Supplementary Material

Supplementary Methods

Immunofluorescence microscopy

For immunofluorescence analysis, cells were grown in 4-well plates in already described conditions. After incubation period, culture media was removed and cells were gently washed twice with cold PBS and fixed in ice-cold 100% Methanol for 10 minutes for vimentin staining or 4% Formol for 3 minutes and subsequently in 100% Methanol for 2 minutes at RT for ϐ-catenin staining. Blocking of non-specific binding was performed by incubation of cells in 1% BSA/PBS for 30 minutes. Cells were incubated with primary antibodies (both from BD Bioscience) against vimentin over night at 4°C or ϐ-catenin for 2 hours at RT and then with secondary rabbit-anti-mouse Alexa Fluor 488 or goat-anti-mouse Alexa Fluor 546 antibodies (Invitrogen), respectively, for 1 hour. Nuclear counterstaining was performed by 1% Hoechst for 10 minutes. Cells were examined using a LEICA Microsystems DFC480 fluorescent microscope with a Digital Camera System.

Live cell microscopy

HK-2 cells were plated on collagen type I (10 µg/ml) and fibronectin (30 µg/ml) - coated 48-well plate as previously described[1]and incubated for 24 hours in above described conditions. Prior to imaging, wells were completely filled with adequate medium and the plate was sealed using silicon grease and a glass plate. Images were

acquired using a 10×0.5 NA Plan objective lens and a 0.5 NA ELWD condenser with a Zeiss Axiocam camera on a Zeiss Axiovert 200 M microscope in climate-controlled incubator. A robotic stage (Zeiss MCU 28) with linear position feedback encoders was used to collect images at different stage positions over time. All electronic microscope functions were controlled using Axiovision software (Zeiss). Phase-contrast images were taken in rapid succession at multiple positions (3 positions/well for each condition), evenly distributed over the chambers to exclude differences due to experimental variation, at 3 minutes intervals for 5 hours.

Analysis of cell migration in time-lapse movies

To analyze cell behavior during migration, software was written in Matlab (Mathworks) by de Rooij J. and Danuser G.[1] that automatically segments phase-contrast images based on pixel intensity and determines the presence of nuclei (centroids) based on phase-density, size and shape. The velocity was calculated as the displacement (µm) over three consecutive frames, divided by the elapsed time (3 minutes). The persistence is defined as the ratio of the vectorial distance travelled to the total path length described by the cell. Detection fidelity in our experiments was over 80%, which was confirmed by eye for each individual time-lapse. To distinguish single cells from clustered cells in this program, areas occupied by the cells were determined by edge detection and overlaid with the detected nuclei to determine if one (single cell) or more (clustered cells) nuclei were present in a detected cell-area. Only cells that were faithfully tracked for at least six consecutive frames and stayed ñsingleò during that period of time were taken in consideration.

Expression of fusion protein GST-RBD Raf1 in *Escherichia coli*

The plasmid pGEX-RBD, encoding the Ras binding domain of Raf-1 fused to glutathione-S-transferase (GST-RBD) (provided by Dr. P. Crespo from the University of Cantabria, Spain) was transfected into *E. coli* (DH5Ŭ) and the GST-RBD expression was induced by 1 mM isopropyl-ɓ-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C. Transformed *E. coli* were harvested and resuspended in a solution containing 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM PMSF and protease inhibitor cocktail (Sigma Aldrich). Cells were lysed in the same buffer containing 0.5% Triton X-100 by sonication and centrifuged at 10.000 rpm at 4°C for 10 minutes. Glutathione Sepharose 4B beads (Amersham Bioscience) were washed thoroughly with PBS and incubated with the supernatant containing GST-RBD for 2 hours at 4°C. Beads coupled with GST-RBD were washed 3 times with PBS and resuspended in PBS (pH 7.4) buffer containing 20% glycerol and 0.5 mM PMSF. Adequate amount of bound GST-RBD was eluted by boiling in SDS sample buffer and subjected to SDS-PAGE electrophoresis and Coomassie blue staining. The band corresponding to the fusion protein was quantified by Quantity One software (Bio-Rad Laboratories) by comparing its intensity to the band intensities of known amounts of bovine serum albumin (BSA) run on the same gel.

Western blot analysis

Total cell lysates were obtained by washing the cell monolayer with cold PBS, scraping and suspending in lysis buffer (125 mM Tris (pH 6.8), 2%SDS, 2 mM PMSF and protease inhibitor cocktail). For the analysis of pAkt and pErk1/2 expression, after adequate treatments for 30 and 60 minutes, cells were collected in lysis buffer (20 mM

Tris (pH 7.5), 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 0.25% Na-deoxycholate, 1 mM EDTA, 10% glycerol, 1 mM Na_3VO_4, 2 mM PMSF and protease inhibitor cocktail). Kidney tissue was homogenized in lysis buffer (20 mM Tris (pH 7.4), 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 2 mM Na_3VO_4, 50 mM ϐ-glycerophosphate, 10 mM NaPPi, 100 U/ml aprotinin, 2 mM PMSF) using a polytron homogenizer. Homogenized tissue was incubated 20 minutes on ice and the supernatant was collected after centrifugation at 12.500 rpm at 4°C for 10 minutes. Protein concentration was determined using a DC protein assay kit (Bio-Rad). 20 μg (or 80-100 μg for NMDAR1 detection) of proteins were electrophoresed on 8%, 10%, 12% or 15% SDS-PAGE gels, as appropriate, and transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were probed with primary antibodies against E-cadherin (1:2500; BD Biosciences), NMDAR1 (1μg/ml; BD Biosciences), Snail1 (1:1000; Abcam), phospho-Smad2/3 (Ser 423/425) (1:1000) and Histone 1 (1:1000; Santa Cruz Biotechnology), α-SMA (1:24000) and α-tubulin (1:5000; Sigma), phospho-specific Akt (Ser 473) (1:2000) and total Akt (1:1000), phospho-specific Erk-1/2 (Thr 202, Tyr 204) (1:2000) and total Erk-1/2 (1:1000) (Cell Signaling Technology) or Ras (1:1000; Upstate Biotechnology) over night at 4ºC. Horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:10000. The immunoreaction was visualized using chemiluminescent kits EZ ECL (Biological Industries) or ECL Advanced (Amersham Biosciences). Images were digitally acquired by VersaDoc Imaging system Model 4000 (Bio-Rad). Positive immunoreactive bands were quantified by densitometry and compared with the expression of adequate loading control.

Semi-quantitative PCR and real time PCR

Total RNA was extracted from cultured cells or from whole kidney tissue with TRIzol reagent (Sigma Aldrich) and the final concentrations were determined by Nanodrop (ND-1000) spectrophotometer. Reverse transcription was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) according to the manufacturer instructions. Real time PCR amplification with gene-specific primers for mouse Ǔ-SMA and collagen I was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix. Forty cycles at 95°C for 15 seconds and 60ºC for 1 minute were performed. Every experiment was carried out three times, all samples were amplified in triplicate and data were normalized using mouse GAPDH as an endogenous control. Relative mRNA levels were calculated and expressed as fold induction over contralateral controls (value=1.0). For semi-quantitative PCR analysis of NMDAR subunits in HK-2 cells, the following oligonucleotide primers were used: human NMDA R1 (sense 5ô-AGA CGT GGG TTC GGT ATC AG-3ô and antisense 5ô-AGG ACC CAT CAG TGT CCT TG-3ô), NMDA R2A (sense 5ô-GTC CTT CTC CGA CTG TGA GC-3ô and antisense 5ô-ACT GCC CGT TGA TAG ACC AC-3ô), NMDA R2B (sense 5ô-GCC TGA GCG ACA AAA AGT TC-3ô and antisense 5ô-CAT CTC CCC ATC TCC AAA GA-3ô), NMDA R2C (sense 5ô-CGC TGG TCT TCA ACA ACT CA-3ô and antisense 5ô-GTC CTT GCC TGC CAT GTA GT-3ô), NMDA R2D (sense 5ô-TTC ACC ATT GGG AAA TCC AT-3ô and antisense 5ô-GGA TAG TTG CTG CGG ATG TT-3ô) and human GAPDH internal control (sense 5ô-GAA GGT GAA GGT CGG AGT-3ô and antisense 5ô-GAA GAT GGT GAT GGG ATT TC-3ô). 20 εL reactions contained 10x reaction buffer (2 εL), $MgCl_2$ (50 mM, 0.6 εL), dNTPôs (2 mM,

2 εL), sterile water (10.2 εL), Taq Polymerase (0.2 εL), NMDAR subunit primers (10 εM, 1 εL), GAPDH primers (5 εM, 1 εL), cDNA (1 εL). Reactions were run for 40 cycles in a termocycler Techne TC-412 at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1minute. Experiments were performed in triplicate and representative samples were presented.

Morphometric analysis of interstitial fibrosis and immunohistohemistry

For histological examination, paraffin-embedded kidney sections were stained with Masson-Trichrome and Sirius Red for interstitial collagen content. After deparaffinization and hydratation, sections of kidneys were incubated in picrosirius red solution (1% Sirius red in saturated picric acid) for 30 minutes at RT. This was followed by dehydratation in absolute ethanol. Quantification of collagen content was made by determining the % of staining area in 20 randomly chosen fields (x400) using Image-Pro Plus Software. Data are expressed as positive stained area vs. total analyzed area. Samples from each animal were examined in a blind manner.

Immunostaining for E-cadherin, Ŭ-SMA and FSP1 was carried out on 4 εm thick tissue sections that were deparafinized through xylene and rehydrated through graded ethanol concentrations (100%, 96%, 90% and 70%) and distilled water. For Ŭ-SMA staining, tissue sections were incubated with trypsin for 30 minutes at 37ºC and for E-cadherin and FSP1 staining, antigen retrieval was done in 10 mM citrate buffer (pH6) for 10 minutes. Endogenous peroxidase quenching (30 minutes incubation in 0.3% (v/v) H_2O_2/PBS)) was followed by blocking of non-specific binding for 1 hour at RT with 4% BSA/PBS. All primary antibodies were incubated overnight at 4ºC. After washing in

PBS, slides were treated with the corresponding anti-IgG biotinylated secondary antibody (Amersham Bioscience, Buckinghamshire, England/or Vector Laboratories) followed by the avidin-biotin-peroxidase complex (Dako, Dako Diagnósticos, Barcelona, Spain/ or Vector Laboratories) and 3,3ô-diaminobenzidine as chromogen. Sections were counterstained with Carazziôs hematoxylin. Negative controls were performed by incubation with non-specific immunoglobulin of the same isotype as the primary antibody but with the omission of primary antibody. After immunostaining, slides were dehydrated, cleared in xylene and mounted with DPX permanent mounting medium.

Viability assay

An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, according to the method previously described[2], was performed on treated and untreated HK-2 cells for 24, 48 and 72 hours in order to determine the range of NMDA concentrations to be used.

Measurements of cell volume

For cell volume measurements, HK-2 cells were cultured in 6-well plates in media with or without NMDA (0.5 mM). After 2 hours and 24 hours, cells were washed with PBS, trypsinized and resuspended in the culture media. Cell volume was measured in Coulter Counter (Model Z2).

Supplementary Figure Legends

Supplementary Figure 1. Effects of NMDA treatment on cell viability and cell volume. Number of viable HK2 cells determined by MTT assay after treatment with various concentrations of NMDA at different time points (A, B). Data are presented as a percentage of control (means values ± SEM). None of the concentrations tested had statistically significant effect on cell viability except very high concentration of 10 mM NMDA (B) *P<0.05 vs. control. (C) NMDA treatment (2 hours) did not have statistically significant effect on the volume of HK2 cells. Cell volume was measured by Coulter. Histograms show means values ± SEM.

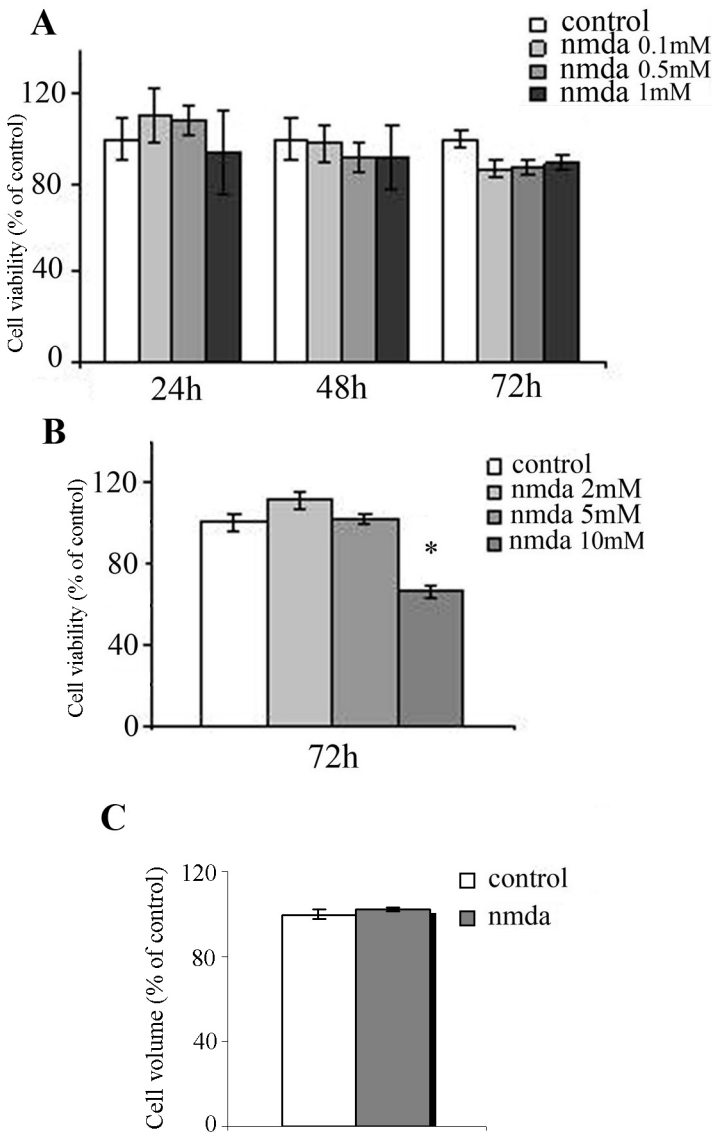
Supplementary Figure 2. NMDA reduced basal and TGF-ß1-stimulated cell velocity and persistence on different matrices. HK-2 cells were plated on Collagen I (Cn) or Fibronectin (Fn)-coated 48-well plates and incubated with different treatments as described in Methods. Cellôs migratory behavior was recorded by time-lapse video microscopy during a 5 hours period (3 minutes frame interval) after 24 hours of treatment. Cell persistence and velocity were quantified using Matlab software. Persistence is defined as the ratio of the vectorial distance traveled to the total path length described by the cell. Cell densities of all populations were equivalent (not shown). (A) Histograms represent the average persistence during 0-300 minutes period after 24 hours of treatment. Values are average cell persistence (averaging values from all time-points during 0-300 minutes) ± SEM. (B) Average cell velocities of cells (avg. values ± SEM)

grown on Coll I and Fn followed over the 0-300 minutes period after 24 hours of treatment. *P<0.05 vs. control; #P<0.05 vs.TGF-β1.

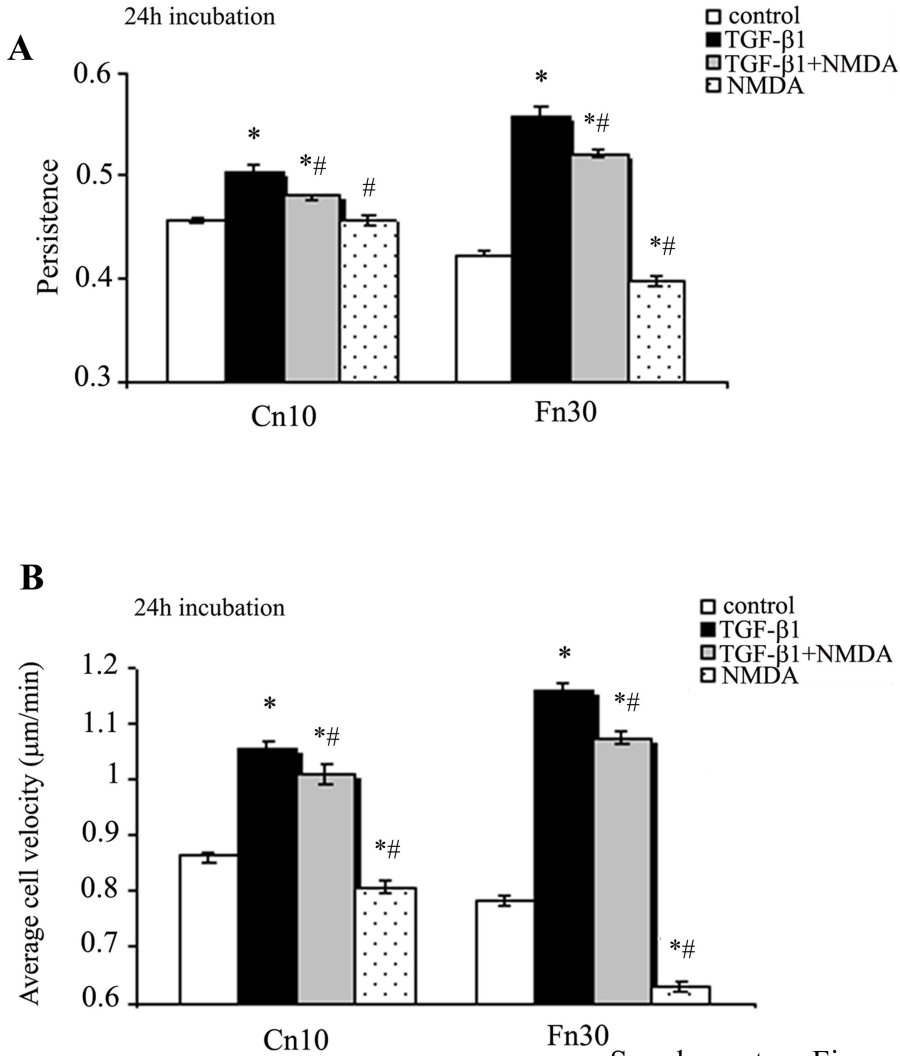
Supplementary References

1. de Rooij J, Kerstens A, Danuser G, Schwartz MA, Waterman-Storer CM: Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol* 171:153-164, 2005

2. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47:936-942, 1987



Supplementary Figure 1



Supplementary Figure 2