Podocyte Glutamatergic Signaling Contributes to the Function of the Glomerular Filtration Barrier

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ABSTRACT
Podocytes possess the complete machinery for glutamatergic signaling, raising the possibility that neuron-like signaling contributes to glomerular function. To test this, we studied mice and cells lacking Rab3A, a small GTPase that regulates glutamate exocytosis. In addition, we blocked the glutamate ionotropic N-methyl-d-aspartate receptor (NMDAR) with specific antagonists. In mice, the absence of Rab3A and blockade of NMDAR both associated with an increased urinary albumin/creatinine ratio. In humans, NMDAR blockade, obtained by addition of ketamine to general anesthesia, also had an albuminuric effect. In vitro, Rab3A-null podocytes displayed a dysregulated release of glutamate with higher rates of spontaneous exocytosis, explained by a reduction in Rab3A effectors resulting in freedom of vesicles from the actin cytoskeleton. In addition, NMDAR antagonism led to profound cytoskeletal remodeling and redistribution of nephrin in cultured podocytes; the addition of the agonist NMDA reversed these changes. In summary, these results suggest that glutamatergic signaling driven by podocytes contributes to the integrity of the glomerular filtration barrier and that derangements in this signaling may lead to proteinuric renal diseases.


It is widely recognized that most glomerular diseases are characterized by defects of the filtration barrier, where podocytes play a central role. Mutations of single podocyte proteins have been found at the basis of human nephrotic syndromes, and podocyte deletion of the same molecules causes heavy proteinuria in experimental models.2–8 Podocytes are highly ramified cells: From the cell body depart a number of primary processes, further originating secondary foot processes. Starting from these features, it has been demonstrated that podocytes share numerous similarities with neurons: They both are terminally differentiated cells, have a common cytoskeletal organization, and have a common machinery of process formation.9 Furthermore, a number of expression-restricted proteins, such as nephrin,2 Neph1 and Neph2,10 GLEPP1,11 CAT3 and EAAT2,12 synaptopodin,13

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Glutamate exocytosis is a property of mature podocytes. (A) Developmental appearance of Rab3A and Rabphilin-3A. Compared with nephrin and synaptopodin, expressed in developing glomerular capillaries in newborn mice, Rab3A and rabphilin-3A stainings are completely negative (top, 1-d-old mouse). Both Rab3A and rabphilin-3A are positive in completely mature glomerular structures of a 30-d-old mouse (bottom). Indirect immunofluorescence: nephrin, bar = 100 μm; synaptopodin, Rab3A, and rabphilin-3A, bar = 50 μm; bottom, bar = 50 μm. (B) Glutamate exocytosis during podocyte differentiation. Glutamate was measured in the culture supernatant as expression of NADH generation at the beginning of the experiment (0) and every 10 min up to 40 min (time points on the x axis). Data come from three different sets of experiments, and results are expressed as percentage variation from the baseline (y axis, mean ± SD). (a) Spontaneous (□) and α-LTX–stimulated (■) glutamate exocytosis do not take place in undifferentiated podocytes grown at 33°C in presence of γ-IFN (see the Concise Methods section). (b) Differentiated podocytes display spontaneous (□) glutamate release at 40 min. Nanomolar α-LTX addition causes glutamate release at increasing concentrations (■). As it occurs in neuronal cells,20,21 regulated exocytosis is greater than spontaneous glutamate release (*P = 0.05; **P < 0.01). Magnifications: ×200 in A, top, nephrin; ×630 in A, top, synaptopodin, Rab3A, and rabphilin-3A; ×400 in A, bottom.

RESULTS

Glutamate Release Is a Property of Mature Podocytes

We first looked at the expression of Rab3A, a marker of mature synapses,18 and its effector rabphilin-3A in developing glomeruli of newborn mice. Different from other podocyte molecules, such as nephrin and synaptopodin, which are fully expressed by day 1 after birth, Rab3A and rabphilin-3A were still negative in developing glomeruli of 1-d-old mice and appeared subsequently, mainly by day 30, in mature glomeruli (Figure 1A), where podocyte location was confirmed by podocin co-labeling (Supplemental Figure 2).

These data suggested Rab3A and rabphilin-3A are part of a system needed by mature glomeruli; therefore, we analyzed glutamate release during podocyte differentiation by using a conditionally immortalized podocyte cell line,19 derived from the H-2Kb-tsA58 transgenic mouse, in which the SV40 large T antigen is both temperature and γ-IFN dependent.

In neurons, spontaneous release typically occurs with a rate of one to two vesicles per minute per release site,20 whereas evoked release occurs at a rate of >100 vesicles/s.21 We evoked stimulated exocytosis by α-latrotoxin (α-LTX), a presynaptic neurotoxin widely used to trigger synaptic vesicles exocytosis.22

Undifferentiated podocytes, maintained at 33°C with γ-IFN, are proliferating cells that never exhibit a podocyte phenotype. In up to 40-min observation, these cells did not show glutamate release at 40 min. Nanomolar α-LTX−stimulated (●) glutamate release. As it occurs in neuronal cells,20,21 regulated exocytosis is greater than spontaneous glutamate release (*P = 0.05; **P < 0.01). Magnifications: ×200 in A, top, nephrin; ×630 in A, top, synaptopodin, Rab3A, and rabphilin-3A; ×400 in A, bottom.

Figure 1. Glutamate exocytosis is a property of mature podocytes. (A) Developmental appearance of Rab3A and Rabphilin-3A. Compared with nephrin and synaptopodin, expressed in developing glomerular capillaries in newborn mice, Rab3A and rabphilin-3A stainings are completely negative (top, 1-d-old mouse). Both Rab3A and rabphilin-3A are positive in completely mature glomerular structures of a 30-d-old mouse (bottom). Indirect immunofluorescence: nephrin, bar = 100 μm; synaptopodin, Rab3A, and rabphilin-3A, bar = 50 μm; bottom, bar = 50 μm. (B) Glutamate exocytosis during podocyte differentiation. Glutamate was measured in the culture supernatant as expression of NADH generation at the beginning of the experiment (0) and every 10 min up to 40 min (time points on the x axis). Data come from three different sets of experiments, and results are expressed as percentage variation from the baseline (y axis, mean ± SD). (a) Spontaneous (□) and α-LTX–stimulated (■) glutamate exocytosis do not take place in undifferentiated podocytes grown at 33°C in presence of γ-IFN (see the Concise Methods section). (b) Differentiated podocytes display spontaneous (□) glutamate release at 40 min. Nanomolar α-LTX addition causes glutamate release at increasing concentrations (■). As it occurs in neuronal cells,20,21 regulated exocytosis is greater than spontaneous glutamate release (*P = 0.05; **P < 0.01). Magnifications: ×200 in A, top, nephrin; ×630 in A, top, synaptopodin, Rab3A, and rabphilin-3A; ×400 in A, bottom.

drebrin,14 and Sam68-like-MP2,15 specifically belong to the podocyte and the neuron.

Our group has contributed to this line of research, initially by describing in podocytes the presence of Rab3A, a small GTPase that is mostly enriched in synaptic vesicles because it tightly modulates highly regulated exocytosis by acting through a number of effector molecules, including rabphilin-3A and Synapsin-I.16 After finding that in podocytes, as it occurs in neurons, Rab3A associates to glutamate-containing vesicles along cell processes, we discovered that podocytes are equipped with a complete neuron-like glutamatergic signaling system.17 We described that podocytes possess functional synaptic-like microvesicles and renal glomeruli express cognate glutamate transporters and receptors. These properties strengthened the analogies between podocytes and neurons and offered a rational interpretation to the biochemical similarity of foot process and synaptic adhesion complexes17; how-ever, the role played by glutamate signaling in podocytes remained unanswered, and nothing was known about its relevance to podocyte and glomerular homeostasis. To get more details on the requirement of this neuron-like system of signaling by podocytes, we first conducted a preliminary analysis on its temporal appearance during podocyte differentiation. Then we studied conditions in which it was altered, on the vesicle and on the receptor side. The vesicular component was analyzed by studying the consequences of the absence of Rab3A. On the receptor side, we antagonized the ionotropic N-methyl-D-aspartate receptor (NMDAR), that we found present in human and rodent glomeruli, as well as in podocyte cell cultures.17 Both Rab3A and NMDAR1 glomerular synthesis were also confirmed by in situ hybridization (Supplemental Figure 1) and by microarray expression data.17

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any glutamate release, either spontaneous or stimulated (Figure 1B, a).

By growing 15 d at 37°C without γ-IFN, cells stop to divide and differentiate to a mature podocyte phenotype. Their rate of spontaneous glutamate release was very mild and not measurable until 40 min. Addition of α-LTX elicited an outburst of glutamate, measurable at 10 min and progressively increasing (Figure 1B, b).

Rab3A-Knockout Model

Then we analyzed the possible appearance of a renal phenotype in Rab3A-null mice. Actually, Rab3A absence from podocytes became symptomatic by 3 mo of age, when Rab3A-knockout (KO) mice displayed macroalbuminuria (albuminuria/creatininuria ratio \( \frac{U_{\text{Alb}}}{U_{\text{Creat}}} \) = 302 ± 38 μg/mg; mean ± SD) that persisted over time, whereas lower values were detectable in wild-type (WT) mice (\( \frac{U_{\text{Alb}}}{U_{\text{Creat}}} \) = 157 ± 33 μg/mg; \( P = 0.03 \); Figure 2A).

KO and WT 3-mo-old kidneys did not show abnormalities by light microscopy (Figure 2B, a and b, e and f), whereas transmission electron microscopy demonstrated segmental areas of podocyte foot process effacement in KO glomeruli (Figure 2B, c; Supplemental Figure 3). By scanning electron microscopy, KO podocytes showed a disorganized, less ordered foot process structure (Figure 2B, d) than WT cells (Figure 2B, h). Focal and segmental decrease of podocyte proteins was detectable by immunofluorescence in KO glomeruli (Supplemental Figure 4, A through F) but was not accompanied by reduction of podocyte number (Supplemental Figure 4G). Overall, podocyte changes in Rab3A-KO mice offered a morphologic support to the evidence of macroalbuminuria; therefore, to understand the consequences of Rab3A absence in podocytes, we compared glutamate exocytosis in primary podocytes from Rab3A-WT and KO animals.

A mild spontaneous release of glutamate from WT podocytes became detectable only after 40 min, and nanomolar concentrations of α-LTX (2.5 nM) caused a glutamate release measurable at 10 min and increasing with time (Figure 3A, a). Instead, KO podocytes demonstrated a spontaneous glutamate release already detectable at 10 min and gradually decreasing with time, but they did not show any response after α-LTX application (Figure 3A, b).

These results were confirmed by experiments in which recycling vesicles were monitored by the styryl dye FM1-43. Styrly dyes reversibly insert into the surface of lipid membranes and have no fluorescence properties in aqueous solution but become intensely fluorescent on membrane binding, allowing labeling of recycling vesicles that is detectable by fluorescence microscopy. In 20-min observation, WT podocytes did not become fluorescent when the dye was added alone, whereas a bright fluorescence signal appeared after α-LTX addition. Different from WT cells, KO podocytes became immediately fluorescent after addition of the dye, but the subsequent addition of α-LTX did not produce changes in cell fluorescence (Figure 3B). To analyze further the different behavior of WT and KO podocytes, we studied the expression of Rab3A effectors rabinphilin-3A and Synapsin-I. Rab3A exerts its role by switching

![Figure 2.](https://www.jasn.org)
between a GDP-cytosolic form to a GTP vesicle–linked form, which interacts with multiple effectors. When GTP-Rab3A recruits rabphilin-3A and Synapsin-I, they control vesicle motility by regulating vesicle-actin association. Rabphilin-3A accomplishes this task by interacting with α-actinin,23 and Synapsin-I acts by switching between a phosphorylated and a dephosphorylated form, respectively releasing or anchoring vesicles to actin.24

Compared with WT cells, Rab3A-KO podocytes displayed decreased expression of both effectors (Figure 4, A through C), but despite total Synapsin-I reduction, KO podocytes had more phosphorylated Synapsin-I than WT cells (Figure 4D).
Rabphilin-3A and Synapsin-I downregulation were confirmed by immunofluorescence in KO glomeruli (Figure 4, E and F), and higher expression of phospho-Synapsin-I was confirmed in KO animals by immunogold electron microscopy (Supplementary Figure 5).

**NMDAR Blockade as a Model of Receptor Alteration**

To block the NMDAR, we alternatively used norketamine hydrochloride or dizocilpine maleate (MK-801), which are selective, noncompetitive antagonists that act by binding to the phencyclidine binding site located within the ion channel, thereby preventing Ca$^{2+}$ flux. We first applied the antagonists on cultured cells, namely primary podocytes from Balb/c mice and the differentiated podocyte cell line.

Either norketamine or MK-801, applied for 30 min at the concentration of 50 and 10 μM, respectively, was sufficient to cause a profound remodeling of podocyte cytoskeleton, as evidenced by almost complete disappearance of actin stress fibers and myosin-IIA redistribution (Figure 5A, a, b, d, and e). These changes were accompanied by disappearance of nephrin from podocyte processes (Figure 5A, g and h) and, similar to neuronal cells, by an increased expression of NMDAR1 (Figure 5B). We were able almost to completely reverse cytoskeletal remodeling and nephrin redistribution when incubation with norketamine was followed by addition of the agonist NMDA, applied at a concentration of 50 μM for 15 min (Figure 5A, c, f, and i).

NMDAR is a calcium channel tightly linked to the actin cytoskeleton. At the synapse, NMDAR activation leads to intracellular calcium rise that activates calmodulin-dependent protein kinase II (CaMKII) by phosphorylation. In turn, phospho-CaMKII phosphorylates a series of downstream mole-

![Figure 5.](image-url)

**Figure 5.** NMDAR in vitro blockade. (A) Effects on the cytoskeleton and nephrin expression. (a, b, d, e, g, and h) Compared with cells incubated with medium (a, d, and g), application of the NMDAR antagonist norketamine hydrochloride produces remodeling of the actin (b) and myosin II A (e) cytoskeleton and redistribution of nephrin (h), which disappears from cell processes. (c, f, and i) The addition, after the antagonist, of the agonist NMDA, abolishes almost completely the agonist’s effects. Phalloidin-TRITC (a through c); indirect immunofluorescence (d through i); bars = 50 μm. (B) Effects on NMDAR1 expression. (a and b) Compared with cells incubated with medium (a), addition of norketamine hydrochloride (b) determines an increased expression of the NMDAR1. Indirect immunofluorescence, bars = 50 μm. (c) Cells incubated with medium (lanes 1 through 3) express less NMDAR1 (band of approximately 100 kD) than cells treated by norketamine hydrochloride (lane 4). The lower band of 50 kD represents tubulin (loading control). (C) Downstream effects of the NMDAR blockade. Compared with cells incubated with medium (a and b, lane 1), phosphorylated CaMKII (a) and phosphorylated cofilin (b) are reduced, as compared with total CaMKII and total cofilin, after 15 (lane 2) and 30 min (lane 3) of treatment with norketamine hydrochloride. The graphs show a densitometric representation of Western blot results obtained from three experiments, where the ratio of lane 1 is taken at the arbitrary value of 100. Magnification, ×400.
cules, including cofilin, thereby regulating actin dynamics. Western blot analysis of CaMKII and cofilin in cultured podocytes, after 15 and 30 min of incubation with the NMDAR antagonist, showed at both time points a reduced CaMKII phosphorylation and reduced phosphorylation of cofilin (Figure 5C), likely responsible for actin remodeling.

To establish whether the NMDAR blockade had a direct effect on glomerular permeability, we measured differences in convective glomerular albumin permeability (PAlb) on isolated rat glomeruli incubated with either norketamine or MK801 at various concentrations and time points. Both antagonists, particularly at the same concentrations and times used on cultured cells, were able to increase significantly glomerular PAlb (Figure 6, A through C). The effect of norketamine was completely reversed when glomeruli were subsequently incubated with NMDA (50 μM for 15 min), whereas only a mild effect was achieved when NMDA followed the more potent MK-801 (Figure 6D); therefore, we moved to the in vivo approach, by intraperitoneal norketamine injection in 16 Balb/c mice for 3 days at a concentration of 0.3 mg/100 g, which is at least 20 times less than the anesthetic dosage and one 10th the concentration required to induce psychotic symptoms in rodents. Sixteen control mice were administered an injection of saline. 

U_{Alb}/U_{Creat}, evaluated on 24-h urine, increased after norketamine treatment (Figure 7A). Histology did not show major glomerular alterations; however, immunofluorescence demonstrated a globally decreased expression of nephrin (Figure 7B) in treated animals.

Norketamine hydrochloride is the major metabolite of ketamine, a general anesthetic optionally included in many routine protocols. A prospective pilot study was conducted to verify whether also in humans its application could be related to U_{Alb}/U_{Creat} differences. According to the inclusion criteria (see the Concise Methods section), 43 patients were initially enrolled. Three patients were excluded because of per-procedure hemodynamic instability. A final cohort of 40 patients was analyzed. Ketamine+ and ketamine− groups were comparable in terms of age, body mass index, estimated GFR, baseline U_{Alb}/U_{Creat}, and mean arterial pressure at H0 (before anesthesia) and H1 (1 h after anesthesia; Table 1). Percentage of celioscopic surgery (68% in ketamine+ versus 71% in ketamine−; P = 0.89), use of isoflurane for anesthesia maintenance (33 versus 29%; P = 0.99), and aminoglycosides (13 versus 12%; P = 0.89) were comparable as well. Although not reaching statistical significance (P < 0.16), ketamine+ subjects had higher H1/H0 of U_{Alb}/U_{Creat} than ketamine−, with a median multiplication factor in U_{Alb}/U_{Creat} between H0 and H1 of 9.0 (4.3 to 15.6) in ketamine+ and 4.2 (2.7 to 8.8) in ketamine− (Figure 8).

Figure 6. (A and B) Effects of NMDAR blockade on PAlb. Application of growing dosages of norketamine hydrochloride (A) and MK-801 (B) on isolated glomeruli (see the Concise Methods section) has a dosage-dependent effect on PAlb, compared with the application of medium alone (Ctrl). (C) The same concentrations used in the in vitro experiments produce effects at different times: 50 μM norketamine (□) changes PAlb when applied for at least 30 min, whereas 10 μM of the more potent MK-801 (■) becomes effective already after 10 min. (D) The addition of the antagonist NMDA at a dosage of 50 μM, and applied for 15 min, completely abolishes norketamine effects, whereas it only mildly reduces the action of MK-801. *P < 0.005 versus Ctrl; **P < 0.001 versus Ctrl.
DISCUSSION

Glutamate is the major excitatory neurotransmitter in the central nervous system, and its signaling at synapses has been extensively studied. Meanwhile, evidence gathered during the past decade has shown the existence of functional glutamate signaling in several non-neuronal cells, such as osteoblasts, pancreatic islet cells, and megakaryocytes. We previously observed that podocytes are equipped with the necessary vesicular and receptor apparatus to use glutamatergic transmission, and here we show that glutamatergic signaling driven by podocytes seems relevant to the maintenance of glomerular filter integrity, because its dysregulation is accompanied by podocyte alterations and increased albuminuria.

The question arising is why cells other than neurons would use glutamate for signaling. As for the glomerulus, we know that the entire blood volume passes the filter every 5 to 6 min, meaning that the glomerular barrier is constantly exposed to continuous physical (BP) and chemical (blood content) microenvironmental changes; therefore, effectiveness in maintaining filter homeostasis highly depends on efficient intercellular communication. Glutamate perfectly fits the need because, different from other types of intercellular communication, it has the advantage of being highly regulated.

Table 1. Clinical features

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ketamine+ (n = 20)</th>
<th>Ketamine− (n = 20)</th>
<th>P</th>
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<tr>
<td>Age (yr)</td>
<td>39 (32 to 50)</td>
<td>42 (32 to 50)</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>22.5 (20.6 to 26.2)</td>
<td>22.3 (20.5 to 26.2)</td>
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<tr>
<td>Baseline creatininemia (µmol/L)</td>
<td>61.0 (56.8 to 64.2)</td>
<td>59.5 (52.0 to 64.0)</td>
<td>0.86</td>
</tr>
<tr>
<td>MDRD eGFR (ml/min per 1.73 m²)</td>
<td>100 (95 to 117)</td>
<td>107 (89 to 125)</td>
<td>0.79</td>
</tr>
<tr>
<td>H0 MAP (mmHg)</td>
<td>97 (88 to 103)</td>
<td>93 (80 to 110)</td>
<td>0.37</td>
</tr>
<tr>
<td>H1 MAP (mmHg)</td>
<td>83 (73 to 90)</td>
<td>83 (72 to 93)</td>
<td>0.97</td>
</tr>
<tr>
<td>H0 UAlb/UCreat (mg/mmol)</td>
<td>0.8 (0.5 to 1.3)</td>
<td>0.7 (0.5 to 1.5)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

*Characteristics of patients, according to the use of ketamine. Data are median (25th to 75th percentiles). Values in Ketamine+ and Ketamine− groups are compared by Mann-Whitney U test. eGFR, estimated GFR; MAP, mean arterial pressure.
Processes of highly regulated exocytosis involve a complex interplay among numerous proteins that coordinate vesicle transport, docking, priming, fusion, and recycling events, most of them subjected to fine modulation. Among modulating factors, one of the best studied is Rab3A, a versatile catalyst that tightly controls vesicle release probability.33 In neurons, Rab3A is an important late element in the establishment of the mature characteristics of presynaptic terminals.18 Rab3A is also considered a marker of maturation in cultured pancreatic rudiments41; therefore, its appearance in 9th and 90th percentiles, respectively.

Figure 8. Effects of ketamine administration during general anesthesia. The graph illustrates the change after anesthesia (H1/H0) of the UAlb/UCreat values (mg/mmol) in patients who received ketamine (ketamine+) compared with those who did not (ketamine−). The horizontal lines inside the boxes represent median values, whereas bottom and top edges of the boxes represent the 25th and 75th percentiles and bottom and top whiskers reach the 10th and 90th percentiles, respectively.

Absence of Rab3A from KO glomeruli manifests itself with a relatively mild renal phenotype, witnessed by steady macroalbuminuria, which is in keeping with the very mild neurologic phenotype of these animals.35 but, different from neurons, where synapse morphology looks intact,36 podocytes display alterations detectable by transmission electron microscopy and scanning electron microscopy. Immunofluorescence integrates these findings, showing focal segmental decrease of podocyte proteins and providing molecular support to the evidence of macroalbuminuria. From these data, we can therefore argue that a tight modulation of glutamate exocytosis, such as the one performed by Rab3A, may be required for the correct functioning of the filtration barrier.

Our in vitro studies seem to help in understanding the type of alteration determined by Rab3A absence. Different from WT cells, Rab3A-KO podocytes display a sustained spontane-
was shown that the very same pathway plays an important role in dendritic spine remodeling and specifically requires NMDAR, because analogue experiments performed on AMPA receptors do not have the same effects.50

The specificity of effects caused by NMDAR blockers and the similarity of events occurring in podocytes and neurons seem further proved by their reversibility after addition of the specific agonist NMDA and by the rebound NMDAR increase. This NMDAR increase is so rapid that it can be achieved only by trafficking of the receptor itself along the cytoskeleton; at least in neurons, actin remodeling is thought to serve exactly to this purpose.51

Together with these features, podocytes specifically displayed disappearance of nephrin from podocyte processes, a variation that prompted our subsequent experiments on isolated glomeruli and in vivo. Changes in nephrin expression have been shown to accompany or even precede most forms of experimental and human proteinuric diseases,52 and, indeed, nephrin changes were the only podocyte alterations accompanying the increased albuminuria in norketamine-treated animals. Although the extremely low dosage and duration of norketamine treatment in vivo guarantee the absence of major neurologic consequences and stand for the action of norketamine on the glomerulus, the experiments performed on isolated glomeruli are those providing evidence of the direct effect of NMDAR antagonists on glomerular filtration. The usefulness of the method has been repeatedly proved in different experimental settings.53,54 In this work it even confirmed the predicted stronger, less reversible action of MK-801.

In summary, our data seem to provide evidence in favor of the hypothesis that glutamatergic signaling driven by podocytes is relevant to the integrity of the filtering barrier, because its derangements are accompanied by podocyte changes and increased albuminuria. Analogue results have been shown in other cells, such as osteoblasts, in which NMDAR antagonists inhibit differentiation and matrix mineralization,55 leading us to speculate that highly specialized cells, requiring continuous modulation of their activity, rely on the spatial and temporal precision of glutamate to finely tune their most regulated functions. Although preliminary and conducted on a limited number of cases, the human study adds to our observations an initial evidence of the potential importance of this system in the clinical setting and certainly suggests the usefulness of further investigation in this direction.

CONCISE METHODS

Animal Models
All rodent protocols strictly adhered to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Milan University Institutional Care and Ethical Treatment Committee. All animals were housed on a 12-h light/dark cycle, and allowed free access to food and water. Balb/c mice and homozygous H-2Kb-tsA58 transgenic mice were acquired from Charles River Laboratories Italia (Calco, Lecco, Italy). Sprague-Dawley rats were from Harlan (S. Pietro al Natisone, Udine, Italy).

Rab3A KO mice (Rab3A-KO; Rab3A+/−; B6;129S-Rab3atm1Sud/j) and the appropriate WT (Rab3A-WT, Rab3A+/+; B6;129SF2/J) were from Jackson Laboratory (Bar Harbor, ME). Backcrosses were generated, and the offspring were genotyped using the following primers: KO forward GCC GCA CGG AGA AGA AGA ATA GG; KO reverse CGG TGG GCT CTA TGG CTT CTG A (amplification product 900 bp); WT forward GCG CAA CCC AGG TCC ACA CA; WT reverse ACG CAC AAG CCT CCC GCA AG (amplification product 210 bp). Genotyping of the H-2Kβ-tsA58 transgenic was conducted by the following primers: forward GTG ACA CCA CAG AAG TG TGT GTC GCT GCC ATT GTA TTC.

Podocyte Cell Cultures
For primary cultures, kidneys were taken from 7- to 10-d-old rodents, as described previously.17 Briefly, glomeruli were isolated by sieving, then seeded in culture flasks (Corning, Sigma-Aldrich, Milan, Italy) precoated with collagen type IV (Sigma-Aldrich) at 37°C in 5% CO2 atmosphere. After 1 wk, first-passage podocytes were separated from glomeruli by an additional sieving through the 36-μm mesh. Second-passage podocytes were seeded on flasks and thermostored coverslips (Nunc, VWR Int., Milan, Italy). Cell characterization was performed by morphology and immunofluorescence, using podocyte (nephrin, podocin), epithelial (cytokeratins), smooth muscle (α-smooth muscle actin), and endothelial cell (CD31) markers.

For the conditionally immortalized cell line, we followed with minor modifications the procedure originally published by Mundel et al. Briefly, glomeruli were isolated from kidneys of adult (10-wk-old) transgenic H-2Kβ-tsA58 mice by the same procedure as described already and grown first in standard medium at 37°C. Second-passage cells were replated and propagated at 33°C in medium containing 20 μM recombinant mouse γ-IFN (Sigma-Aldrich). Limiting dilution (10.0, 1.0, and 0.1 cells/vial) of these cells was followed by characterization, and only five clones expressing WT1 and nephrin on >90% of cells were selected and propagated. For initiation of differentiation, cells were thermo-shifted to 37°C and maintained in medium without γ-IFN.

Spontaneous and Stimulated Glutamate Release Assay
As described previously,17,56 glutamate release was detected by an enzymatic assay based on the following reaction that occurs in the presence of glutamate dehydrogenase: Glutamate− + NAD+/H2O → ketoglutarate2− + NADH + NAD4+/H+ (all reagents from Sigma-Aldrich).

In brief, podocytes were plated and grown to semiconfluence. Before measurements, cells were thoroughly washed and incubated for 1 h at 37°C in DMEM-F12 buffer containing 1 mM MgCl2 and 20 mM HEPES. The medium was further supplemented with glutamate dehydrogenase (60 U/ml) and NAD+(1 mM) and incubated for 5 min. Then, either medium (to evaluate spontaneous exocytosis) or 2.5 mM α-LTX was added, and spectrophotometric increase of OD due to increase of NADH was monitored at 340 nm every 10 min. Results were expressed as percentage variations from the baseline.
In Vivo Monitoring of Recycling Vesicles
The styryl dye FM1–43 (Invitrogen, S. Giuliano Milanese, Italy), which has no fluorescence properties in aqueous solution but becomes fluorescent on membrane binding, allows the labeling of recycling vesicles that is easily detectable by fluorescence microscopy.57 In our experiments, the dye was added to the cell medium at a 2-μM concentration. Cells were maintained under observation for the whole duration of the experiment by the AxioVert 40 microscope (Zeiss, Arse, Mi, Italy) equipped for immunofluorescence. Dye fluorescence (wave length excitation 479; emission 598) of cells incubated in medium and after addition of 2.5 nM α-LTX was recorded by a digital videocamera (AxioCam MRC; Zeiss), with acquisition parameters settled by the “live” module of the software AxioVision (Zeiss).

Animal Studies
See supplemental material.

Immunofluorescence Studies
See supplemental material.

Immunogold Electron Microscopy
An indirect immunogold labeling procedure was performed on ultrathin frozen kidney sections, as described previously.16 Briefly, after blocking, the material was incubated with the primary antibody followed by the proper secondary gold-conjugated goat anti-rabbit or goat anti-mouse IgG (H+L) 12 nm (Jackson ImmunoResearch Europe, Suffolk, UK).

Specificity of antibody labeling was demonstrated by the lack of staining after substituting proper control Igs (Invitrogen) for the primary antibody.

Western Blot
See supplemental material.

Convective Glomerular PAlb Test
As described previously,58 Sprague-Dawley rat glomeruli were isolated by sieving from the renal cortex in isotonic PBS, and 5 g/dl BSA was added to the medium as an oncotic agent. Glomeruli were videotaped through an inverted microscope before and after the medium exchange from 5 to 1 g/dl BSA, used to create an oncotic gradient across the basement membrane, that resulted in a measurable glomerular volume change [ΔV = (Vfinal − Vinitial)/Vinitial], which could be measured off-line by a video-based image analysis software (Sigma Scan Pro) that determines the area of the glomerulus in the two-dimensional space and calculates the volume by a mathematical formula.

Because there is a direct relationship between the increase in glomerular volume (ΔV) and the oncotic gradient (ΔΠ) applied across the capillary wall, this principle was applied to calculate σAlb, using the ratio of ΔV of experimental to ΔV of control glomeruli in response to identical oncotic gradients: σAlb = ΔV_{experimental}/ΔV_{control}. Convective PAlb was therefore defined as (1 − σAlb) to describe the movement of albumin consequent to water flow. When σAlb is 0, albumin moves at the same rate as water and PAlb is 1.0. Alternatively, when σAlb is 1.0, albumin cannot cross the membrane with water and PAlb is 0. It is assumed that the experiment is positive to PAlb test when values are ≥0.5.

NMDAR Blockade
The glutamate receptor antagonist norketamine hydrochloride (Tocris Bioscience, Bristol, UK) was diluted into the cell medium at the concentration of 50 μM for 30 min, whereas the more potent antagonist MK801 (Tocris Bioscience) was added at a concentration of 10 μM. In some experiments, the agonist NMDA (Tocris Bioscience) was added at a concentration of 50 μM for 15 min.

Human Study
The human study design was in accordance to the Declaration of Helsinki. After informed consent to the study, consecutive patients scheduled for gynecologic surgery were prospectively included during a period of 2 mo. All patients were orally premedicated with 1.5 mg/kg hydroxyzine, then randomly assigned to receive intraoperative low-dose hydromorphone (intravenous bolus of 0.15 mg/kg; ketamine + group) for preemptive analgesic effect or nothing (ketamine– group), during standardized induction of general anesthesia (0.3 μg/kg sufentanil, 2 to 3 mg/kg propofol, and 0.5 mg/kg atracurium, followed by maintenance with 6 to 9 mg/kg propofol or 1.0 to 1.5% isoflurane in 50% N2O/O2), for 30 min. Both protocols were routinely in use.

Exclusion criteria were age (20 to 60 yr) and admittance for celioscopic or transvaginal surgery. Patients presenting preexisting stages 3 through 5 chronic kidney disease (estimated GFR using the four-variable Modification of Diet in Renal Disease [MDRD] study equation <60 ml/min per 1.73 m2), baseline UAlb/UCreat >3.39 mg/mmol, diabetes, renin-angiotensin-aldosterone blocker medication, or preexisting allergy to ketamine were not included in the study.

Exclusion criteria were the use of hydroxyethyl starch and preoperative hemodynamic instability (defined by fluid loading >1000 ml crystalloid/30 min and/or use of vasopressors). Data included patients’ age and body mass index. Surgical route, type and volume of fluid infusion, drugs used for anesthesia maintenance, and antibiotic prophylaxis regimen were additionally collected. Noninvasive mean arterial pressure was measured before (H0) and 1 h after (H1) ketamine administration. UAlb (immunonephelometry, Dade Behring BNII) and UCreat (colorimetric Jaffe method, Beckman CX) were measured on urine samples collected by urinary catheterization at H0 and H1, before any postoperative analgesic medication was given.

Primary objective of this pilot study was to compare UAlb/UCreat difference between H0 and H1 in ketamine + and ketamine − groups. Descriptive analyses were performed using median values (25th to 75th percentiles) for continuous variables and percentages for categorical variables. Values were compared with Mann-Whitney U test or Fisher exact test, as appropriate.

ACKNOWLEDGMENTS
We thank Guido Brusini for technical assistance and Vincent Compere, MD, PhD, and Sophie Claeyssens, MD, for contribution to the human study.
DISCLOSURES
None.

REFERENCES
Supplementary Material

Supplementary Methods

Animal studies
24 hour urine samples, to assess albumin (mouse albumin ELISA-kit, Bethyl Laboratories) and creatinine (Sentinel Kit, Sentinel, Milan, Italy) values, were obtained by placing the animals in metabolic cages. These measurements allowed calculation of UAlb/UCreat, which is a better measure of glomerular permeability than urinary albumin alone, especially in mice.

To collect renal tissue, animals were sacrificed either by decapitation, or anaesthetized by cloralium hydrate and perfused by cold PBS. In both cases, kidneys were taken and material for routine light microscopy stainings was fixed in 4% buffered paraformaldehyde, dehydrated, and paraffin-embedded. For transmission electron microscopy, 1mm³ renal cortex pieces were fixed in a mixture of paraformaldehyde, glutaraldehyde, and phosphate buffer, and embedded in resin. For scanning electron microscopy, pieces of cortex were fixed in a mixture of glutaraldehyde 2% and paraformaldehyde 4% in phosphate buffer 0,12 M for 6 h, fixed in a mixture of osmium tetroxide 1% and sodium cacodylate buffer 0,12M for 2 h, dehydrated in a graded ethanol series and dried in hexamethyldisilazane (all reagents from Electron Microscopy Sciences, Società Italiana Chimici, Roma, Italy). Then the pieces were sputter-coated with gold (Edwards S150A sputter coater). For immunofluorescence, the unfixed renal tissue was embedded in OCT (optimum cutting temperature cryoembedding matrix) (Tissue-Tek, Electron Microscopy Sciences), snap-frozen in a mixture of isopentane and dry ice, and stored at –80°C. For immunogold electron microscopy on ultracyrosections, tissue was fixed in a mixture of paraformaldehyde, glutaraldehyde, and phosphate buffer, soaked in saccarose 18% and frozen on the top of specimen pins (Electron Microscopy Sciences).

Immunofluorescence studies
Apart from F-actin, directly detected by Phalloidin–TRITC (Sigma-Aldrich), an indirect immunofluorescence method was applied on 5μm-thick acetone-fixed tissue cryosections and on acetone-fixed cultured cells.

The following primary antibodies were used for the study: rabbit anti-mouse nephrin (intracellular domain) (#035, provided by H. Holthofer, Dublin City University, Ireland), mouse anti-synaptopodin (Progen, Heidelberg, Germany), rabbit anti-podocin (Sigma-Aldrich), rabbit anti-WT1 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ZO-1 (Invitrogen, S.
 Giuliano Milanese, Italy), rabbit anti-alpha-actinin-4 (LifeSpan Biosciences, Seattle, WA, USA), mouse anti-smooth muscle actin alpha isoform (α-SMA, Invitrogen), rat anti-mouse CD31/PECAM (Abcam, Cambridge, UK), mouse anti-pan cytokeratin (Abcam), rabbit anti-non muscle myosin IIA (Abcam), rabbit anti-NMDA1 receptor (Abcam), mouse anti-Rab3A (Synaptic System), rabbit anti-rabphilin-3A (BD Transduction Laboratories, Milan, Italy), rabbit anti-Synapsin-I (Sigma-Aldrich), rabbit anti-phospho-(S603)-Synapsin-I (Sigma-Aldrich), rabbit anti-CaMKII (Abcam), rabbit anti-phospho-(T286)-CaMKII (Abcam), rabbit anti-cofilin (Abcam), rabbit anti-phospho-(S3)-cofilin (Abcam), rabbit anti-alpha-tubulin (Abcam).

As secondary fluorescent-labelled antibodies, we used the following: Alexa Fluor 488 (or 546) goat anti-rabbit IgG, Alexa Fluor 488 (or 546) goat anti-mouse IgG highly cross adsorbed, and Alexa Fluor 488 chicken anti-rat IgG highly cross adsorbed (all from Invitrogen).

Specificity of Ab labelling was demonstrated by the lack of staining after substituting proper control immunoglobulins (rabbit primary Ab isotype control and mouse primary Ab isotype control, both from Invitrogen, and Rat IgG1 negative control, from Serotec, Kidlington Oxford, UK) for the primary antibodies.

Slides were mounted with VectaShield aqueous mounting medium (Vector Laboratories, DBA Italia SRL, Milan, Italy).

Images were acquired by a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital videocamera and immunofluorescence apparatus (Carl Zeiss SpA), and recorded by AxioVision software 4.3.

**Western Blot**

Glomeruli and cells were lysed in RIPA buffer, protein lysates were separated on a SDS-PAGE and transferred by electroblotting on a PVDF membrane (ImmunBlot PVDF membrane, BioRad Laboratories Inc., CA, USA). After blocking, each membrane was incubated with the primary antibody, followed by the proper HRP (horseradish peroxidase)-conjugated secondary antibody, and positive reaction products were identified by chemiluminescence (BM Chemiluminescence Western Blotting Kit, Roche). Images were digitally acquired by Chemidoc XRS instrument (Bio-Rad, Milan, Italy).
Supplementary Figure legends

Suppl.Fig 1. In situ hybridization

In situ hybridization demonstrates synthesis of Rab3A and NMDAR1 in glomeruli from a 3 month old Balb/c mouse (left panels), and negative controls (right panels) obtained by sense probes (630X, scale bars 20µm).

In situ hybridization was performed as described. Briefly, tissues were deparaffinized and rehydrated, then permeabilized and post-fixed. Sections were then acetylated by acetic-anidride/triethanolamine, washed, and incubated with prehybridization solution. Biotin-labelled probes were applied overnight in a water-saturated atmosphere. The following probes were used:

Rab3A antisense: GCA TAG ACT TCA AGG TCA AAA CCA TCT ACC GCA ACG ACA AGA GGA TCA AGC TGC AGA TCT; Rab3A sense: AGA TCT GCA GCT TGA TCC TCT TGT CGT TGC GGT AGA TGG TTT TGA CCT TGA AGT CTA TGC.

Considering that the NMDAR1 subunit has eight splicing variants, we used the same probes as Laurie et al, specifically designed to span all subunits and detect total NMDAR1 mRNA:

NMDAR1 antisense: GCT CTT GGA AGA TAC AGC TCA ACG CCA CTT CTG TCA CCC ACA AGC; NMDAR1 sense: GCT TGT GGG TGA CAG AAG TGG CGT TGA GCT GTA TCT TCC AAG AGC.

After hybridization, stringency washes were performed with SSC (standard sodium citrate) 4X, SSC 2X-50% formamide at 45°C, and again with SSC 1X at room temperature. Specimens were then incubated with alkaline phosphatase-labelled streptavidin and the reaction developed with Fast Red (all reagents from Sigma, nucleotides from Eurofins MWG Operon, Ebersberg, Germany).

Suppl.Fig 2. Double stainings

Fig 2A: Double staining immunofluorescence reveals glomerular areas co-stained by Rab3A (in red) and podocin (green), as shown by the yellow labelling on the merge picture (400X, scale bars 50µm).

Fig 2B: Podocin (green) and rabphilin-3A (red) co-labelling is revealed by the yellow glomerular staining after image merging (400X, scale bars 50µm).

Double staining was obtained by repeating twice the indirect immunofluorescence procedure described in the Concise Methods.
Suppl.Fig 3. Podocyte foot process effacement
Both images (scale bars 1μm) from a Rab3A-KO mouse clearly show areas of foot process effacement.

Suppl.Fig 4. Three month old Rab3A-KO and WT mice: immunofluorescence analysis of podocyte proteins.
Immunofluorescence demonstrates segmental loss of the podocyte markers nephrin (4A), podocin (4B), synaptopodin (4C), alpha-actinin4 (4D), and ZO-1 (4E), indicated by white arrows, in glomeruli of 3 month old Rab3A-KO mice (upper panels) and absent from glomeruli of corresponding WT animals (lower panels) (all images 400X, scale bars 50μm, but WT-synaptopodin in Fig 4C, taken at 200X, scale bar 100μm).
Quantification of data, obtained from 5 mice per strain and 30 glomeruli per specimen, is represented in Fig 4F, where podocyte markers have been evaluated either as percentage of the glomerular area occupied by the staining, and as percentage of glomeruli with segmental loss of staining.
These alterations were not accompanied by variations of podocyte number in KO glomeruli compared to the WT, as shown by Fig 4G, where the graph summarizes the quantitativa data obtained from calculation of the number of WT-1 positive cells per glomerulus (representative immunofluorescence, lower panel, 400X, scale bars 50μm) in 3 mice per strain and 30 glomeruli per tissue.
Quantitative evaluations were performed on digitalized images by using appropriate macros (essentially formed by color threshold procedure and filtering) applied on glomerular areas selected as region of interests (ROI). The software (AxioVision 4.7 Quantification Modules, Zeiss) was programmed to automatically calculate percentages of the area or number of particles per ROI, according to the specific needs.

Suppl.Fig 5. Immunogold electron microscopy
Fig 5A: In a WT mouse glomerulus, Synapsin-1, detected by immunogold electron microscopy (upper panel, scale bar 200nm), displays several dots of positivity in the foot processes, as indicated by the red arrows, whereas phospho-Synapsin-1 (lower panel, scale bar 500nm) can not be detected (scale bar 200nm).
Fig 5B: Only a single dot of Synapsin-I positivity (upper panel, scale bar 200nm) can be observed in this tissue from a KO animal, whereas phospho-Synapsin-I appears relatively more represented (lower panel, scale bar 200nm), as evidenced in both figures by the red arrows.
Supplementary References


2. Laurie DG, Seeburg PH: Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. J Neurosci 14: 3180-3194, 1994
Suppl. Fig 1

Rab3A – antisense probe

Rab3A – sense probe

NMDAR1 – antisense probe

NMDAR1 – sense probe
Suppl.Fig 2A
Suppl.Fig 2B
Suppl. Fig 4A
Suppl. Fig 4B
Suppl. Fig 4C
Suppl.Fig 4D
Suppl. Fig 4F

Suppl. Fig. 4G
Suppl. Fig 5A