see Fletcher and Barnes, 1998). Within the CNS, the 5-HT_{3A} receptor is highly expressed in the area postrema, the caudate nucleus, the hippocampus and the amygdala (Barnes and Sharp, 1999).

The membrane distribution of the 5-HT₃ receptor together with the localization of psychopharmacological drugs within the membrane may thus be crucial for receptor function and its modulation by psychopharmacological drugs. In the present study, we therefore investigated to what extent lipid raft integrity contributes to the functional properties of the 5-HT₃ receptor and to the functional antagonistic effects of antidepressants at this ligand-gated ion channel. Lipid raft impairment induced by the cholesterol depleting agents methyl- β -cyclodextrine (M β CD) or simvastatin (Sim) markedly affected 5-HT₃ receptor function. However, the modulation of 5-HT evoked cation currents at the 5-HT₃ receptor by the antidepressants desipramine (DMI) and fluoxetine (Fluox) was retained after cholesterol depletion.

Materials and Methods

Chemicals and drugs. Desipramine (DMI) and fluoxetine (Fluox) were obtained from Sigma-Aldrich (Deisenhofen, Germany). For stock solutions DMI and Fluox (10 mM) were dissolved in H₂O.

Cell cultures. N1E-115 mouse neuroblastoma cells naturally expressing the 5-HT_{3A} receptor were obtained from ATCC LGC Promochem, Wesel, Germany. HEK 293 cells stably expressing the human 5-HT_{3A} receptor were constructed as described previously (Lankiewicz, *et al* 1998). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Invitrogen), 1% sodium pyruvate (Invitrogen) and antibiotics (Invitrogen). Cells were reseeded onto fresh Petri dishes (Josef Peske, Aindlingen-Arnhofen, Germany) twice a week after treatment with 1% trypsin-EDTA (Invitrogen). Cells were cultured at 37°C, 5% CO₂ and 95% humidity.

Electrophysiological recordings. Cells were kept in DMEM without FCS for 12 h prior to the recordings. Serotonin (5-HT) induced inward Na⁺-currents were recorded from lifted cells in the whole-cell voltage-clamp configuration under visual control using an inverted microscope (Zeiss, Jena, Germany) as described previously (Rammes, *et al* 2004). N1E-115 cells were kept in a bath solution containing 162 mM NaCl, 5.3 mM KCl, 0.67 mM Na₂HPO₄, 0.22 mM KH₂PO₄, 15 mM HEPES and 5.6 mM glucose. The bath solution for HEK 293 cells stably expressing the human 5-HT_{3A} receptor consisted of 140 mM NaCl, 2.8 mM KCl, 10 mM HEPES and 5.6 mM glucose. Both solutions were adjusted to pH 7.2 with NaOH. Patch electrodes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a horizontal pipette puller (Zeitz-Instruments, Augsburg, Germany) to yield pipettes with a resistance of 3-6 MΩ. Pipettes were filled with a solution containing 130 mM CsCl, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, 2 mM K-ATP, 0.2 mM Tris GTP, 10 mM glucose, pH of 7.2, which was adjusted with CsOH. After the whole-cell configuration was established, the cells were lifted and 30 μM 5-HT for N1E-115 cells or 10 μM 5-HT for HEK

293 cells stably expressing the human 5-HT_{3A} receptor were applied using a fast superfusion device. The respective concentrations of 5-HT were applied since these were used for the determination of the IC₅₀ value for the inhibition of the 5-HT response by psychopharmacological drugs in our previous study, which was in the low micromolar range (Rammes, *et al* 2004). The respective concentrations of DMI and Fluox used to modulate 5-HT₃ receptor function during electrophysiological recordings were chosen according to the IC₅₀ values of our previous study (Eisensamer, *et al* 2003) and are achieved in the brain (Baumann, *et al* 1983; Karson, *et al* 1993). For control experiments a piezo translator-driven double-barreled application pipette was used to expose the lifted cell either to 5-HT-free or 5-HT-containing solution. A 2 s 5-HT pulse was delivered every 90 s. DMI and Fluox were diluted with bath solution to the desired concentration. Current signals were recorded at a holding potential of -50mV with an EPC-9 amplifier (Heka, Lamprecht, Germany) and were analysed using the Heka 8.5 PulseFit and IgorPro v. 5.04B (Wavemetrics, Lake Oswego, OR, USA) software on a Power Macintosh G3 computer. Only results from stable cells showing at least 50% recovery of responses to 5-HT following the removal of drugs entered the final analysis. To compensate for this effect the % antagonism at each concentration was based on both the control and the recovery current by assuming a linear time course for the rundown. The peak amplitude and charge evoked by 5-HT in the absence of cholesterol depleting compounds or antidepressants was set as 100%. Data were analyzed by the t-test for paired samples.

Sucrose density gradient centrifugation. Two subconfluent Petri dishes (10 cm in diameter) of cells were washed twice in ice-cold PBS (Invitrogen) before resuspension in 800 µl of high-salt HEPES buffer (20 mM HEPES, 5 mM EDTA, 1 M NaCl, pH 7.4) supplemented with a protease-inhibitor cocktail (Sigma-Aldrich). After homogenization and sonification, the suspension was diluted 1:1 with an 80% sucrose stock solution prepared in high-salt HEPES buffer to a final concentration of 40% sucrose. This solution was put to the bottom of a centrifuge tube (Beckman Coulter, Fullerton, CA, USA) and overlaid with 1400 µl of 35% sucrose and finally with 1000 µl of 5% sucrose (both in low-salt HEPES buffer: 20 mM HEPES, 5 mM EDTA, 0.5 M NaCl, pH

7.4). The samples were ultracentrifuged at 40,000 rpm (164,326 x g on average) in a Beckman SW60 rotor for 4 h at 4°C. Thereafter, 10 fractions à 400 µl were taken from the top to the bottom of the gradient.

Western Blotting. Equal volumes of sucrose gradient fractions were adjusted 1:5 with sample buffer (Laemmli: 0.31 M TRIS, 25 mM EDTA, 0.5 M DTT, 10% SDS, 500 µl/ml glycerine, 0.33 mg/ml bromophenol blue, pH 6.8) and denaturated by heating 5 min at 95°C. SDS/PAGE was performed on 12% gels. After transfer to a nitrocellulose membrane (Whatman, Dassel, Germany) and blocking in TBS-T supplemented with 5% milk powder, blots were probed with specific antibodies to the following antigens: flotillin-1 (mouse monoclonal, BD Biosciences, Heidelberg, Germany, dilution 1:1000), caveolin (rabbit polyclonal, BD Biosciences, dilution 1:5000) and 5-HT₃ receptor (rabbit polyclonal, a generous gift from Günter Gisselmann, Lehrstuhl für Zellphysiologie, Ruhr-Universität Bochum, Germany, dilution 1:5000). After incubation with horseradish peroxidase-coupled secondary antibodies, the specific antibody binding was visualized by ECL chemiluminescence (Amersham Biosciences, Freiburg, Germany).

Immunocytochemistry. Cells were cultured on poly-L-lysine coated 4-chamber culture slides (BD Biosciences). Cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Afterwards, permeabilization was carried out with 0.2% Triton X-100 in PBS for 5 min. Blocking in PBS supplemented with 5% BSA for 2 h was followed by incubation with primary antibodies over night at 4°C (anti-5-HT₃ receptor rabbit polyclonal, Calbiochem, Gibbstown, NJ, USA, dilution 1:25; flotillin-1 mouse monoclonal, BD Biosciences, dilution 1:100) in PBS supplemented with 5% BSA and 0.1% Triton X-100. Secondary antibody incubation (Cy3 anti-mouse, Dianova, Hamburg, Germany and biotin-SP-conjugated anti-rabbit, Dianova) in PBS supplemented with 5% BSA and 0.1% Triton X-100 was performed for 1 h at room temperature and followed by tertiary antibody treatment (avidin-conjugated Alexa Flour 488, Molecular Probes, Eugene, OR, USA) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Dianova) for one further hour at room temperature (in PBS supplemented with 5% BSA and 0.1% Triton X-

100). Culture slides were examined using confocal laser microscopy (Olympus FV 1000D, Hamburg, Germany). Quantitative colocalization data were analyzed by one-way ANOVA.

Cholesterol depletion. For cholesterol depletion cells were either treated with methyl- β -cyclodextrine (M β CD, Sigma-Aldrich) or simvastatin (Sim, Sigma-Aldrich). When M β CD was used, cells were incubated with 0.5 mM up to 7.5 mM for 12 h at 37°C in serum-free DMEM. In order to maintain cell viability and to retain the possibility to obtain functional data by whole-cell voltage-clamp recordings we chose a rather low concentration (0.5 mM) coupled with a prolonged application time (12 h), because serotonin evoked cation currents could not be recorded any more after treatment of cells with higher concentrations of M β CD. For Sim induced cholesterol depletion, cells were kept in serum-free DMEM for 12 h before Sim was added at a concentration of 0.5 μ M for 24 h at 37°C.

Preparation of cell membranes for cholesterol/protein measurements. For cholesterol or protein quantification, one Petri dish (10 cm in diameter) of cells was first washed twice in ice-cold PBS before resuspension in 300 μ l of high-salt HEPES buffer supplemented with protease-inhibitor cocktail prior to homogenization and sonification. The suspension was then centrifuged for 10 min at 1000 x g at 4°C. The supernatant was again centrifuged at 8000 x g for further 10 min at 4°C. This second supernatant was assayed for cholesterol and protein concentrations.

Cholesterol and protein assay. Cell cholesterol concentrations were determined colorimetrically with a commercial assay kit (Boehringer, Mannheim, Germany) according to the manufacturer's recommendations. Cell protein concentrations were determined by a modified method of Lowry, *et al* 1951. Data were analyzed using Student's t-test.

Results

Effects of cholesterol depletion on 5-HT₃ receptor function

Because withdrawal of cholesterol from cell membranes by means of methyl- β -cyclodextrine (M β CD) is a commonly used approach for lipid raft disruption, we investigated whether cholesterol depletion by M β CD affects 5-HT₃ receptor function using whole-cell voltage-clamp recordings. Treatment of N1E-115 cells kept in DMEM without fetal calf serum (FCS) with 0.5 mM M β CD for 12 h markedly reduced the peak amplitude and increased onset, desensitization and deactivation kinetics of serotonin evoked cation currents under cholesterol depleting conditions, whereas charge was less affected (**Figure 1a, Table 1**). 0.5 mM was the maximum dose of M β CD which still allowed whole-cell voltage-clamp recordings (data not shown).

In order to verify lipid raft impairment under these experimental conditions we quantified cholesterol levels in membranes of N1E-115 cells, which were reduced to 43.9% by treatment with M β CD (**Figure 2a**). Moreover, immunocytochemistry revealed a more diffuse distribution of the lipid raft marker protein flotillin-1 upon cholesterol depletion by M β CD (**Figure 2b**). The apparent increase in signal intensity of flotillin-1 following M β CD treatment in immunocytochemistry was not related to an altered expression of this protein as shown by Western blot analysis (**Figure 2c**). Apparently, treatment with M β CD under these experimental conditions affects both lipid raft integrity and 5-HT₃ receptor function.

When cells were kept in DMEM supplemented with 10% FCS, M β CD induced cholesterol depletion was prevented due to the presence of cholesterol in FCS (data not shown). Nevertheless, M β CD treatment reduced the amplitude, although to a smaller degree than under cholesterol depleting conditions, and, in contrast to cholesterol depleting conditions, accelerated receptor desensitization and deactivation (**Figure 1b, Table 1**).

As an alternative approach for cholesterol depletion we used the hydroxyl-methylglutaryl-coenzyme A reductase inhibitor simvastatin (Sim), which inhibits endogenous cholesterol

synthesis. Treatment with 0.5 μ M Sim for 24 h reduced serotonin evoked cation currents in a similar way as did cholesterol depletion by M β CD (**Table 1**).

Effects of cholesterol depletion on the antagonistic effects of antidepressants at the 5-HT₃ receptor

To address the question of whether impairment of lipid raft integrity also affects the modulation of the 5-HT₃ receptor by antidepressants we studied the impact of cholesterol depletion on the functional antagonistic effects of desipramine (DMI) and fluoxetine (Fluox) at this ligand-gated ion channel.

The tricyclic antidepressant DMI reduced the peak amplitude and charge of serotonin evoked cation currents and accelerated the decay in N1E-115 cells as reported previously (Eisensamer, *et al* 2003) (**Figure 3a, 3d, Table 2**). Although treatment with 0.5 mM M β CD for 12 h markedly diminished 5-HT₃ receptor currents, the DMI-induced inhibition and kinetic changes were still retained following cholesterol depletion by M β CD (**Figure 3b, 3d, Table 2**). Also after treatment with 0.5 μ M Sim for 24 h, DMI still reduced serotonin evoked cation currents and accelerated receptor kinetics in N1E-115 cells with similar potency as after incubation with M β CD (**Figure 3c, 3d, Table 2**). Moreover, the antagonistic effect of DMI on serotonin evoked cation currents was maintained following M β CD treatment also in HEK 293 cells stably expressing the human 5-HT_{3A} receptor (data not shown). Concerning other antidepressant compounds, comparable findings were obtained, when the selective serotonin reuptake inhibitor Fluox was applied. The antagonistic potency against the peak amplitude and charge and the Fluox-induced acceleration of receptor desensitization could be observed both in control experiments (**Figure 4a, 4c, Table 3**) and under conditions of M β CD-induced cholesterol depletion (**Figure 4b, 4c, Table 3**).

Differential distribution of the 5-HT₃ receptor and flotillin-1 in sucrose density gradients and in immunocytochemistry

In view of the persistent antagonistic effects of antidepressants at the 5-HT₃ receptor even under cholesterol depleted conditions we investigated to what extent cholesterol depletion leads to lipid

raft disruption. Although treatment with 0.5 mM M β CD for 12 h markedly reduced membrane cholesterol levels and affected flotillin-1 distribution in immunocytochemistry, flotillin-1 still remained in the low buoyant density fractions (LBD) of sucrose gradients under these experimental conditions (**Figure 5a**). A 15 fold higher concentration of M β CD (7.5 mM) was required to shift flotillin-1 to the high buoyant density fractions (HBD) (**Figure 5a**). The same results were obtained for the caveolar raft marker protein caveolin (**Figure 5b**). Also the 5-HT₃ receptor was predominantly detected in the LBD fractions as shown previously (Eisensamer, *et al* 2005) (**Figure 5c**). Whereas treatment with 0.5 mM M β CD did not change the membrane distribution pattern of the receptor, 7.5 mM M β CD shifted the 5-HT₃ receptor to the HBD fractions (**Figure 5c**). Thus, only concentrations of M β CD, which are far above those needed for significant cholesterol depletion and which do not allow whole-cell voltage-clamp recordings any more, induced lipid raft disruption, which can reliably be detected in sucrose density gradients.

The persistence of the antagonistic effects of DMI and Fluox at the 5-HT₃ receptor following cholesterol depletion might therefore be due either to incomplete lipid raft disruption or to the possibility that these antagonistic effects are conferred through 5-HT₃ receptors outside of raft-like domains. We therefore reinvestigated the membrane localization of the 5-HT₃ receptor and flotillin-1 by means of immunocytochemistry. Whereas our Western blot analysis of sucrose density gradients indicated a localization of both the 5-HT₃ receptor and flotillin-1 within the LBD fractions (**Figure 5**), immunocytochemistry revealed a rather low degree of colocalization of these two proteins (21.9%, **Figure 6**), which suggests that 5-HT₃ receptor localization within the membrane is not restricted to raft-like domains. These findings argue for the hypothesis that 5-HT₃ receptor-mediated neuronal activity and the allosteric modulation of 5-HT₃ receptor function by antidepressants are determined both by raft and non-raft 5-HT₃ receptors.

Discussion

Our study revealed that lipid raft integrity is important for physiological 5-HT₃ receptor function, whereas it is not a prerequisite for the antagonistic properties of the antidepressants DMI and Fluox at this ligand-gated ion channel.

An impact of lipid raft integrity for ligand-gated ion channel function has been shown for a variety of receptors (for review see Allen, *et al* 2007). Lipid rafts are important for the maintenance of synaptic morphology and density, whereas lipid raft disruption reduced e.g. the stability of lipid raft associated surface AMPA receptors of rat hippocampal neurons (Hering, *et al* 2003). Specifically, GABA_A (Sooksawate and Simmonds, 2001) and nicotinic acetylcholine receptor function (Barrantes, 2007) has been shown to be affected by the cholesterol content of the membrane. Cholesterol depletion resulted in a reduced ligand binding and G-protein coupling of the serotonin 1A (5-HT_{1A}) receptor in bovine hippocampal membranes (Pucadyil and Chattopadhyay, 2004). Moreover, currents of the vanilloid receptor subtype 1 of rat dorsal root ganglia were diminished after cholesterol depletion (Liu, *et al* 2006).

To impair lipid raft function we used the well established cholesterol depleting agent M β CD in N1E-115 mouse neuroblastoma cells and HEK 293 cells stably expressing the human 5-HT_{3A} receptor. Our experimental conditions still allowed the characterization of serotonin evoked cation currents through 5-HT₃ receptors, although the cholesterol content of cell membranes was reduced to less than 50%. The issue of “lipid raft disruption” by M β CD is controversially discussed, because no firm guidelines exist how to properly prove lipid raft disruption. Only partial removal of cholesterol from cell membranes can be achieved by M β CD under conditions that retain cell viability (Christian, *et al* 1997; Kilsdonk, *et al* 1995; Yancey, *et al* 1996; for review see London, 2005), which is in line with our results. It would therefore be preferable to refer to “lipid raft impairment” rather than to complete “lipid raft disruption”. However, the existence of different raft populations with a differential sensitivity to cholesterol depletion cannot be completely excluded.

To rule out potential direct pharmacological effects of M β CD on 5-HT evoked cation currents independent from cholesterol depletion N1E-115 cells were kept in medium supplemented with 10% FCS before and during M β CD treatment thereby preventing cholesterol depletion. The effects of M β CD on 5-HT evoked cation currents under these experimental conditions were far less pronounced and clearly distinguishable from those of cholesterol depletion.

As another approach to show lipid raft impairment under our experimental conditions we investigated the membrane distribution of the lipid raft marker protein flotillin-1 by means of immunocytochemistry. Treatment with M β CD caused a more diffuse membrane distribution of flotillin-1 along with an apparent signal enhancement of flotillin-1, which was not due to a higher expression of the protein but might reflect an increased availability of the flotillin-1 epitope for detection by the respective antibody. This phenomenon of increased immunoreactivity of lipid raft associated proteins following M β CD treatment has already been observed previously. For example, the enhancement of cannabinoid 1 (CB1) receptor immunoreactivity expressed in rat C6 glioma cells following M β CD treatment has also been suggested to be dependent on an increased accessibility of protein binding sites to specific antibodies (Bari, *et al* 2008). Thus, cholesterol depletion under these experimental conditions affects lipid raft integrity, although a complete lipid raft disruption remains questionable. We therefore titrated the concentration of M β CD necessary to shift flotillin-1 to the HBD fractions of sucrose density gradients and we found that an at least 15-fold higher concentration of M β CD was required, which does not allow whole-cell voltage-clamp recordings anymore, due to severe cell damage. These results suggest that impairment of lipid rafts by cholesterol depletion is not necessarily detected by sucrose density gradients under experimental conditions needed to perform functional investigations such as electrophysiological recordings.

With regard to 5-HT₃ receptor function, there was a significant reduction in peak amplitude and charge of serotonin evoked cation currents after depletion of cholesterol with 0.5 mM M β CD in spite of a prolongation of receptor desensitization and deactivation. Simvastatin, through its ability to inhibit the synthesis of endogenous cholesterol, is considered as an alternative approach

for lipid raft impairment (Ponce, *et al* 2008; Sjögren, *et al* 2006) and exerted similar effects on serotonin evoked cation currents. The pronounced reduction of the peak amplitude after cholesterol depletion by M β CD was indeed due to cholesterol depletion, since acute application of 0.5 mM M β CD did not affect the peak amplitude but prolonged the decay for deactivation kinetics thus increasing the charge (data not shown). These findings support the hypothesis that the function of the 5-HT₃ receptor largely depends on the cholesterol content of the membrane, even if there is no complete raft disruption under cholesterol depleted conditions. Nevertheless, lipid raft integrity appears to be important for physiological receptor function in view of the localization of this ligand-gated ion channel in raft-like domains in sucrose density gradients (Eisensamer, *et al* 2005).

Since certain antidepressants are enriched in LBD fractions of sucrose density gradients and their concentrations within LBD fractions are related to their antagonistic potency against 5-HT₃ receptors (Eisensamer, *et al* 2005), it might be hypothesized that lipid raft integrity is a major determinant for the allosteric modulation of the 5-HT₃ receptor channel by antidepressants. Unexpectedly, the antagonistic potency of antidepressants against serotonin evoked cation currents was retained following cholesterol depletion by either M β CD or Sim in spite of a pronounced overall reduction of serotonin evoked currents. This is further underlined by the observation that both DMI and Fluox, which are enriched in LBD fractions of sucrose density gradients (Eisensamer, *et al* 2005), accelerated receptor desensitization even under conditions of cholesterol depletion. These findings challenge the hypothesis that 5-HT₃ receptor integration within lipid rafts is an indispensable prerequisite for the non-competitive antagonistic effects of antidepressants or antipsychotics at the 5-HT₃ receptor (Eisensamer, *et al* 2003; Rammes, *et al* 2004) and that these effects are predominantly conferred through an interaction with 5-HT₃ receptors within raft-like domains.

In view of this apparent discrepancy we reinvestigated the colocalization of the 5-HT₃ receptor with the lipid raft marker protein flotillin-1 both by means of Western blots of sucrose density gradient fractions and by immunocytochemistry. We could replicate our previous findings that

both the 5-HT₃ receptor protein and flotillin-1 are predominantly found in the LBD fractions of sucrose density gradients (Eisensamer, *et al* 2005), which is compatible with a localization within raft-like domains. Treatment with high concentrations of M β CD shifted both the 5-HT₃ receptor protein and flotillin-1 to the HBD fractions, which was not observed with 0.5 mM M β CD in spite of an effect of this concentration on membrane cholesterol content and redistribution of flotillin-1 in immunocytochemistry. In contrast to the findings obtained with sucrose density gradients, immunocytochemistry revealed only a coincidental degree of colocalization of the 5-HT₃ receptor and flotillin-1 in the absence of cholesterol depleting agents, which suggests that only a minor proportion of 5-HT₃ receptors is constantly present in lipid rafts.

Because lipid rafts are believed to be highly dynamic structures, the determination of whether a distinct protein is raft associated or not is a technical challenge, which largely relies on the respective technique employed. Several studies report a lower rate of colocalization of “raft-candidate proteins” and even an underestimation of raft association by means of immunocytochemistry when compared with biochemical methods such as sucrose density gradient centrifugation (for review see Kusumi and Suzuki, 2005), which is in line with our results. For example, the membrane localization of glycosylphosphatidylinositol (GPI)-anchored proteins such as the folate receptor seems to be related to multimerization processes, which, in turn, were influenced by the chemical fixation procedure in immunocytochemistry (Mayor, *et al* 1994). As another example, also the sodium-taurocholate cotransporting polypeptide in mouse liver yielded divergent results in immunocytochemical colocalization with caveolin-1 and Western blots of sucrose density gradient fractions (Molina, *et al* 2008).

Possible explanations for these discrepancies between immunofluorescence colocalization studies and biochemical techniques are the liability of the respective results to subtle variations in experimental protocols and the observation that transient and dynamic interactions with lipid rafts are often missed (Kusumi and Suzuki, 2005). As such, detection of raft or non-raft association of proteins in a dynamic system may be preferentially detected by the respective method employed and, therefore, a combination of different methodological approaches as applied in our study gives

a more realistic picture than just a single method. Our observations that the functional antagonistic effects of antidepressants at the 5-HT₃ receptor are retained in spite of cholesterol depletion together with the results from immunocytochemistry argue against the hypothesis that receptor integration within lipid rafts is the major determinant for these modulatory effects.

In conclusion, 5-HT₃ receptors appear to be raft and non-raft associated within a dynamic system. This differential membrane localization may play a role for receptor function. However, lipid raft integrity is not a prerequisite for the allosteric modulation of this ligand-gated ion channel by antidepressants.

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Titles and legends to figures

Figure 1

Treatment with M β CD decreases serotonin evoked cation currents in N1E-115 cells.

(a) Effect of M β CD under cholesterol depleting conditions. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s. The left panel shows representative recordings of an untreated cell (control; without FCS) and of a cell pretreated with 0.5 mM M β CD for 12 h (M β CD). The left panel shows currents of a representative experiment, the right panel shows the same recording normalized to control.

(b) Effect of M β CD in the presence of FCS avoiding cholesterol depletion. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s. The left panel shows representative recordings of an untreated cell in the presence of FCS (+ FCS) and of a cell pretreated with 0.5 mM M β CD for 12 h (+ FCS + M β CD). Cells were kept in DMEM with FCS for 12 h both during control and during M β CD incubation prior to the recordings. The left panel shows currents of a representative experiment, the right panel shows the same recording normalized to the untreated cell + FCS.

Figure 2

Treatment with M β CD impairs lipid raft integrity in N1E-115 cells.

(a) Reduction of cholesterol content of membranes following M β CD treatment. One proportion of living cells remained untreated (control), the other proportion was treated with 0.5 mM M β CD for 12 h. Cells were then resuspended in high-salt HEPES buffer, homogenized and sonificated. After a two-step centrifugation, the second supernatant enriched with membranes was quantified for cholesterol and protein content. Results represent the mean \pm SEM of three independent experiments. The asterisk indicates a significant difference from control experiments ($P < 0.05$).

(b) Treatment with M β CD induces a more diffuse distribution of flotillin-1. After fixation and permeabilization cells were immunostained with an antibody against flotillin-1 (red spots), cell nuclei were stained with DAPI (blue). Immunofluorescence was detected by confocal microscopy

at a 60 fold magnification. Prior to immunocytochemistry one proportion of living cells was pretreated with 0.5 mM M β CD for 12 h, the other proportion remained untreated (control). Representative images of three independent experiments are shown.

(c) The more diffuse distribution of flotillin-1 in immunocytochemistry following M β CD treatment is not due to an altered expression of flotillin-1. Equal amounts of membrane homogenates were analyzed by SDS-PAGE. Replicate gels were immunoblotted with an antibody against flotillin-1. The blot shows a representative experiment of three independent experiments with untreated cells (control) and cells pretreated with 0.5 mM M β CD for 12 h.

Figure 3

The modulation of serotonin evoked currents by DMI is retained after M β CD or Sim treatment of N1E-115 cells.

(a) Antagonistic effect of DMI at the 5-HT₃ receptor. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s in the absence or presence of 1 μ M DMI. The upper bars indicate the presence of 5-HT, the lower bar indicates the presence of DMI. Representative recordings before and after application of DMI and subsequent washout are shown.

(b) Antagonistic effect of DMI after M β CD treatment. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s in the absence or presence of 1 μ M DMI. The upper bars indicate the presence of 5-HT, the lower bar indicates the presence of DMI. Representative recordings before and after application of DMI and subsequent washout are shown. Cells were treated with 0.5 mM M β CD for 12 h prior to the recordings.

(c) Antagonistic effect of DMI after Sim treatment. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s in the absence or presence of 1 μ M DMI. The upper bars indicate the presence of 5-HT, the lower bar indicates the presence of DMI. Representative recordings before and after application of DMI and subsequent washout are shown. Cells were treated with 0.5 μ M Sim for 24 h prior to the recordings.

(d) Peak amplitude and charge of controls (white bars) and after pretreatment with 0.5 mM M β CD for 12 h (black bars) or 0.5 μ M Sim for 24 h (grey bars) in the presence of DMI of six independent experiments in relation to values in the absence of DMI, which are set as 100%. Data are shown as mean \pm SEM % of control. DMI reduces peak amplitude and charge to a similar degree in all three experimental conditions.

Figure 4

The modulation of serotonin evoked currents by Fluox is retained after M β CD treatment of N1E-115 cells.

(a) Antagonistic effect of Fluox at the 5-HT₃ receptor. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s in the absence or presence of 3 μ M Fluox. The upper bars indicate the presence of 5-HT, the lower bar indicates the presence of Fluox. Representative recordings before and after application of Fluox and subsequent washout are shown.

(b) Antagonistic effect of Fluox after M β CD treatment. Cation currents were recorded in a whole-cell voltage-clamp configuration. 30 μ M 5-HT were applied for 2 s in the absence or presence of 3 μ M Fluox. The upper bars indicate the presence of 5-HT, the lower bar indicates the presence of Fluox. Representative recordings before and after application of Fluox and subsequent washout are shown. Cells were treated with 0.5 mM M β CD for 12 h prior to the recordings.

(c) Peak amplitude and charge of controls (white bars) and after pretreatment with 0.5 mM M β CD for 12 h (black bars) in the presence of Fluox of six independent experiments in relation to values in the absence of Fluox, which are set as 100%. Data are shown as mean \pm SEM % of control. Fluox reduces peak amplitude and charge to a similar degree in both experimental conditions.

Figure 5

Treatment with high concentrations of M β CD shifts flotillin-1, caveolin and the 5-HT₃ receptor to the high buoyant density fractions (HBD) in sucrose gradients of HEK 293 cells stably expressing

the human 5-HT_{3A} receptor. Living cells were treated with M β CD for 12 h at different concentrations (0.5 and 7.5 mM); one proportion of the cells remained untreated (control). Cells were then homogenized, sonificated in high-salt HEPES buffer and fractionated by sucrose gradient density centrifugation. Thereafter, ten fractions were collected from the top to the bottom of the gradient and analyzed by SDS-PAGE. Replicate gels were immunoblotted with antibodies against flotillin-1 **(a)**, caveolin **(b)** and the 5-HT₃ receptor **(c)**. The blots shown are representative gradients out of three independent experiments. LBD: low buoyant density fractions; HBD: high buoyant density fractions.

Figure 6

Low degree of colocalization of the 5-HT₃ receptor with the lipid raft marker protein flotillin-1 in N1E-115 cells using immunofluorescence.

(a) Cells were fixed, permeabilized and immunostained with an antibody against flotillin-1 (red spots) and against the 5-HT₃ receptor (green spots). Cell nuclei were stained in blue with DAPI. Immunofluorescence was recorded at a 60 fold magnification and a 6 fold zoom. Colocalization of flotillin-1 and the 5-HT₃ receptor is indicated by the superposition of red and green fluorescence in form of yellow spots (merge). Representative images out of 5 independent experiments are shown.

(b) Magnification of the merged images from the yellow box in **(a)** without DAPI. Intensity profiles along the blue lines of the red channel (flotillin-1) and the green channel (5-HT₃ receptor) show only a low degree of colocalization of flotillin-1 and the 5-HT₃ receptor (white arrow).

(c) The proportion of double labeled spots within one cell (flotillin-1 labeled (flotillin-1⁺) and 5-HT₃ receptor labeled (5-HT₃⁺) was quantified in relation to the total number of flotillin-1⁺ spots to assess the relative percentage of raft-associated 5-HT₃ receptors (raft). This was compared with the proportion of 5-HT₃ receptor⁺/flotillin-1 non-labeled (flotillin-1⁻) spots in relation to total 5-HT₃ receptor⁺ spots to quantify the relative percentage of non-raft-associated 5-HT₃ receptors (non-raft). The relative percentage of raft-5-HT₃ receptors was significantly lower (21.9 \pm 2.3%)

in comparison to that of non-raft-5-HT₃ receptors ($78.2 \pm 3.6\%$) as indicated by one-way ANOVA analysis of the results from 5 independent experiments ($F_{(1)} = 161.96$; *** $p \leq 0.001$). There was no difference in immunofluorescence patterns between cells kept in DMEM supplemented with 10% FCS compared to cells kept in serum-free DMEM for 12 h prior to immunocytochemistry (data not shown).

Titles and legends to tables

Table 1 Effect of M β CD on serotonin evoked cation currents of N1E-115 cells.

Time constants (τ) of onset (τ_{on}), desensitization (τ_{des}) and offset (τ_{off}), peak amplitudes and charges of 5-HT evoked cation currents of N1E-115 cells under the following conditions: control: cells were kept in DMEM without FCS for 12 h; +M β CD: cells were treated with M β CD 0.5 mM for 12 h in DMEM without FCS; +Sim: cells were treated with Sim 0.5 μ M for 24 h in DMEM without FCS; +FCS: cells were kept in DMEM supplemented with 10% FCS; +FCS+M β CD: cells were kept in DMEM supplemented with 10% FCS and were treated with M β CD 0.5 mM for 12 h. Results are shown as the mean \pm SEM of 10 independent experiments for control, +M β CD, +FCS, +FCS+M β CD, respectively with the exception of +Sim (n = 6).

Table 2 Effect of M β CD and Sim on the modulation of 5-HT₃ receptor kinetics by DMI.

Time constants (τ) of onset (τ_{on}), desensitization (τ_{des}) and offset (τ_{off}) of 5-HT evoked cation currents of N1E-115 cells under the following conditions: DMI: application of DMI, DMI+M β CD: application of DMI to cells pretreated with M β CD 0.5 mM, DMI+Sim: application of DMI to cells pretreated with Sim 0.5 μ M. Results are shown as mean \pm SEM % of control (see Fig. 3d) of 6 independent experiments.

Table 3 Effect of M β CD on the modulation of 5-HT₃ receptor kinetics by Fluox.

Time constants (τ) of onset (τ_{on}), desensitization (τ_{des}) and offset (τ_{off}) of 5-HT evoked cation currents of N1E-115 cells under the following conditions: Fluox: application of Fluox, Fluox+M β CD: application of Fluox to cells pretreated with M β CD 0.5 mM. Results are shown as mean \pm SEM % of control (see Fig. 4c) of 6 independent experiments.

Table 1 Effect of M β CD on serotonin evoked cation currents of N1E-115 cells.

	<i>control</i>	<i>+MβCD</i>	<i>+Sim</i>	<i>+FCS</i>	<i>+FCS + MβCD</i>
τ_{on} [ms]	26.6 \pm 4.4	80.7 \pm 17.5 (303.4%) [#]	26.0 \pm 3.8 (97.7%) [#]	19.8 \pm 1.7	21.8 \pm 2.4 (110.1%)*
τ_{des} [ms]	546.0 \pm 30.0	1429.4 \pm 196.4 (261.8%) [#]	1215.8 \pm 166.1 (222.7%) [#]	1337.8 \pm 216.1	876.1 \pm 92.5 (65.5%)*
τ_{off} [ms]	1938.0 \pm 65.3	4010.2 \pm 262.1 (206.9%) [#]	3480.2 \pm 281.2 (179.6%) [#]	3715.5 \pm 150.5	3091.9 \pm 172.6 (83.2%)*
<i>peak</i> [pA]	-714.7 \pm 60.1	-242.9 \pm 51.0 (34.0%) [#]	-363.1 \pm 111.3 (50.8%) [#]	-1845.3 \pm 127.4	-1262.5 \pm 116.7 (68.4%)*
<i>charge</i> [pC]	-1695 \pm 502.7	-1395 \pm 371.8 (82.3%) [#]	-1229 \pm 500.8 (72.5%) [#]	-8295 \pm 808.2	-3123 \pm 623.9 (37.6%)*

percent of control

* percent of +FCS

Table 2 Effect of M β CD and Sim on the modulation of 5-HT₃ receptor kinetics by DMI.

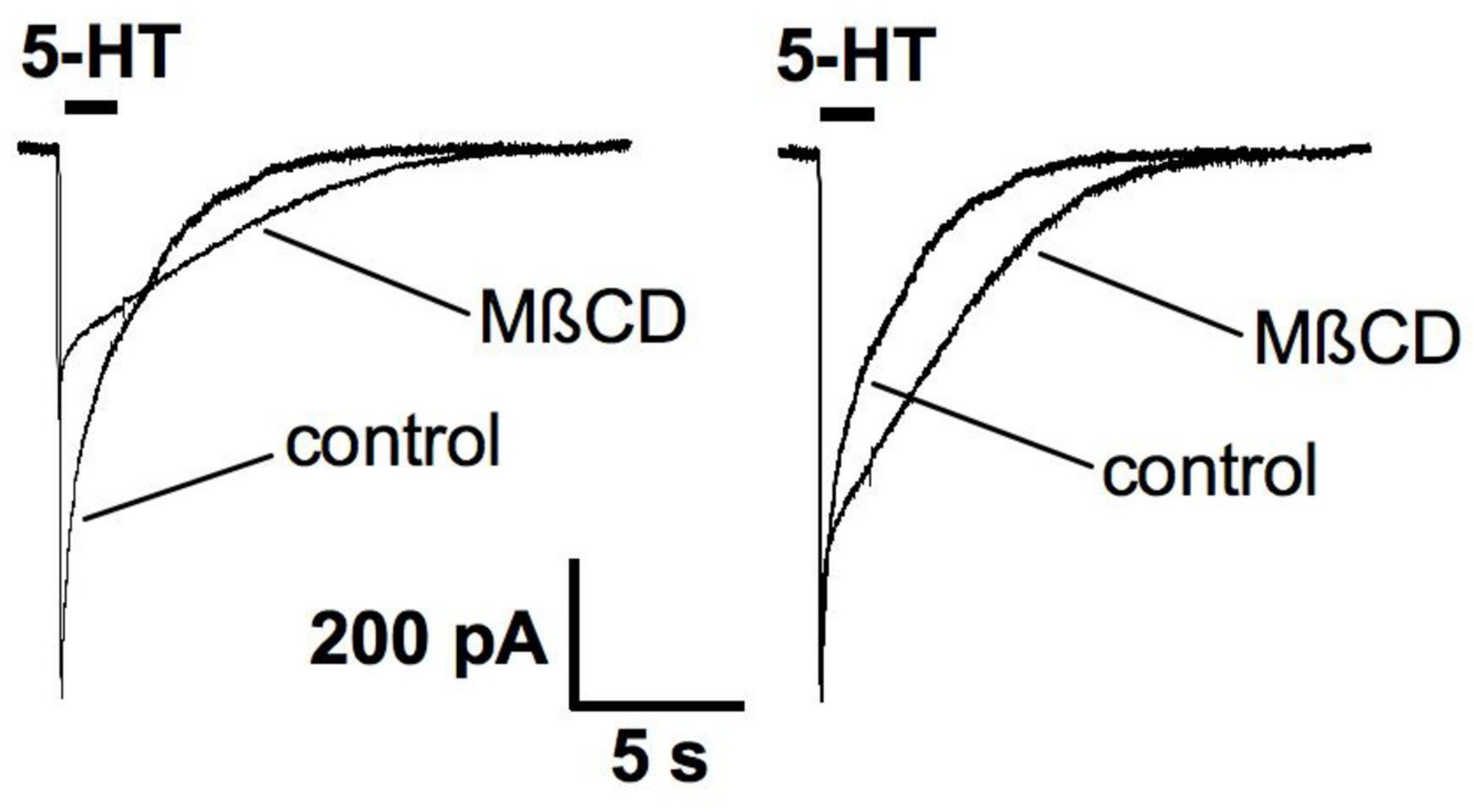
	<i>DMI</i>	<i>DMI</i> <i>+MβCD</i>	<i>DMI</i> <i>+Sim</i>
τ_{on} %	162.2 \pm 1.7	156.5 \pm 9.0	160.7 \pm 11.0
τ_{des} %	77.9 \pm 2.1	79.1 \pm 2.8	78.7 \pm 16.5
τ_{off} %	89.9 \pm 18.1	60.9 \pm 16.1	54.9 \pm 8.1

Table 3 Effect of M β CD on the modulation of 5-HT₃ receptor kinetics by Fluox.

	<i>Fluox</i>	<i>Fluox</i> <i>+MβCD</i>
τ_{on} %	98.1 \pm 5.1	100.6 \pm 3.9
τ_{des} %	96.1 \pm 12.9	84.6 \pm 1.3
τ_{off} %	47.3 \pm 1.2	54.0 \pm 4.2

Fig. 1

a



b

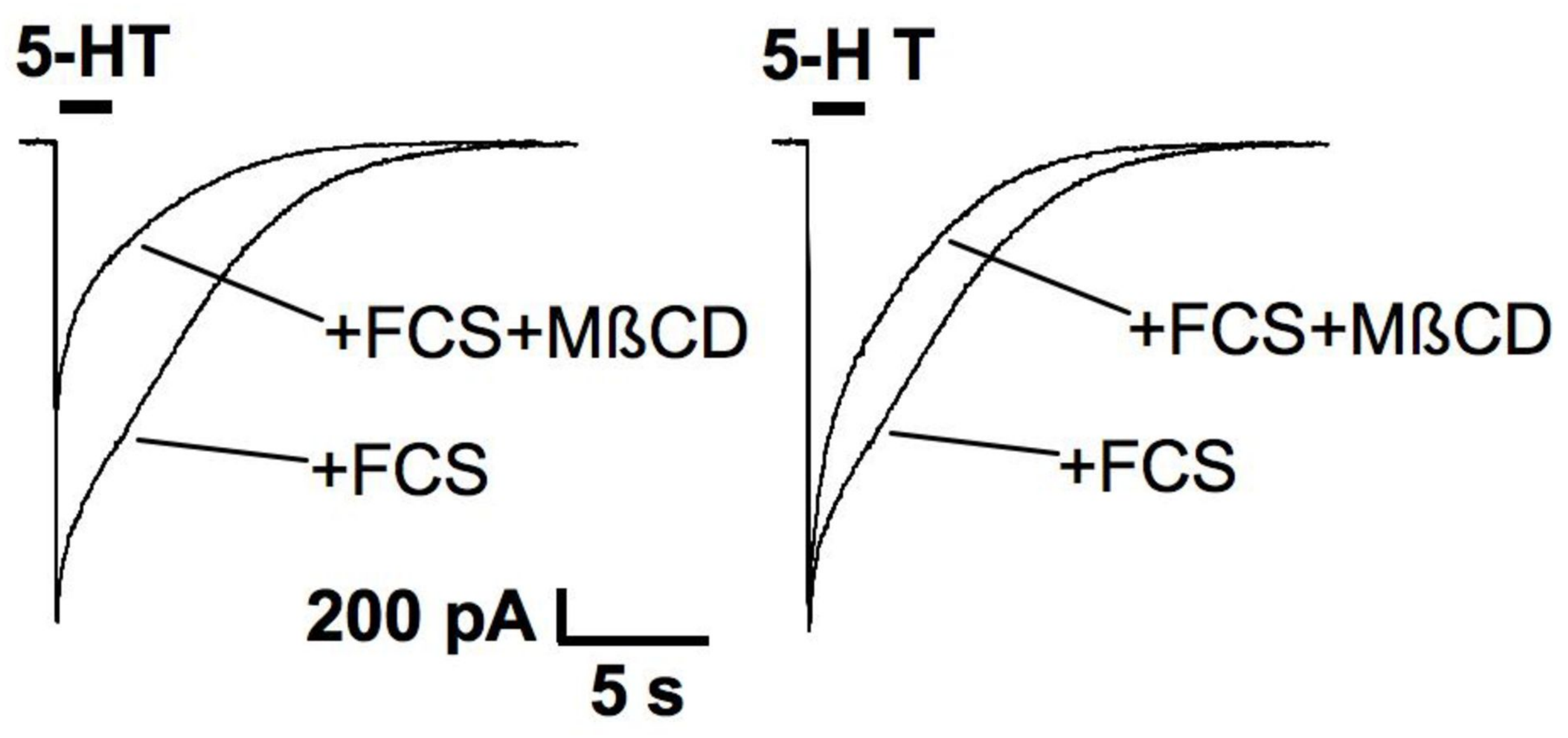
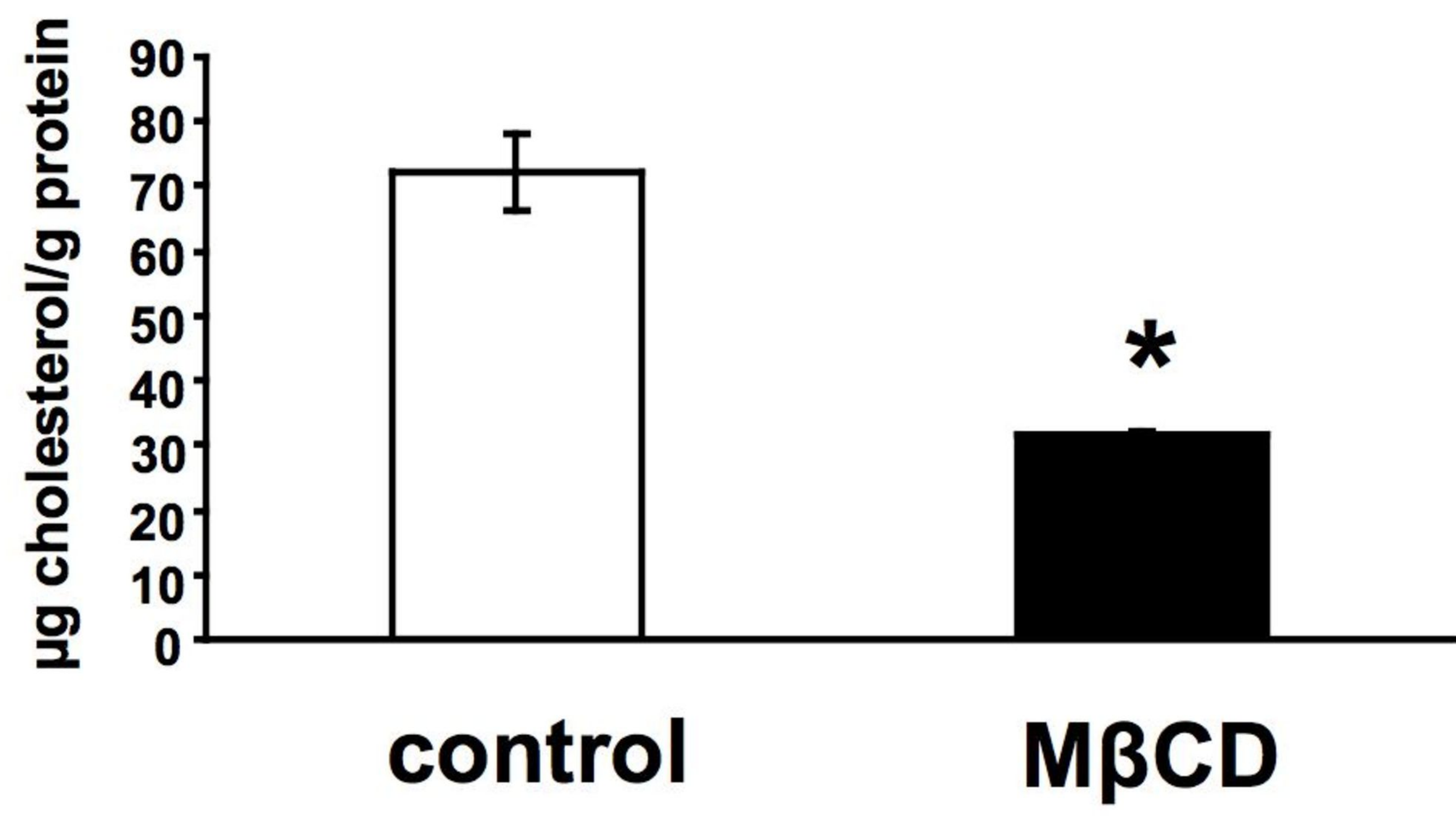
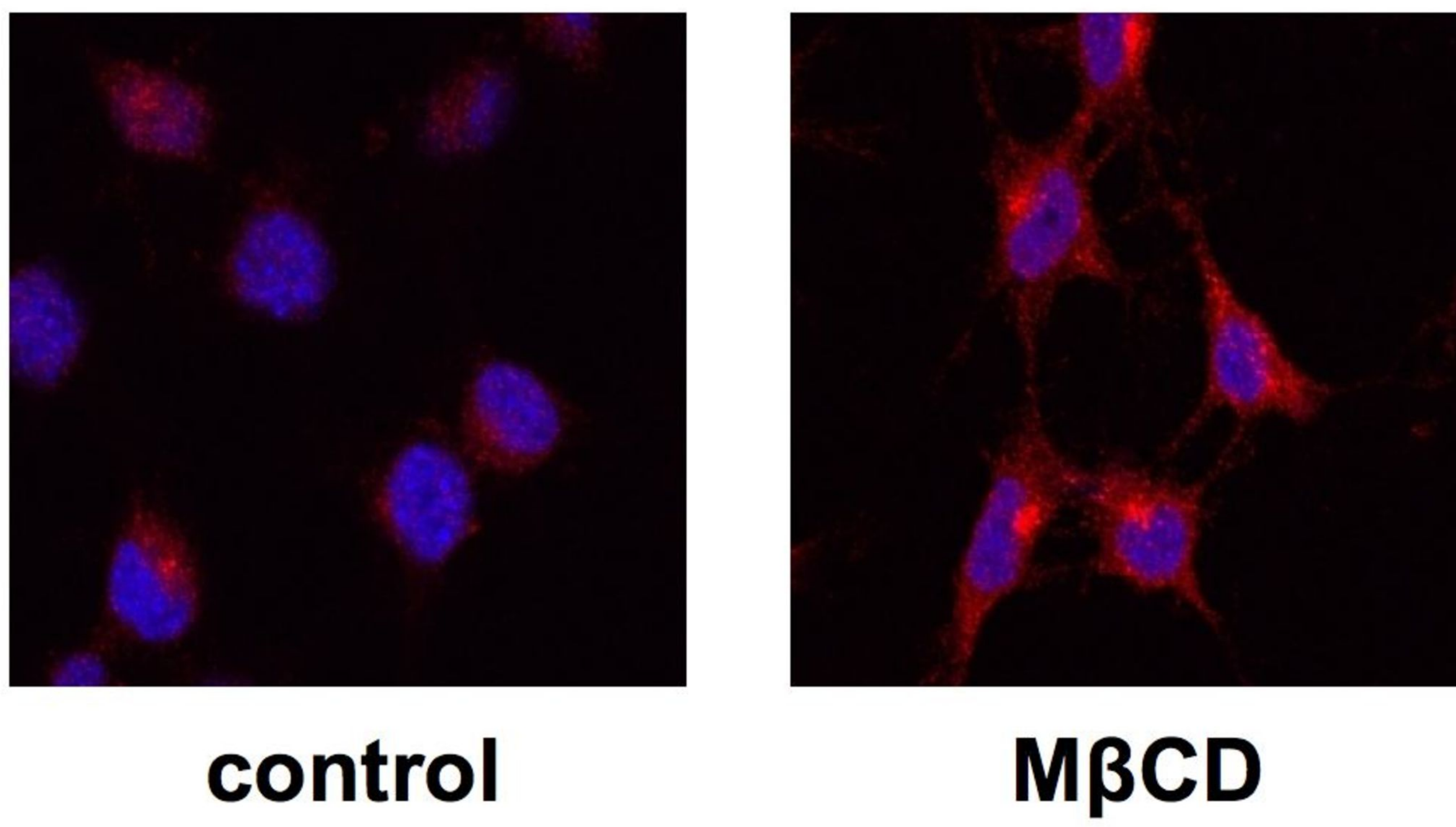


Figure 2

a



b

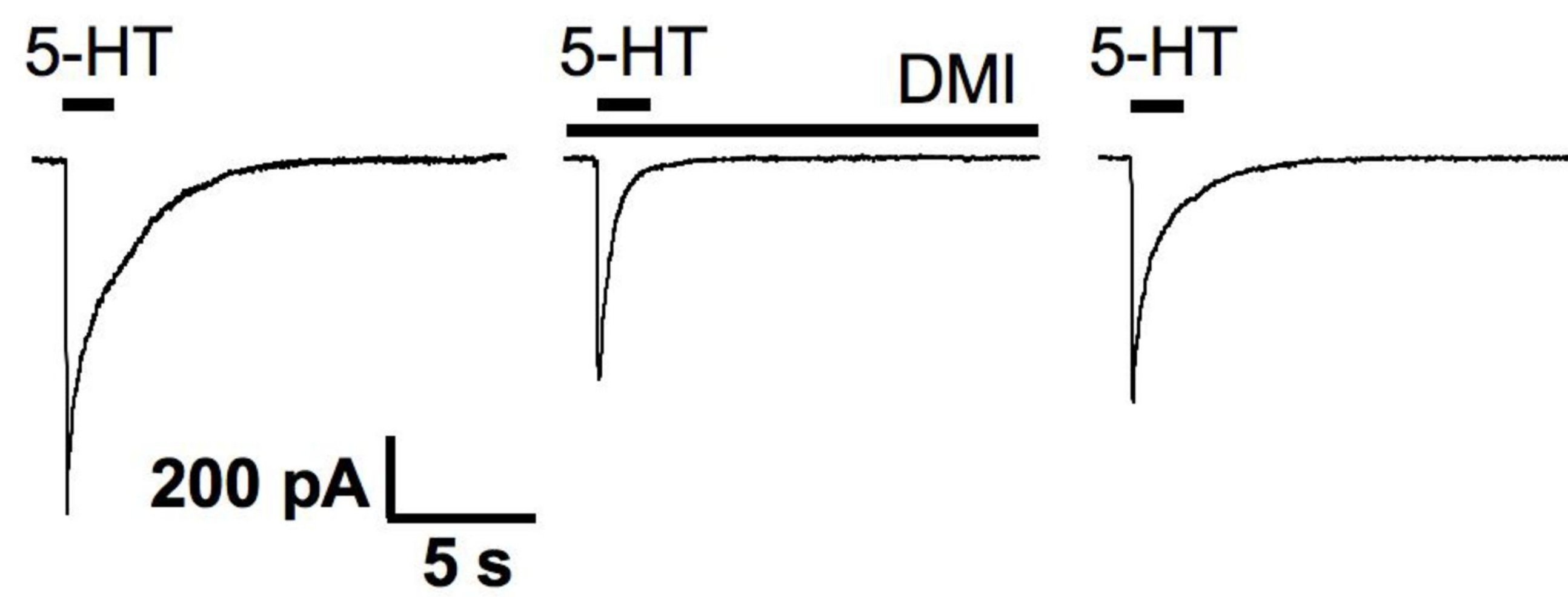


c

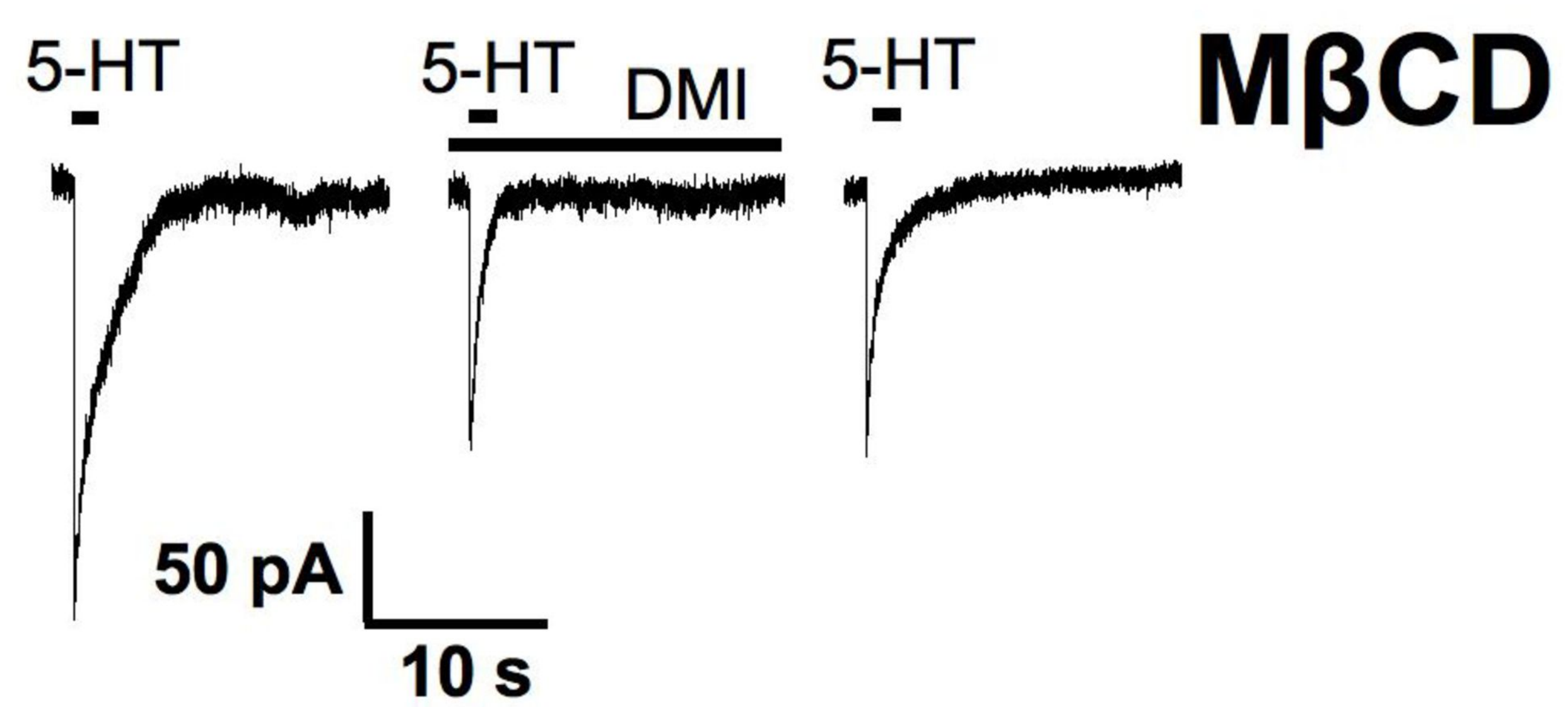


Fig. 3

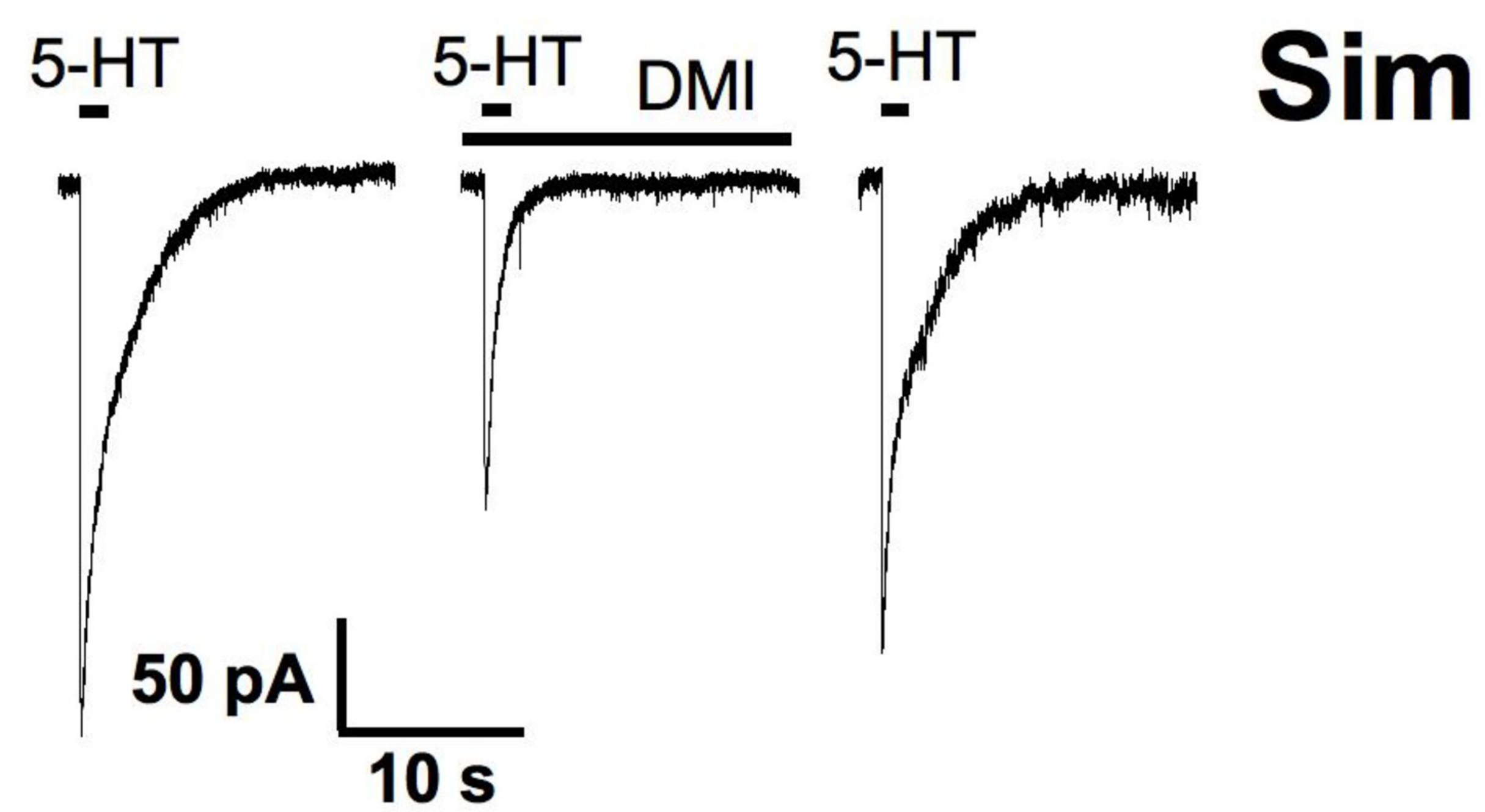
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b



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d

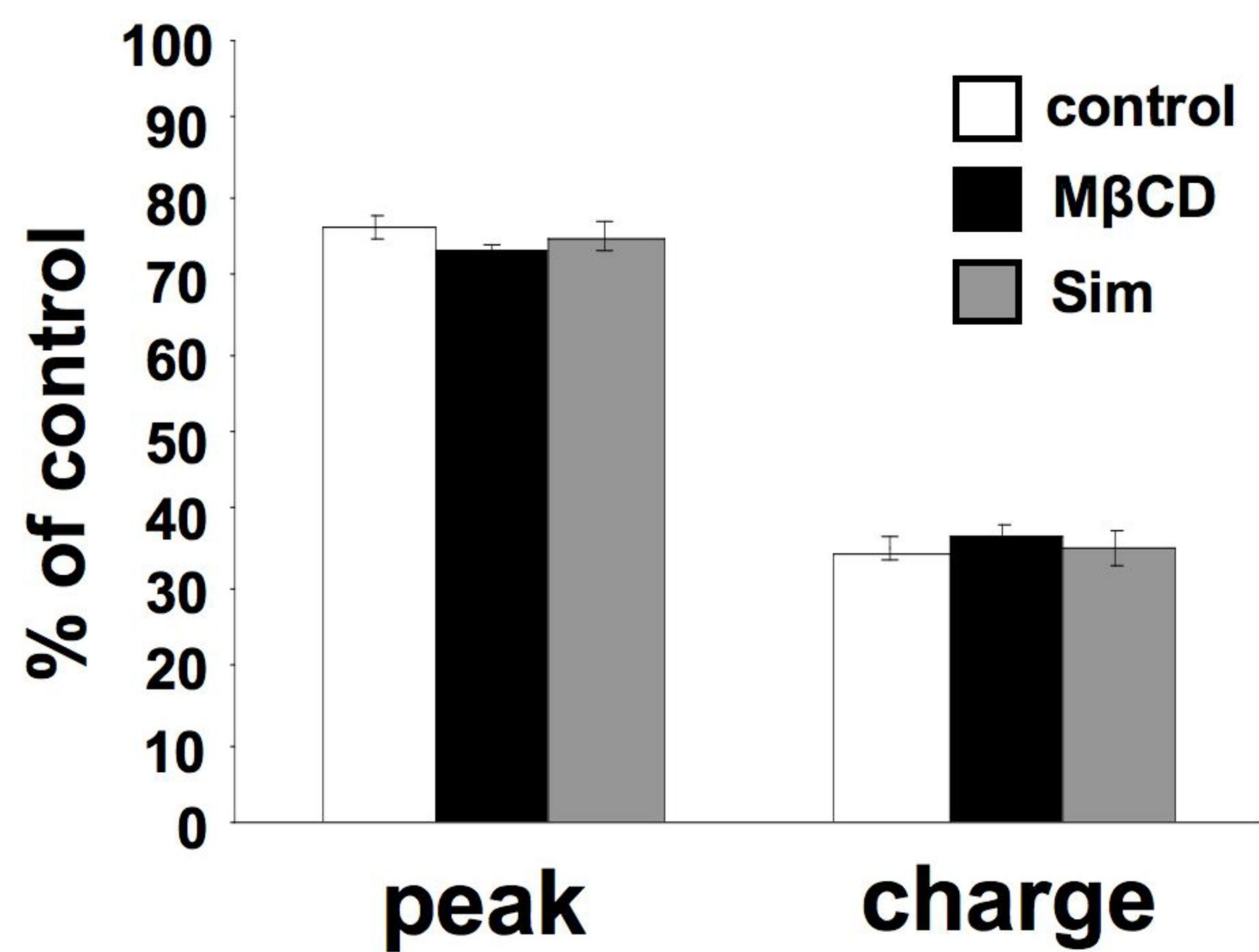
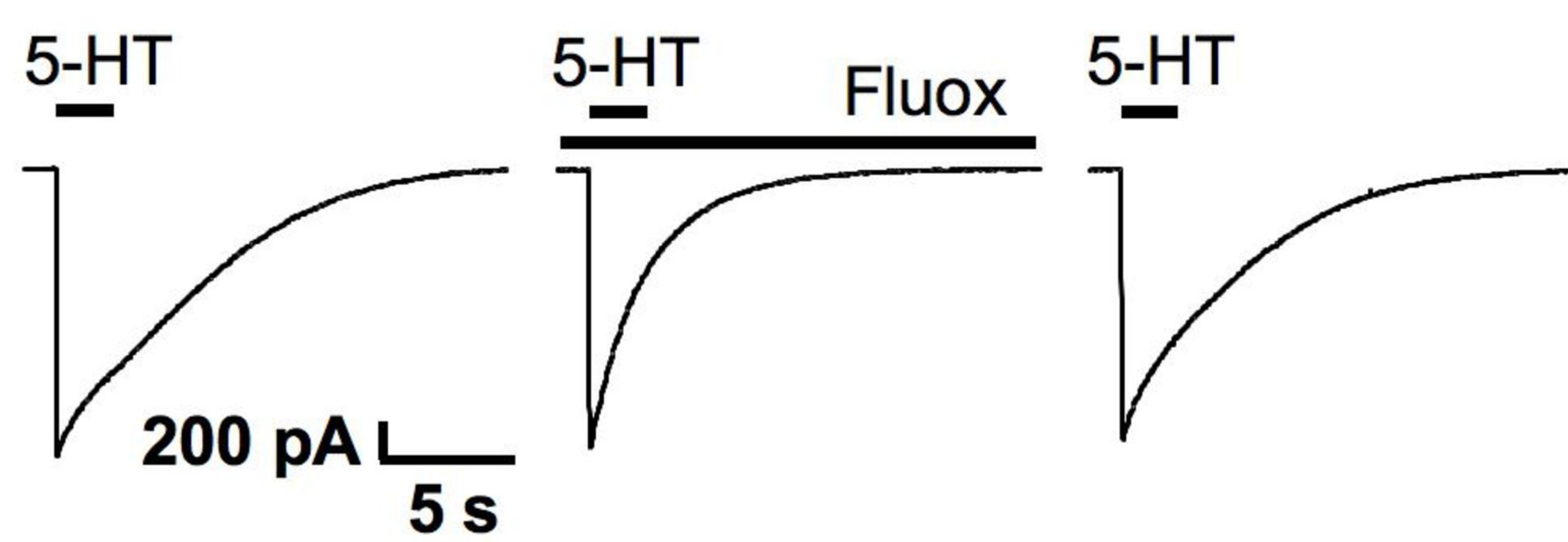
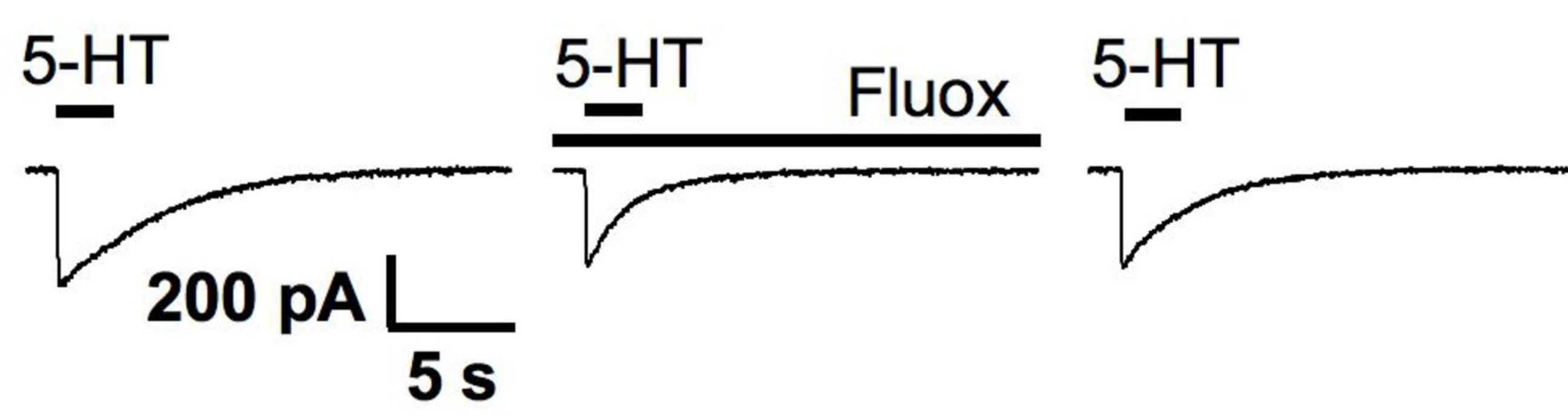


Fig. 4

a



b



c

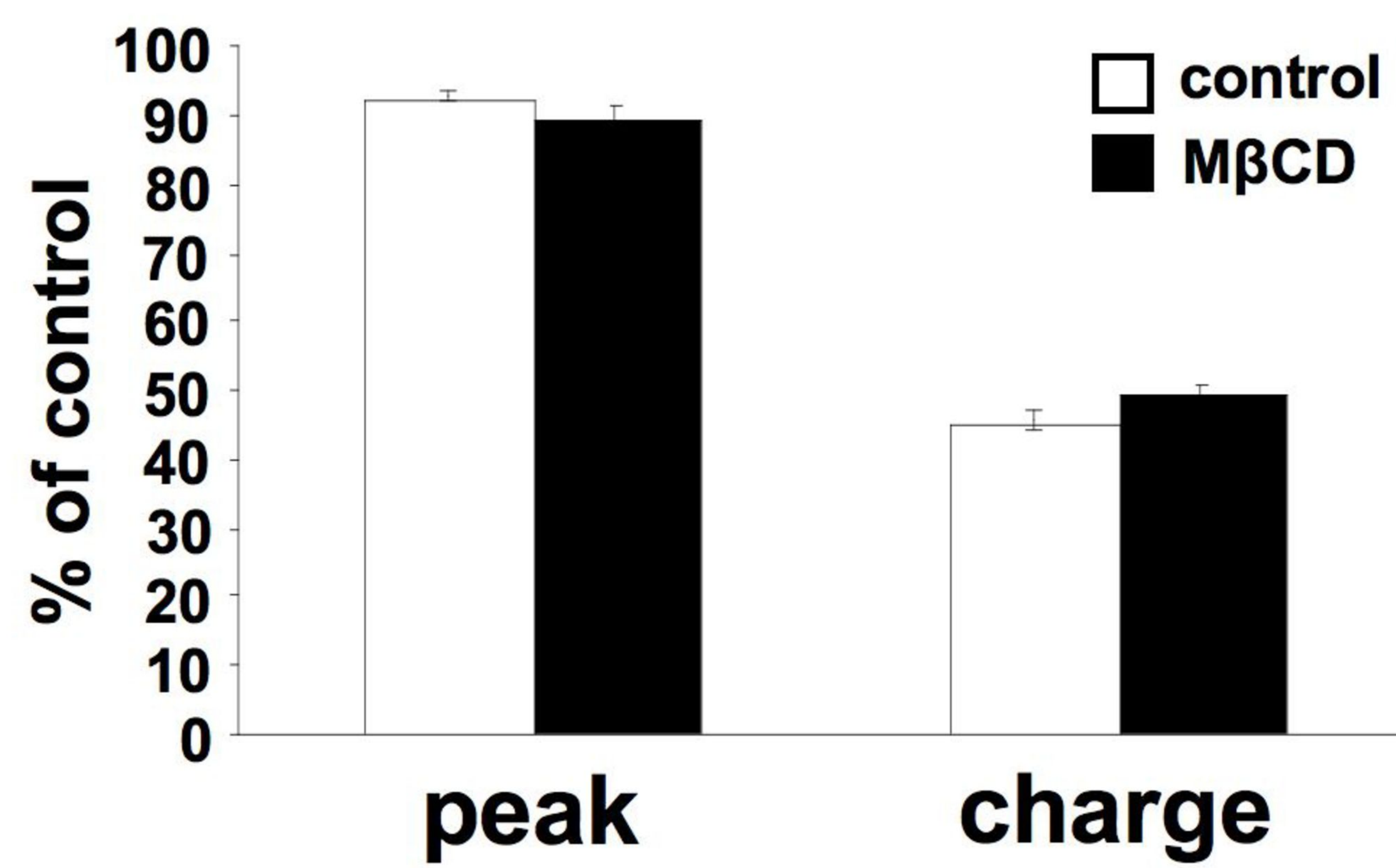
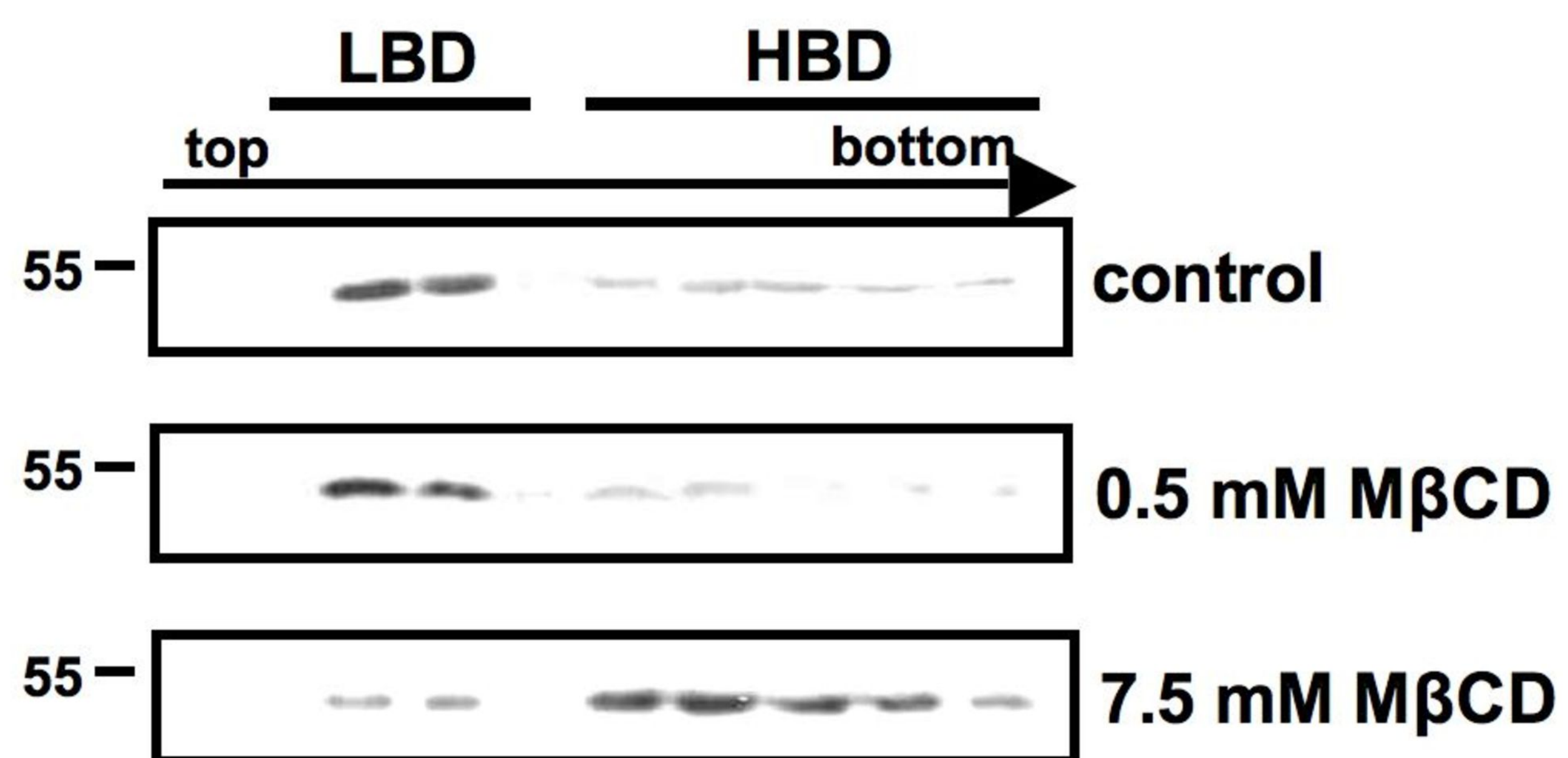


Figure 5

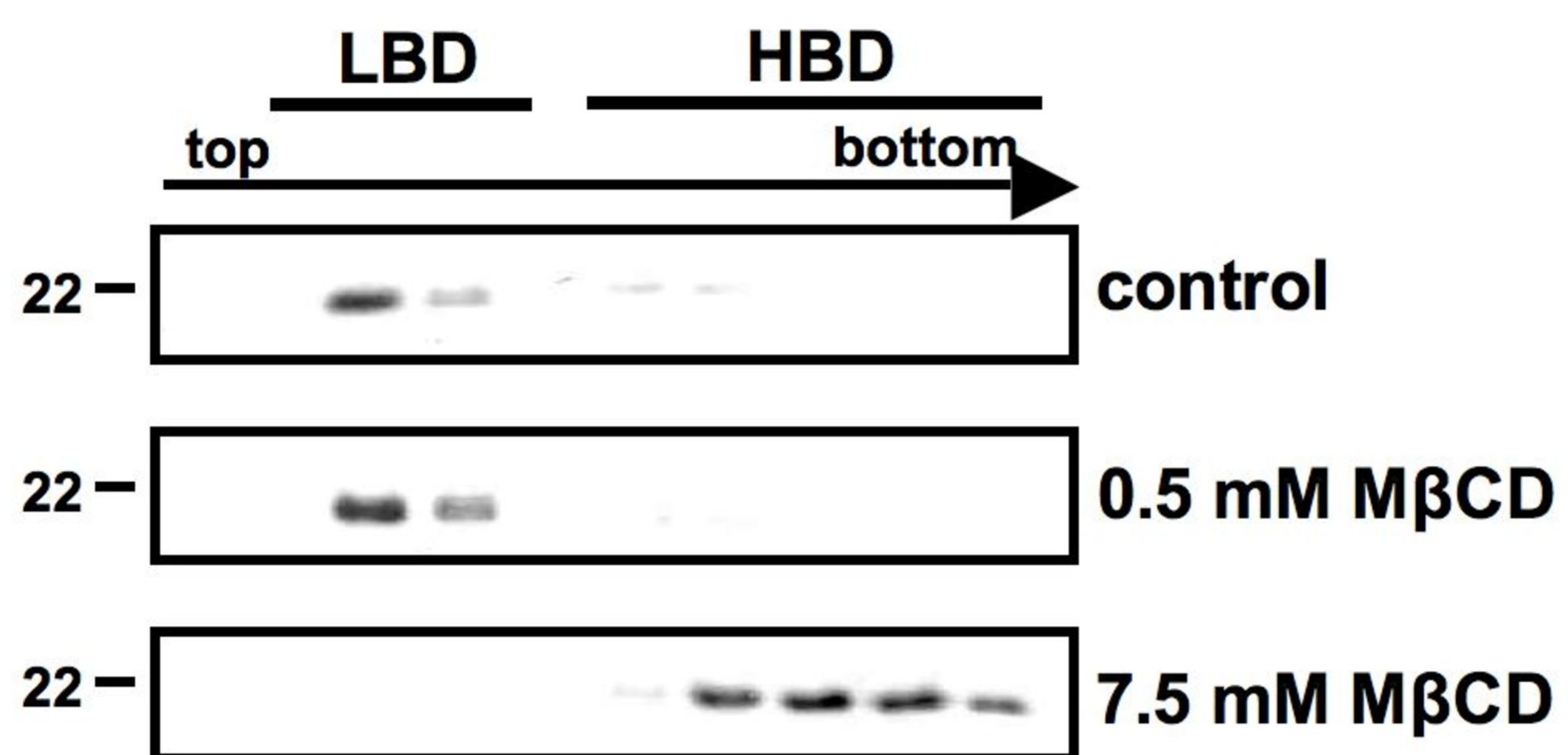
a

flotillin-1



b

caveolin



c

5-HT₃ receptor

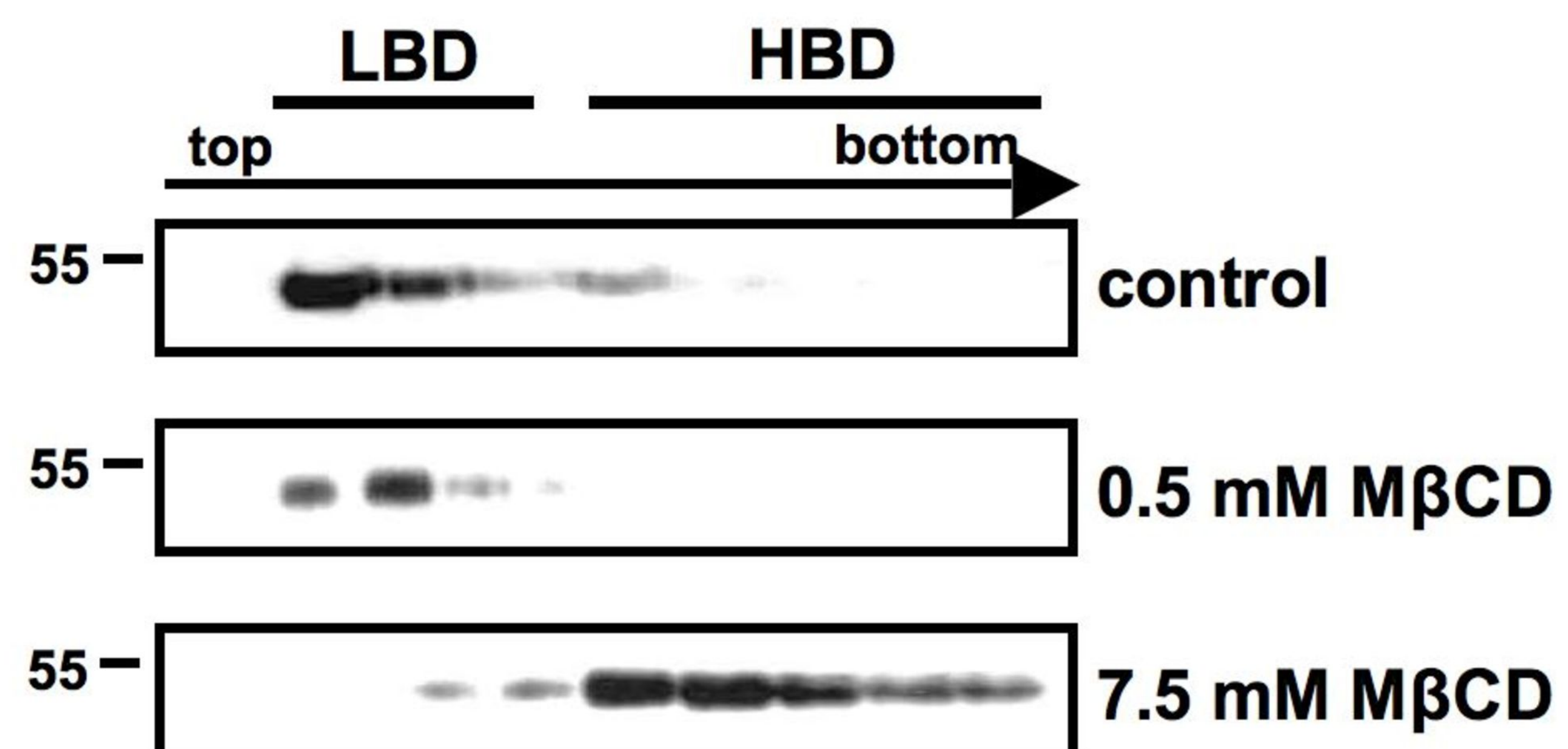
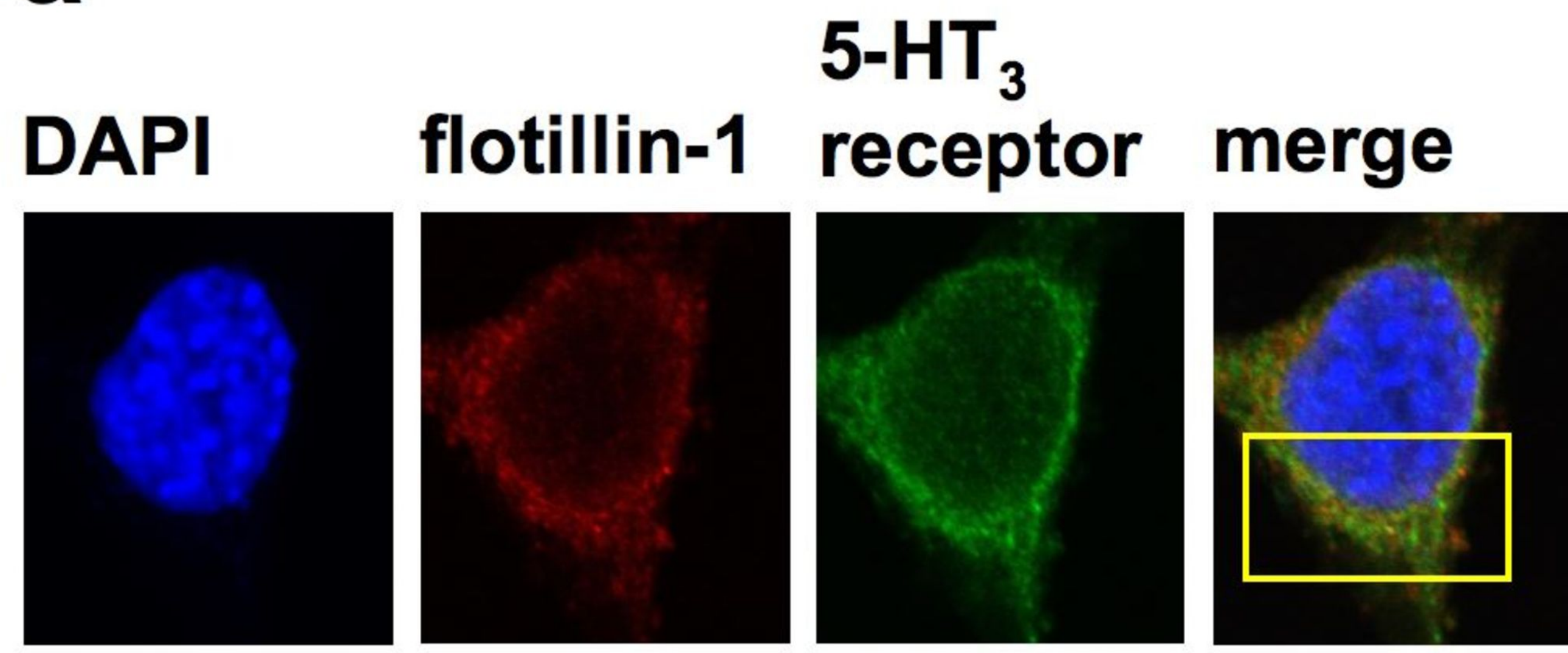
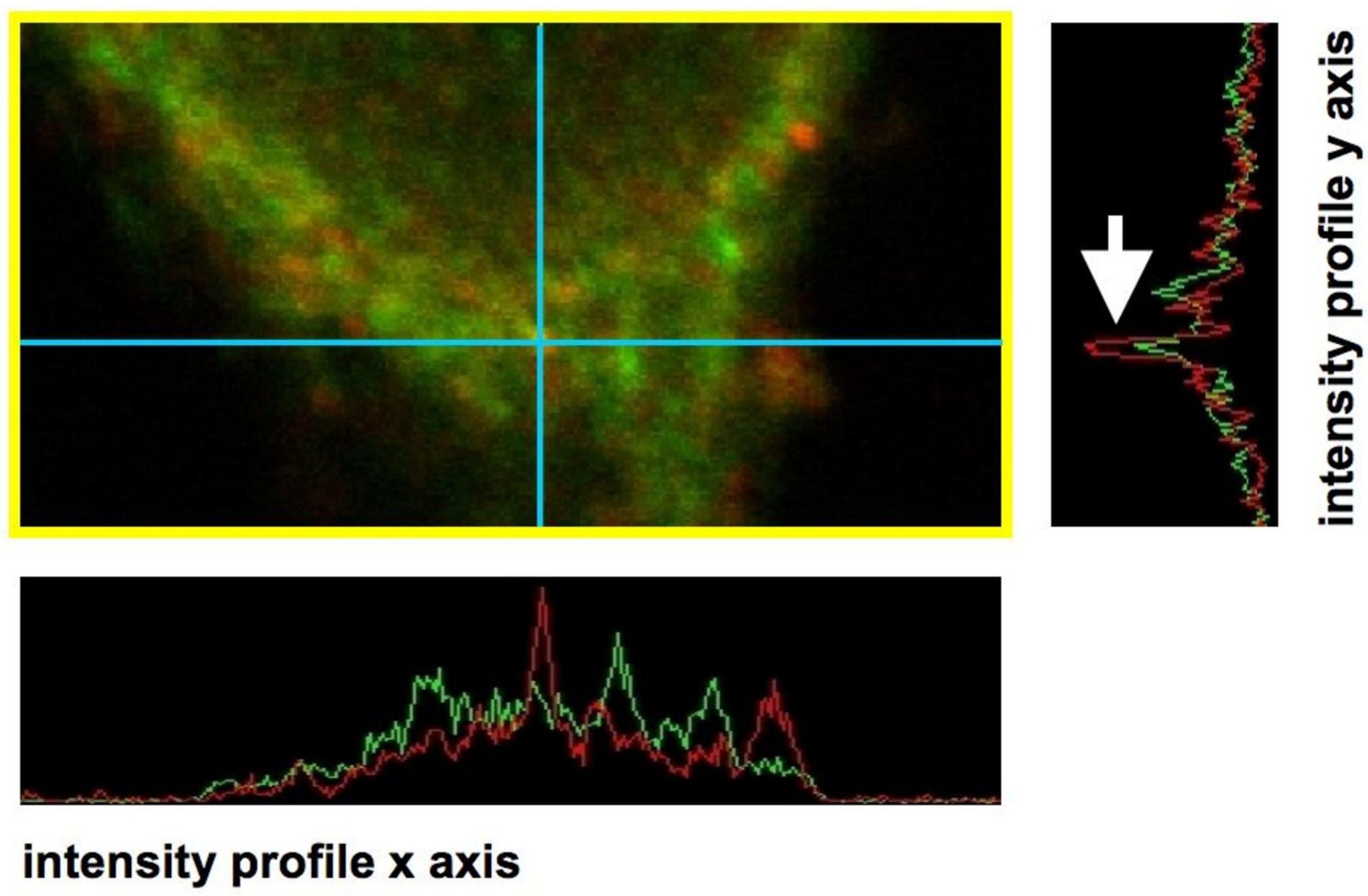


Figure 6

a



b



c

