NMDA RECEPTORS MEDIATE CONTRACTILE RESPONSES IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

Vidyanand Anaparti 1,4, Ramses Ilarraza 1, Kanami Orihara 1, Gerald L. Stelmack 2,4, Oluwaseun O. Ojo 2,4, Thomas H. Mahood 2,4, Helmut Unruh 3,5, Andrew J Halayko 2,3,4* and Redwan Moqbel 1,4‡

1 Department of Immunology, University of Manitoba, Winnipeg, Canada
2 Department of Physiology and Pathophysiology, University of Manitoba, Winnipeg, Canada
3 Department of Internal Medicine, University of Manitoba, Winnipeg, Canada
4 Biology of Breathing Group, Manitoba Institute of Child Health, Winnipeg, Canada
5 Section of Thoracic Surgery, University of Manitoba, Winnipeg, Canada
‡ Deceased

*Corresponding author: Dr. Andrew J. Halayko, Department of Physiology and Pathophysiology, University of Manitoba, Room 605 John Buhler Research Centre, 715 McDermot Avenue, Winnipeg, Manitoba, Canada R3E 3P4. Email – andrew.halayko@umanitoba.ca

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ABSTRACT

Human airway smooth muscle (HASM) exhibits enhanced contractility in asthma. Inflammation is associated with airway hypercontractility, but factors that underpin these features are not fully elucidated. Glutamate toxicity associated with increased plasma glutamate concentrations was observed in airway inflammation, suggesting that multi-subunit glutamate receptors, $N$-methyl-$D$-aspartate receptors (NMDA-R) contribute to airway hyperreactivity. We tested the hypothesis that HASM expresses NMDA-R subunits that can form functional receptors to mediate contractile responses to specific extracellular ligands. In cultured HASM cells we measured NMDA-R subunit mRNA and protein abundance by quantitative PCR (qPCR), immunoblotting, flow cytometry and epifluorescence immunocytochemistry. We measured mRNA for a number of NMDA-R subunits, including the obligatory NR1 subunit, which we confirmed to be present as a protein. *In vitro* and *ex vivo* functional NMDA-R activation in HASM cells was measured using intracellular calcium flux (Fura-2AM), collagen gel contraction assays and murine thin cut lung slices (TCLS). NMDA, a pharmacological glutamate analog, induced cytosolic calcium mobilization in cultured HASM cells. We detected 3 different temporal patterns of calcium response, suggesting presence of heterogeneous myocyte subpopulations. NMDA-R activation also induced airway contraction in murine TCLS and soft collagen gels seeded with HASM cells. Responses in cells, lung slices and collagen gels were mediated by NMDA-R as they could be blocked by D-AP5, a specific NMDA-R inhibitor. In summary, we reveal the presence of NMDA-R in HASM that mediate contractile responses via glutamatergic mechanisms. These findings suggest that accumulation of glutamate-like ligands for NMDA-R associated with airway inflammation contribute directly to airway hyperreactivity.
Airway smooth muscle (ASM) encircles the airways (23), providing mechanical support and regulating airway caliber (3). Inflammation-induced alterations in ASM structure and function contribute to variable bronchial airflow obstruction and persistent airway hyperresponsiveness (AHR) in asthma (2, 18, 21, 22). In the airways, multiple mechanisms regulate ASM phenotype and function, including neuroimmune interactions (16, 54), primarily mediated through the release of various neuromediators such as glutamate.

Glutamate, a predominant excitatory neurotransmitter in the brain (1) signals via various glutamate receptor classes, broadly categorized as metabotropic (mGlu-Rs) and ionotrophic (iGlu-Rs). Best studied among the iGluRs are N-methyl-D-aspartate receptors (NMDA-R), associated with neurodevelopmental and neurodegenerative processes (1, 6). They exist as transmembrane channels permeable to cations, particularly Ca$^{2+}$ (1, 6, 53). Functional NMDA-R are multimeric protein complexes comprised of two obligatory NR1 subunits (GRIN1) and two NR2 (GRIN2A-D) subunits or a combination of NR2 and NR3 (GRIN3A-B) subunits (53). Subunit composition of the receptor determines its specific physiological and pharmacological properties (1, 6, 53).

Classically, glutamate is released from presynaptic nerve endings during excitatory neurotransmission. However, recent evidence suggests immune cells like neutrophils (8), dendritic cells (32), macrophages (39) and platelets (31) release glutamate endogenously. Additionally, functional glutamatergic signaling and NMDA-R expression has been reported in various pulmonary cells and tissues, including alveolar macrophages (12), alveolar Type II cells (45), and the trachea and airways (12, 14, 25). Nevertheless, information is limited about the functional relevance for NMDA-R in the respiratory system. In vitro and in vivo observations by
Said et al (40, 41) suggest an involvement of glutamate and NMDA-R in lung injury and inflammation. In perfused guinea pig tracheal segments NMDA induces edema, augments resting muscle tone and enhances airway responsiveness to cholinergic agonists (12, 40, 41). Strapkova et al (48) have suggested that NMDA-R contribute to airway hyperreactivity as augmentation in ovalbumin-induced tracheal responses to acetylcholine (ACh) are observed in animals that receive NMDA, a pharmacological glutamate analog specific to NMDA-R. Ligand binding to NMDA-R can also promote oxidative stress and lung inflammation in a rat model for experimental sepsis, via a pathway that is blocked by a non-competitive NMDA-R antagonist, MK-801 (10, 11). Despite these reports, there has been no evidence for a direct role of glutamatergic signaling via NMDA-R in human ASM.

Considering the primary role of ASM in controlling airway responses in asthma (34, 47), we tested the hypothesis that human ASM (HASM) expresses NMDA-R subunits that can form functional receptors to mediate contractile responses to specific extracellular ligands. In this study, using cultured HASM cells we profile NMDA-R subunit expression using qPCR, immunoblotting, flow cytometry, and immunocytochemistry. We further assessed the capacity of NMDA-R to mediate contractile responses of HASM to the glutamate analogue, NMDA in the presence and absence of the NMDA-R inhibitor D-AP5, by measuring: intracellular Ca$^{2+}$ mobilization in Fura-2-loaded HASM cells; contraction of soft collagen gels seeded with HASM cells; and, airway narrowing using video-microscopy and murine thin cut lung slices.
METHODS

Culture of ASM cells and stimulation

In our study, we used senescence-resistant HASM cell lines that have been described elsewhere (44, 52). HASM cells were isolated from 2\textsuperscript{nd} to 4\textsuperscript{th} generation macroscopically healthy bronchial segments obtained as biopsies from patients undergoing surgical resection for lung carcinoma according to procedures approved by the Human Research Ethics Board, University of Manitoba, Winnipeg, Canada. Cells were grown to monolayer confluence in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmBH) and antibiotics (Penicillin-Streptomycin; Lonza Inc). To promote expression of a contractile ASM phenotype, at confluence cultures were serum-deprived for up to 48h in serum-free Ham’s F12K medium supplemented with antibiotics and insulin-transferrin-selenium (ITS, Lonza Inc) (15). Cell culture plasticware was purchased from Nunc (Thermo Fisher Scientific, NY).

Quantitative PCR Analysis (qPCR) and RT-PCR

Total cellular RNA was isolated using E.Z.N.A Total RNA Isolation Kit (Omega Biotek), as per the manufacturer’s instructions. Reverse transcription for cDNA synthesis was done using qScript\textsuperscript{TM} cDNA supermix containing an equimolar concentration of oligo dT’s and random hexamers (Quanta Biosciences, MD) using 200\textmu g of DNase-treated total RNA (Omega Biotek). The mRNA expression level for NMDA-R subunits in HASM cells was determined using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, MD) and Roche LightCycler 480 qPCR system. The sequence for oligonucleotide primers used and their product sizes are summarized in Table 1. Subunit-specific primers were designed across intron/exon boundaries...
using IDT primer quest software (www.idt.com) while specificity was determined using NCBI Primer Blast and subsequent melt curve analysis (Roche LightCycler 480). We used 18s rRNA detection as a reference standard for normalization. Absolute quantification of mRNA for NMDA-R subunits was determined by standard curve method, where standards for all the target genes were created from Human Universal Reference (HUR) total RNA (Clontech Laboratories Inc, CA) as purified PCR products and quantified. To quantify the exact copy number of each target gene in test samples, a standard curve was generated using a 7-point 10-fold dilution series over 6 orders of magnitude (1 x 10^7 to 1 x 10^1) using quantified concentrations of the standards. Standard curves with a slope of 3.6 ± 0.3 and efficiency range of 1.8 – 2.0 (equivalent to 90-100% amplification efficiency) were considered for analysis. Aliquots of cDNA equivalent to 5ng of total RNA samples were used for each real-time PCR reaction. The mRNA expression levels were normalized to 18S rRNA levels for each sample. Data was represented as copy number/ng RNA normalized to 18s. The mRNA isolated from SK-N-SH cells (a human neuroblastoma cell line) was used as a positive control (38).

To determine NR1 expression in mouse lungs and brain, tissues were homogenized and total RNA was isolated using RNeasy Mini Kit (Qiagen Inc). First strand cDNA synthesis was done in a 20µl using 1µg RNA as template. Gene of interest was amplified in a 25µl reaction (as per manufacturer’s instructions) using EconoTaq PLUS GREEN 2X master mix (Lucigen) using primers specifically designed for mouse NR1 subunit (no. of PCR cycles = 35; primer sequence and amplicon size is indicated in Table 1). The amount of RNA used for PCR amplification was ~3 times higher in mouse lungs (1µg) compared to mouse brain (300ng), considering less abundance of NR1 in murine lung tissues.
**Immunoblotting**

Protein lysates were prepared in RIPA lysis buffer (composition: 40mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Iqepal, 1% deoxycholic acid, 1X PMSF, 1X Protease inhibitor cocktail (SIGMAFAST™, Sigma-Aldrich, St.Louis, MO, US) and concentration was determined using BCA kit (EMD Millipore). Proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membrane. Blots were blocked in blocking buffer (5% Non-fat powdered milk diluted in Tris-buffered saline containing 0.1% Tween-20) for 60min at room temperature. Next, membranes were incubated at 4°C overnight with goat polyclonal anti-NR1 antibody (1:250, Santacruz Biotechnology Inc) diluted in blocking buffer. After 3 washes (10min each) with 1X Tris-buffered saline-Tween 20 (TBST) buffer, membranes were incubated with HRP-conjugated secondary anti-goat IgG (1:15,000 dilution in blocking buffer) for 60min at room temperature. Blots were washed 6 times (10 min each) with 1X-TBST buffer and developed by Clarity™ Western enhanced chemiluminescence substrate (Bio-Rad Laboratories, CA).

**Immunocytochemistry (ICC)**

To visualize NR1 expression by immunofluorescence, HASM cells were cultured in 4-well Lab-Tek II chamber slides (Nunc) for 96h. Cells were washed 3 times (5min each) with 1X cytoskeleton buffer (CSB; 10mM 4-Morpholineethanesulfonic acid, 150mM NaCl, 5mM MgCl₂, 0.9mg/ml glucose, 5mM EGTA, pH 7.0) and fixed for 15min at room temperature using 4% paraformaldehyde (PFA) prepared in 1X CSB. Again, cells were washed 3 times (5min each) with 1X CSB and blocked using 0.5% Gelatin dissolved in 1X phosphate-buffered saline (PBS) for 60min at room temperature. After washing cells with 1X PBS (3 x 5'), anti-NR1 (BD, diluted 1:100 in 0.1% Gelatin in PBS) antibody was added and incubated overnight at 4°C. Isotype-
matched mIgG antibody was used as negative control. Later, cells were washed with 1X PBS (3 x 5') and incubated with Biotinylated-Donkey anti-mouse IgG (H+L) secondary antibody (Jackson Laboratories) for 60min at room temperature. Cells were washed with 1X PBS (3 X 5') and incubated with AlexaFluor 488-conjugated streptavidin (Invitrogen) in dark for 1h at room temperature. Later, cells were washed (3 x 5'; 1X PBS) and coverslips were mounted with ProLong Gold Antifade medium containing DAPI (Molecular Probes). DAPI was used to stain the nuclei. Fluorescent images were captured using an Olympus BX-51 fluorescent confocal microscope.

**Flow cytometry**

For intracellular staining, HASM cells were detached using ice-cold PBS containing 2mM EDTA, washed with PBS and fixed with ice-cold 2% PFA in PBS for 10min. After washing with flow buffer (1% BSA in PBS), cells were permeabilized using 0.1% saponin in flow buffer for 10min at 4°C. After washing cells with flow buffer (2 x 5'), cells were incubated with primary antibodies (anti-NR1; 1:100; goat polyclonal; Santa Cruz Biotechnology) for 1h at 4°C. Cells washed three times with flow buffer, and labeled with AlexaFluor-488 conjugated Donkey anti-goat IgG (H+L) secondary antibody (1:350; Invitrogen) for 1h at 4°C. Goat IgG was used as isotype control. Fluorescence intensity was detected by BD FACS Canto II and data was analyzed by FlowJo software (BD Biosciences, San Jose, CA).

**Intracellular Ca^{2+} flux measurements**

Real-time quantification of intracellular cytosolic Ca^{2+} ([Ca^{2+}]_{i}) was performed using Fura-2AM (Invitrogen, NY), a Ca^{2+}-sensitive ratiometric fluorescent dye (43). For measurement,
cells were grown on Lab-tek coverglass chamber slides (Nunc), washed with HBSS/HEPES buffer containing 0.1% BSA (HHB) and incubated with 5 μg/ml Fura-2 AM (room temperature, 1 hour) in HHB containing 0.01% pluronic acid. After washing, cells were further incubated in HHB (room temperature, 1 hour) containing 0.2mM glycine to facilitate Fura-2AM de-esterification. Real-time changes in $[Ca^{2+}]_i$ were recorded using an Olympus LX-70 inverted epifluorescent microscope (20× objective) coupled to a Nikon CCD camera controlled by NIS imaging software (NIKON Instruments Inc., NY, US). The system was further coupled to a Sutter Instruments Lambda 10-2 filter wheel and controller with repeated 100ms excitation at 340 and 380 nm; emission at 510 nm was recorded continuously for up to 5 minutes after the addition of contractile agonists. The ratio of emission at 510 nm excited by 340- and 380-nm light was converted to $[Ca^{2+}]_i$ values from a calibration curve generated using Ca$^{2+}$ standards and calculated by the method of Grynkiewicz (17). Mean Ca$^{2+}$ flux response was calculated from all the cells present in a whole microscopic field. We also showed responses generated by a whole individual cell, wherever required.

**Collagen gel contraction assay**

Collagen gel lattices were prepared using PureCol collagen solution (Advanced Biomatrix, CA, US) as per manufacturer’s instructions. Briefly, 7ml of collagen solution was diluted in 2ml of 5X DMEM and pH was adjusted to 7.4 using 1N NaOH. Volume was made up to 10ml using sterile dH$_2$O. 500μl of this solution was aliquoted in each well of a 24-well plate and allowed to polymerize at 37°C for 1h. HASM cells were layered on top of the polymerized collagen gels at 0.5x10$^5$ cells/mL in DMEM containing 10% FBS. 24h later, cells were washed with HBSS and serum deprived for 96h. Later, edges of the collagen gel were released from
wells and allowed to contract spontaneously for 2h, after which agonist was added. Shortening of gels was recorded and measured from images obtained using a Canon digital camera at time intervals for up to 6h. Images were analyzed by ImageJ (NCBI) and were normalized as percentage decrease in gel area from baseline. Percentage decrease in gel area represents the contraction ability of HASM cells in response to different conditions.

**Thin Cut Lung Slices (TCLS)**

We used a protocol described by Perez-Zoghbi *et al* (36). Briefly, 8-10wk old female Balb/c mice were euthanized by intra-peritoneal injection of 0.1mL of sodium pentobarbital (protocol approved by the animal ethics committee at the University of Manitoba). After tracheal cannulation, lungs were inflated with ~1.3mL of warm 2% low-melting agarose in 1X HBSS followed by injecting ~0.2mL of air to flush the agarose to the distal alveoli. The lungs were cooled at 4°C to allow agarose polymerization. After opening the thoracic cavity, left and right lobes were removed and sectioned using a vibratome (model EMS-4000; Electron Microscope Sciences, EMS) into slices of ~180 µm thickness starting at the lung periphery. Temperature was maintained between 4-5°C and ice-cold HBSS was used during the process. Slices were maintained (37°C and 5% CO₂) in DMEM supplemented with antibiotic-antimycotic solution (Lonza Inc) and medium was changed every 24h.

**Measurement of airway contraction in murine TCLS**

Murine TCLS slices maintained between 24-72h were used for our experiments. For measuring airway responses, a large cover glass was placed on a plexiglass support and a perfusion chamber was custom-made on the coverglass using silicone (Dow). Lung slices were...
held in place inside the chamber with the help of a small nylon mesh. A hole was made in the middle of nylon mesh to allow microscopic observation of selected airway. A constant perfusion rate was maintained within the chamber using a gravity-fed perfusion chamber connected to a multi-tube manifold, which was controlled by a valve-system. Phase-contrast images of airway narrowing were recorded using an inverted microscope with a 10X objective connected to a CCD camera. VideoSavant 4.0 software (IO Industries Inc) was used for image acquisition and digital recording of images in a time-lapse mode. Recordings were analyzed with "NIH /Scion Image" software (Scion Corp.). The airway lumen area was determined by adding the number of pixels below a selected threshold grey level with respect to time. All experiments were performed at room temperature.

**Statistical analysis**

Data is presented as mean ± SE, unless otherwise stated. Statistical significance was determined by two-way ANOVA with Bonferroni’s post-hoc tests or one-way ANOVA with Bonferroni’s multiple comparison tests, as required by data. *P* values < 0.05 were considered significant.
RESULTS

ASM cells express subunits for complete NMDA-R complexes

To profile NMDA-R subunit expression in cultured HASM cells, we first performed qPCR using subunit-specific primers. We revealed the presence of mRNA for most NMDA-R subunits, including NR1, NR2C, NR2D, NR3A and NR3B, but we found no evidence for NR2A or NR2B. (Fig 1A, n=6). The abundance of mRNA for NMDA-R subunits appeared to be stable as we observed no change when cells were serum-deprived for up to 48h (Fig 1B, n=6). These data indicate that HASM cells express mRNA for all the subunits required to form a functional NMDA-R complex.

To validate our qPCR data we next examined the abundance of protein for the obligatory NR1 subunit, essential for formation of functional NMDA-R complexes. Using immunoblotting (Fig 2A), NR1 was readily detected (~120kDa) in whole cell lysates from HASM cultures. We noted a modest difference in the mobility of the protein band for NR1 from HASM compared to that for our positive control, mouse brain; this is likely due to tissue- and species-specific differences in glycosylation and other post-transcriptional or translational modifications (4, 28, 51). We further assessed NR1 protein abundance in permeabilized HASM cells using flow cytometry (Fig 2B). Fluorescent labeling with an anti-NR1 polyclonal antibody and AlexaFluor488-conjugated secondary antibody revealed significant positive staining, with ~77% of cells exhibiting greater fluorescence compared to cells labeled with isotype-matched control primary antibody. Last, we performed fluorescence cytochemistry using confocal microscopy, revealing labeling for NR1 in perinuclear regions, the cytoplasm and some foci aligned with cell margins that was absent in fixed and permeabilized HASM cells that were fluorescently immunolabelled with isotype-matched control antibody (Fig 2C).
**NMDA-R mediates airway contraction, ex vivo**

We used murine TCLS preparations, and time-lapse video microscopy to measure intact airway narrowing mediated via NMDA-R. For these experiments we used NMDA, because it mimics glutamate pharmacologically but is selective for the ionotropic NMDA-R, whereas glutamate is more promiscuous, being a ligand for both mGluRs and iGluRs. Prior to completing these functional studies, we surveyed NR1 subunit expression in mouse lungs using RT-PCR and detected a ~137bp transcript corresponding to NR1 (Table 1 and Figure 3A). Using a cumulative NMDA dose-response experimental design in the presence of 0.2mM glycine, we measured airway narrowing in TCLS (Fig 3B and 3C). We did not observe any significant reduction in airway lumen area from baseline in response to 0.01 or 0.03mM NMDA, but at the highest concentrations tested (0.1 and 0.5mM) NMDA significantly narrowed airways by 10%. Maximum constriction was achieved with 0.1mM NMDA as no increase was evident using 0.5mM NMDA (not shown), thus for subsequent functional studies we used 0.1mM NMDA. To confirm that NMDA-induced airway narrowing was mediated by NMDA-R, some TCLS were pre-incubated with the selective NMDA-R antagonist, (2R)-amino-5-phosphonopentanoate (D-AP5; 0.5mM). We found that D-AP5 abrogated NMDA-induced airway narrowing (Fig 3C). In all TCLS specimens we also determined active narrowing in response to 1µM methacholine (MCh) as a comparator to that induced by NMDA. We found that NMDA-induced contraction (0.1mM) was physiologically significant, being ~50% of that induced by MCh (Fig. 3C). To further characterize the impact of NMDA on airway contraction, we compared airway narrowing in response to 1µM MCh in TCLS before and after NMDA exposure (Fig 3D). We did not observe any effect of NMDA exposure on MCh-induced airway contraction.
To assess the direct effects of NMDA on HASM contraction, we next used floating collagen gels seeded with HASM cells and measured the decrease in collagen gel area that occurs upon NMDA exposure (Figure 4). Compared to control gels that were not exposed to agonist, NMDA (0.1, 0.5 and 1mM) induced significant contraction of HASM-seeded collagen gels, reaching near-maximum shortening (~20% decrease in gel size) after 60min, a response that was stable for up to 6h (Fig 4A and 4C). Interestingly, all concentrations of NMDA triggered gel contraction of similar magnitude suggesting that using this system NMDA-R mediates HASM cell contraction in an all-or-none manner. To confirm that NMDA-R mediates HASM contraction, we pre-treated some gels with the NMDA-R antagonist, D-AP5, and found that contractile responses were blocked (Figs. 4B and 4C). We also compared collagen gel contraction induced by NMDA (0.1mM) with that of a physiologically relevant positive control, bradykinin (0.1mM) and found that though NMDA-induced contraction was of a slower onset, its magnitude was not different from that induced by bradykinin (Fig 4B). We also tested contractile response to MCh (0.1mM) and to histamine (0.1mM), considered to be physiologically relevant G_q-coupled agonists known to induce robust contraction in intact airways (Fig 4D). MCh-elicited gel contraction was comparable to that induced by bradykinin, and histamine also induced significant contraction, albeit to a lesser degree than other agonists. Further, we showed that contraction of collagen gels over time requires the presence of airway myocytes (Fig 4D). In comparison to gels that harbored HASM cells, cell-free collagen gels demonstrated no more than ~3% reduction in their area over a 5h time period, with gel contraction being almost “0” in the first 3hours. Thus, collagen gels themselves do not undergo any significant physical change, thus confirming that the contractile effect observed in our experiments is mediated by HASM cells.
NMDA-R mediates a heterogeneous pattern of $[\text{Ca}^{2+}]_i$ flux in HASM cells

Upon agonist binding, NMDA-R activation causes influx of extracellular Ca$^{2+}$ in neurons (1), therefore, we used real-time Ca$^{2+}$ imaging to examine whether NMDA-R mediates Ca$^{2+}$ flux in HASM cells. HASM exhibited a significant increase in $[\text{Ca}^{2+}]_i$ in response to both 0.1mM and 0.5mM NMDA, but we observed no change in $[\text{Ca}^{2+}]_i$ in response to 0.01mM NMDA (Figure 5A and 5B). NMDA induced a sustained calcium response over a period of 4 min attaining peak $[\text{Ca}^{2+}]_i$ of ~0.6µM after 120s (Fig 5A and 5B). The elevation in $[\text{Ca}^{2+}]_i$ induced by NMDA was abrogated when cells were pre-treated with D-AP5 (0.5mM), confirming that NMDA-R mediates changes in $[\text{Ca}^{2+}]_i$ (Fig 5C and 5D). We compared response to NMDA with that triggered by 0.1mM bradykinin and found that: time-to-peak $[\text{Ca}^{2+}]_i$ was much slower; peak $[\text{Ca}^{2+}]_i$ was ~65% lower than the acute peak induced by bradykinin; and peak $[\text{Ca}^{2+}]_i$ induced by NMDA was sustained and similar in magnitude to that sustained in response to bradykinin (Fig 5C). Notably, NMDA-induced Ca$^{2+}$ flux in HASM cells was observed only in the presence of glycine (0.2mM), a NMDA-R co-agonist. Without glycine, NMDA failed to evoke any calcium response in our cell preparations (Fig 5D), which confirms the necessity of simultaneous binding of agonist (NMDA) and co-agonist (glycine) to evoke NMDA-R activation. To determine whether calcium mobilization mediated by NMDA-R was reliant on extracellular calcium, we measured changes in $[\text{Ca}^{2+}]_i$ induced by NMDA in cultured HASM cells incubated in Ca$^{2+}$-free buffer. In the absence of extracellular Ca$^{2+}$, we observed no increase in $[\text{Ca}^{2+}]_i$ upon NMDA exposure (Fig 5E). These data indicate that NMDA-R can mediate increase in cytosolic Ca$^{2+}$, and that extracellular Ca$^{2+}$ is required for this effect. To further complement our findings, we examined $[\text{Ca}^{2+}]_i$ elevation in HASM cells when stimulated with endogenous NMDA-R ligand, glutamate
in the presence and absence of co-agonist glycine. We used a physiologically relevant concentration of glutamate (0.1mM) and observed that it evoked significant Ca\(^{2+}\) mobilization (~1.2µM above baseline) in the presence of glycine. Notably, glutamate induced Ca\(^{2+}\) responses were observed even in the absence of glycine; however, the active release of Ca\(^{2+}\) was ~40% less than in the presence of glycine. This suggests that NMDA receptor accounts for 60% of the glycine-dependent glutamate response. Consistent with this, we observed that Ca\(^{2+}\) mobilization by glutamate in the presence of glycine was also blunted by ~40% when cells were treated with the 0.5mM D-AP5 (Fig 5F). Together, these observations validate our findings with NMDA, but also suggest the presence of non-NMDA glutamate receptors in HASM cells.

In all cultures studied, NMDA alone routinely induced an elevation in [Ca\(^{2+}\)]\(_i\) in 75-80% of cells, and there were no significant differences in basal [Ca\(^{2+}\)]\(_i\) between cells. However, in the course of our experiments using real time fluorescent microscopy to measure changes in [Ca\(^{2+}\)]\(_i\), we observed that individual cells exhibited a heterogeneous temporal response pattern that we have classified broadly into four groups: Early Response; Late Response; Phasic Response; and, No Response (Fig 6). Consistent with the mean Ca\(^{2+}\) response curve for the whole culture population (see Fig 5A), ~50% of all cells were of the Late Response group (Fig 6A), displaying a delayed (~60-100s after NMDA exposure) Ca\(^{2+}\) response that was sustained for at least 300s (Fig 6B (iii and iv)). A sub-group we identified as showing a Phasic Response profile (initial acute rise in [Ca\(^{2+}\)]\(_i\) within 20s followed by a second spike 75-100s later) (Fig 6B (vi)), encompassed approximately 13% of all myocytes (Fig 6A). Indeed, some cells even presented 3-5 calcium spikes within the timeframe of measurement (300s) (Fig 6B (v)). We also identified 15% of cultured HASM as exhibiting an Early Response profile characterized by an almost immediate response (10-15s after NMDA exposure) (Fig 6A and 6B (i and ii)). Finally, we
observed that 22% of the total cell population did not show any change basal [Ca\(^{2+}\)]\(_i\) upon
NMDA, thus forming the so-called No Response sub-group (Fig 6A). NMDA-induced increases
in [Ca\(^{2+}\)]\(_i\) in all response sub-groups were blunted by removal of extracellular Ca\(^{2+}\) or selective
antagonism of NMDA-R with D-AP5 (data not shown). Together, these results demonstrate an
inherent heterogeneity in NMDA-R mediated mobilization of cytosolic Ca\(^{2+}\) in sub-groups of
HASMs that is dependent upon extracellular Ca\(^{2+}\), suggesting there could be differential
expression of NMDA-R subunits, regulation of NMDA-R function, or both.


Ours is the first study to provide direct evidence for NMDA-R expression by HASM cells and that these receptors can mediate contraction by inducing an elevation of intracellular Ca$^{2+}$. Using qPCR we show that mRNA for all but two of the known NMDA-R subunits is present in cultured HASMs and we confirm that protein for the obligatory NR1 protein is readily detectable. Our functional analyses show that ligand binding for NMDA-R leads to increased [Ca$^{2+}$], directly induces contraction of HASM cells, and triggers physiologically relevant degrees of airway narrowing ex vivo. Collectively, our data reveals for the first time that requisite subunits can be assembled into functional multimeric NMDA receptor complexes.

As proposed by Haong et al (20), AHR occurs as a consequence of several exogenous and endogenous stimuli, including neurogenic mediators such as glutamate and their receptors like NMDA-R. While glutamate is proposed to be a novel regulator of adaptive T-cell immune responses (27), deregulated ion transport mediated through hyperexcitation of ligand-gated glutamate channels such as NMDA-R either in excitatory or non-excitatory pulmonary cells or tissues is suggested to be an potential mechanism underlying increased airway responsiveness during inflammation (20, 48). Besides glutamate and its pharmacological analog NMDA, NMDA-R can be activated by other physiological ligands such as D-serine, L-alanine, L-cysteine and quinolinic acid (9). The presence of multiple allosteric modulators and distinct subunit composition of NMDA-R appears to determine the functional diversity of the receptor (9, 33). NR2A-D and NR3A-B subunits act as modulatory proteins in the receptor complex (1, 6, 9, 53). While NR2 harbors the glutamate-binding domain, NR1 and NR3 subunits contain the glycine-binding site; glycine functions as a NMDA-R co-agonist. Studies by several groups, including Dickman et al (12), show NR1, NR2C and NR2D subunit expression in rat peripheral lungs.
Consistent with these findings, we observed the expression of these NMDA-R subunits in ASM cells, and we also identified the presence of mRNA for NR3 subunits (NR3A and NR3B). Our studies using cultured HASM cells from adult donors indicated that NMDA-R subunits may be stably expressed by airway myocytes. We found that mRNA abundance was highest for the NR2C, NR2D and NR3B. Differential expression of subunits is consistent with studies in other cell types. For instance, NMDA-R subunit composition and distribution in neurons is plastic and dependent on the CNS developmental stage, neuronal activity and tissue microenvironment (9, 13). Nonetheless, the presence of protein for the NR1 subunit is indispensable for the formation of functional NMDA-R, and our data confirm that both mRNA and protein for NR1 is abundant in lung tissue and HASM cells. Thus, our findings, aligned with that from other groups on multiple cell and tissue types, indicate a need for additional research to determine the role of various receptor subunits in modulating ASM contractile function.

ASM is essential in regulating airway conductance. Its emergence as a phenotypically diverse tissue that is also capable of orchestrating local inflammation and promoting airway remodeling warrants attention as a potential therapeutic target (19). Notably, the multifunctional ability of ASM is controlled, in part, through oscillation patterns for [Ca$$^{2+}$$], which is in turn regulated by various Ca$$^{2+}$$ pumps and membrane ion channels. We examined whether NMDA-R on ASM cells demonstrate a role in mediating changes in cytosolic Ca$$^{2+}$$, an effect that requires extracellular calcium when myocytes are exposed to NMDA. Our studies suggest that NMDA-R may serve as a sarcolemma transmembrane calcium channel in ASM. However, the approach we used, fluorescence microscopy with Fura-2 loaded cells, does not allow direct discrimination of the contribution of Ca$$^{2+}$$ derived from extracellular or intracellular stores, or whether influx of extracellular Ca$$^{2+}$$ may trigger release of intracellular stores, thereby leading to the increases in
[Ca$^{2+}$], that we observed upon ligand binding to NMDA-R. Ca$^{2+}$ released from internal sarcoplasmic reticulum induces ASM contraction, while membrane ion channels are typically thought to replenish calcium stores (37). Our data supports the notion that NMDA-R-mediated changes in cytosolic Ca$^{2+}$ in human ASM cells could contribute directly to airway contraction.

Our experiments revealed that NMDA, only in the presence of glycine, was sufficient to induce maximum increase in [Ca$^{2+}$], which we confirmed using glutamate, a more physiological NMDA-R agonist. NMDA is an amino acid derivative used as a pharmacological analogue of glutamate to specifically distinguish NMDA-R from other glutamate receptors. Considering normal physiological plasma of glutamate concentrations range between 0.05mM and 0.08mM (24, 26), our observations that maximum responses could be induced with 0.1mM NMDA are of physiological relevance. HASM cells, treated with 0.1mM glutamate, exhibited a significant Ca$^{2+}$ mobilization, which was comparable to those induced by 0.1mM NMDA, thereby supporting our results with NMDA and involvement of glutamatergic signaling in airways linked to NMDA receptors. Agonist-binding affinity of NMDA-Rs to NMDA (EC50 35-50µmol L$^{-1}$) is significantly lower than glutamate (EC50 1-3µmol L$^{-1}$) that is further dependent on the receptor subunit configuration (7, 35). During inflammatory conditions such as lung injury, endothelial permeability facilitates extravasation of glutamate towards the inflamed tissues causing an increased local glutamate concentration (24). In this context, the increases in [Ca$^{2+}$], we observed with 0.1mM NMDA and even higher concentrations (0.5mM) are physiologically relevant as they fall within the levels of plasma glutamate levels that have been measured for normal and diseased conditions. More importantly, we found presence of glycine as an essential pre-requisite for NMDA-R mediated Ca$^{2+}$ flux in HASM cells. Cells responded to glutamate even without glycine. Our experiments show that unlike responses to NMDA, glutamate-induced [Ca$^{2+}$],...
responses cannot be blocked completely by D-AP5 suggests likely presence of additional glutamate receptors including mGluRs. In ASM cells, NMDA-induced $\text{Ca}^{2+}$ responses are only a quarter of the magnitude of that induced by a robust GPCR agonist such as bradykinin, but NMDA-R mediates sustained elevation in $[\text{Ca}^{2+}]_i$, suggesting the potential to activate multiple signaling pathways associated with airway contraction. Our experiments do show that inhibiting NMDA-R with D-AP5 prevents all responses triggered by NMDA-R ligands. This includes abolition of all of the diverse temporal patterns for $\text{Ca}^{2+}$ flux that we characterized in HASM cells. Sukkar *et al* (49) suggest that ASM cells of diverse phenotype and with disparate synthetic, contractile and proliferative function are actually representative of a continuum of overlapping cell populations. The heterogeneous intercellular $\text{Ca}^{2+}$ responses mediated by NMDA-R that we observed may reflect a composite of multi-functional behavior that is a fundamental character of ASM cells. Also, existence of spatio-temporal heterogeneity in $\text{Ca}^{2+}$ oscillations between airway myocyte subpopulations to agonists such as MCh and bradykinin described by Sieck *et al* (46) and by us (30) suggests such a phenomenon is not unique to NMDA-R. Thus, future studies that directly correlate ASM functional capacity with $\text{Ca}^{2+}$ flux patterns mediated by NMDA-R, albeit technically challenging, could reveal the full spectrum of control that NMDA-R may contribute to ASM cells.

In humans, increased frequency of $[\text{Ca}^{2+}]_i$ oscillatory waves is believed to be the fundamental event underlying agonist-induced contractions initiated by ASM tissue (37). NMDA-R involvement in promoting airway muscle contractility was previously shown, albeit in animal models (48). These studies used isolated tracheal rings, which do not necessarily represent ASM responses surrounding smaller airways. To further understand the physiological significance of NMDA-R-mediated $[\text{Ca}^{2+}]_i$ influx in ASM cells, we used murine thin cut lung
slices to measure airway contraction, a methodology widely accepted to study the behavior of smaller airways (42). Lung slices retain an *in vivo*-like cellular and tissue microstructure surrounding the airways, which provides an ideal platform to correlate microscopic sub-molecular events with macroscopic responses in a physiologically relevant manner (42). Using cultured airway slices, we show that NMDA-induces airway contraction via pathways that require NMDA-R, as the NMDA-R antagonist D-AP5 blunted airway constriction. In guinea pig tracheal segments, Said *et al* (40) and others (48) showed that NMDA enhances airway contractile responses to MCh. In our experiments, we found no such positive effect of NMDA when we measured murine airway responses to 1µM MCh before and after NMDA treatment. It is not clear why such a discrepancy between those data and ours might exist, but this could be ascribed to innate disparity between larger and smaller airways or to species-to-species variation. Additionally, we observed considerable variation in airway contractile responses to both NMDA and MCh individually, which corresponds to the biological heterogeneity observed across different *in vitro*, *ex vivo* and *in vivo* models used in studying airway behavior.

To further ascertain the involvement of human ASM cells in regulation of airway contraction, we employed a collagen gel assay system as a way to measure contraction at cellular level. We show that 0.1mM NMDA decreased collagen gel area by ~20%, a maximum response that reflects an all-or-none effect as lower concentrations elicited no contraction, whereas higher concentrations did not increase the contractile response. Nonetheless, maximum contraction induced by NMDA-R activation was significant as it mimicked that induced by bradykinin, whose ability to contract HASM-seeded gels was comparable to other Gq-coupled contractile agonists MCh and histamine. Furthermore, contractile responses demonstrated were indeed by HASM cells, as cell-free collagen gels failed to evoke any contraction over time. These
observations concur with that of Bourke et al (5), where authors confirmed that human ASM-mediated collagen gel contraction measured over a time period of 72h, as they saw no physical change in collagen gel lattices that were not seeded with HASM cells. The collagen gel contraction model does have limitations in interpretation to physiological systems, as smooth muscle contraction is generally not rapid, and removal of agonist does not result in relaxation. The gel system does resemble that of Margulis et al and others (5, 29) where diminutive, yet noticeable contractile responses generated during wound healing are portrayed over a period of hours to days. In our experiments, bradykinin demonstrated a peak contractile response only after 30min even though $[Ca^{2+}]$, flux responses were rapid and occurred within 30s of adding NMDA. Peak NMDA-induced contraction in ASM cells occurred 3h after agonist exposure. Our observations in response to bradykinin are comparable to the kinetics demonstrated in the study by Sutcliffe et al (50). Notably, NMDA-induced reduction in collagen gel area was inhibited by D-AP5 indicating that the response measured is mediated via NMDA-R.

Our observations using multiple systems are in accordance with those in different small animal models of asthma reported by Said et al (41) and Strapkova et al (48) in that they support a role for NMDA-R as a contributor to airway hyperreactivity. Together, our studies confirm NMDA-R and glutamatergic signaling to be a potential mechanism for regulating airway contractility, likely through direct effects on $[Ca^{2+}]$ in ASM cells. NMDA-R in ASM cells might have additional functional roles that were not addressed in our work, for example whether in conditions of inflammation NMDA-R subunit expression is altered or pathways that regulate NMDA-R function are altered. In summary, we provide evidence that cultured human ASM cells express functional NMDA-R containing the obligatory NR1 subunit. Activation of NMDA-R on ASM cells results in significant increase in $[Ca^{2+}]$, that mediates airway myocyte contraction.
This presents NMDA-R as a novel regulator of ASM cell function. Nonetheless, the physiological relevance of NMDA-R in healthy and diseased airways needs further investigation.
ACKNOWLEDGEMENTS

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REFERENCES


51. Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, and Huganir RL. Characterization of protein kinase A and protein kinase C phosphorylation of the


**Figure 1:** HASM cells express mRNA for different NMDA-R subunits. qPCR was performed for mRNA isolated from cultured HASM cells. Abundance is expressed as copies/ng RNA normalized to 18S rRNA, (A) Abundance of mRNA for respective NMDA-R subunits in confluent HASM cultures maintained in serum-free medium, for 48h. (B) Time-course for mRNA abundance of NR1, NR2C, NR2D, NR3A and NR3B in confluent HASM cultures in serum-free conditions. Data represent mean values from 6 different experiments performed in triplicate under similar experimental conditions. Error bars are shown as mean ± SE.

**Figure 2:** Presence of protein for the obligatory NR1 subunit in HASM cells. (A) Representative immunoblot from three independent experiments for NR1 (~120kDa band) in protein lysates from HASM cells and from whole mouse brain (Br), which served as a positive control. (B) Histogram showing results from flow cytometry of permeabilized HASM cells for NR1 protein (black filled histogram) and IgG isotype control (open histogram, red line). Compared with matched isotype control cells (dotted-lined open histogram), NR1 was detected on ~77% of cells (filled histogram). The histogram shows % mean fluorescence intensity from 10,000 cells and is representative of 5 experiments. (C) Confocal micrographs showing immunofluorescence for NR1 in HASM cells (ii). Isotype goat IgG antibody was used as negative control (i). Image in (iii) is an enlarged portion of the boxed region in panel (ii). Arrows point to perinuclear (white), cytosolic (orange) and membrane (yellow) staining. DAPI was used to stain nuclei. Images are representative of 5 independent experiments done under similar conditions. Scale bar: 50µm.

**Figure 3:** Agonist-induced NMDA-R activation causes airway contraction. (A) Agarose gel showing products generated by RT-PCR using primers for the obligatory NR1 subunit in mouse
brain (lane 1) and mouse lungs (lane 2). Lane 3 is a negative control (water). The left lane shows size markers as indicated. Equal volume of sample (2.5µl) was loaded in a 2% agarose gel. (B) Representative tracing from a TCLS showing temporal pattern of active narrowing of lumen area of a single airway perfused with increasing concentrations of NMDA. At the end of each study TCLS were perfused with saline for (30 min (wash) then re-stimulated with 1µM MCh. Response to each agonist was recorded for 4 min. Experimental values were normalized as % decrease in baseline (pre-agonist) lumen area (Y-axis). (C) Histogram showing cumulative dose-response patterns for airway narrowing in response to increasing concentrations of NMDA or to 1µM MCh. In some studies TCLS were pre-incubated with 0.5mM D-AP5 prior to stimulation with 0.1mM NMDA (D-AP5/NMDA). Data presented represent the mean ± SE from 13-16 airways obtained from 5 different mice. Data was analyzed by One-way ANOVA. **P<0.01, ***P<0.001 and ns (not significant) compared to baseline). ## P<0.01 for D-AP5/NMDA compared to 0.1mM NMDA. (D) Histogram summarizing the mean ± SE for airway narrowing in response to 1µM MCh before (white) and after (grey) treatment with 0.1mM NMDA. Response to NMDA alone is also shown (black). Airways were perfused with saline for at least 30 minutes between agonist additions to ensure that airway lumen area returned to baseline (unstimulated). Data is the mean from 9 separate airways from 4 different mice, analyzed by One-way ANOVA (ns=not significant).

**Figure 4:** NMDA-R directly mediates contraction of HASM cells. (A) Contraction of collagen gels seeded with HASM cells stimulated with increasing concentration of NMDA or retained in culture medium alone (Control). Data for each time point is the mean ± SE of 3 independent experiments done in triplicate. Comparisons were made using two-way ANOVA with
Bonferroni’s post hoc test. *** P<0.001; and, ** P<0.01 compared to Control at each time point. No statistical difference existed between time-matched values for gels exposed to 0.1mM, 0.5mM or 1mM NMDA. (B) Time response curve showing effect on collagen gel contraction before and after treatment with NMDA-R antagonist D-AP5. Gels were either untreated (Control) (solid dots), pre-treated with 0.5mM D-AP5 for 2h then stimulated with 0.1mM NMDA (open triangles), or treated with 0.1mM NMDA alone (solid squares) then gel area size was measured. As a positive control some gels were treated with 0.1mM bradykinin (open circles). Data for each time point is the mean ± SE of 3 experiments done in triplicate. Comparisons were made using two-way ANOVA with Bonferroni’s post hoc test. ***, P<0.001; and *, P<0.05) compared to Control at each time point. (C) Scatter plots showing percentage decrease in collagen gel area at 3h (left) and 6h (right) in Controls (solid circles), gels treated with 0.1mM NMDA (solid squares) or 0.5mM D-AP5/0.1mM NMDA (solid triangles). Data were obtained from 3 independent experiments. Mean values for each group is designated as a horizontal line. Data were analyzed by One-way ANOVA with Bonferroni’s multiple comparison test. **P<0.01 for 0.1mM NMDA alone compared to Control and # P<0.05 for D-AP5/NMDA compared to 0.1mM NMDA. (D) Time response curve showing contraction in cell free collagen gels (open circles, dotted line), and gels containing HASM cells stimulated with 0.1mM MCh (open diamonds), 0.1mM histamine (open triangles, dashed line) or 0.1mM bradykinin (filled squares). Values are representative of 2 experiments.

**Figure 5: NMDA-R induces Ca^{2+} flux in HASM cells.** (A) Cultured HASM cells were serum-deprived for 48h, loaded with Fura-2AM, then temporal changes in free cytosolic Ca^{2+} concentration ([Ca^{2+}]_{i}) was measured by fluorescence microscopy. Cells were stimulated with
increasing concentrations of NMDA: 0.01mM (open triangles), 0.1mM (solid squares) and 0.5mM (open squares). [Ca^{2+}]_i was recorded for 4 minutes. Basal [Ca^{2+}]_i was determined prior to addition of agonists. The graph shows mean data + SE from 50-60 cells present within the microscopic field from HASM cultures in 4 separate experiments. (B) Histogram showing mean values +SE for basal (white) and maximum [Ca^{2+}]_i observed at 135s when HASM cells were stimulated with 0.01mM (black), 0.1mM (grey) and 0.5mM NMDA (lined). Values were determined from 4 independent experiments done in duplicate and analyzed by one-way ANOVA with Bonferroni’s multiple comparison test: ***P<0.001; *P<0.05; and ns (not significant) compared to basal. (C) Mean temporal tracings showing [Ca^{2+}]_i in HASM cells exposed to NMDA (0.1mM), pretreated with 0.5mM D-AP5 (2h) before exposure to NMDA (0.1mM); or treated with bradykinin (0.1mM). Data is from 5 independent experiments done in duplicate. (D) Histogram showing mean values +SE for basal (white) and maximum [Ca^{2+}]_i observed when HASM cells were stimulated with 0.1mM NMDA in the absence (black) and presence (grey) of 0.2mM glycine. Values were determined from 3 independent experiments and analyzed by one-way ANOVA with Bonferroni’s multiple comparison test: **P<0.01; and ns (not significant) compared to basal. (E) Histogram summarizing mean data +SE for [Ca^{2+}]_i under basal conditions (white), or as maximum [Ca^{2+}]_i in HASM cells treated with NMDA (0.1mM) (black), pre-treated with D-AP5 (0.5mM) prior to NMDA (0.1mM) exposure (grey), or cultures pre-incubated in Ca^{2+}-deficient buffer prior to treatment with NMDA (0.1mM) (lined). Data were obtained from 5 independent experiments done in duplicate and analyzed by One-way ANOVA with Bonferroni’s multiple comparison test. ***, P<0.001 compared to Basal, and ##, P<0.01 compared to NMDA (0.1mM). (F) Histogram summarizing mean data + SE for [Ca^{2+}]_i under basal (white), or as maximum [Ca^{2+}]_i in HASM cells treated with 0.1mM glutamate.
without glycine (black), or with 0.2mM glycine in the absence (grey) and presence of 0.5mM D-
AP5 prior to glutamate (0.1mM) exposure (vertical lined). Data were obtained from 3
independent experiments done in duplicate and analyzed by One-way ANOVA with
Bonferroni’s multiple comparison test. ***, P<0.001; compared to basal, and #, P<0.01
compared to glutamate (0.1mM, + glycine).

Figure 6: HASM cells exhibit heterogeneous temporal patterns for mobilization of intracellular
Ca^{2+} upon NMDA exposure. (A) Histogram showing distribution (%) of cells exhibiting different
temporal responses for Ca^{2+} mobilization in response to 0.1mM NMDA (examples shown in
6B). Tracings from ~30 randomly selected cells in a field were analyzed from 5 independent
experiments done in duplicate and individual cell response was categorized into one of four
groups: Early Responses (white), Late Responses (black), Phasic Responses (grey) or No
Response (lined). Analysis was done using One-way ANOVA with Bonferroni’s multiple
comparison test (B) Tracings showing representative (i, iii and v) and mean (ii, iv and vi) Ca^{2+}
responses upon NMDA stimulation for different cell populations. Following agonist stimulation,
cells showing Early responses (i and ii) mobilized [Ca^{2+}], within 10s, Late responses (iii and iv)
showed a response 100s later and Phasic responses (v and vi) showed Ca^{2+} spikes respectively.
Each mean curve represents data collated from at least 20 individual cells from 5 independent
experiments. Error bars show + SE.

Table 1: Summary of primer sequences used in qPCR – Primers were designed using Primer
Quest (IDT Technologies Inc) and verified by Primer Blast (NCBI). Specific amplification was
confirmed by PCR and one-dimensional agarose gel electrophoresis.
18S, 18S ribosomal RNA; F, forward; R, reverse
D

% lumen area decrease (over baseline)

1μM MCh  
0.1mM NMDA  
1μM MCh

ns  ns
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