Article

Receptor-Mediated Field Effect Transistor Biosensor for Real-Time Monitoring of Glutamate Release from Primary Hippocampal Neurons

Yu-Tao Li, Xin Jin, Lina Tang, Wen-Liang Lv, Mengmeng Xiao, Zhiyong Zhang, Chuan Gao, and Guo-Jun Zhang

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.9b00832 • Publication Date (Web): 30 May 2019

Receptor-Mediated Field Effect Transistor Biosensor for Real-Time Monitoring of Glutamate Release from Primary Hippocampal Neurons

Yu-Tao Li[1, §], Xin Jin[1, §], Lina Tang[1], Wen-Liang Lv[2], Meng-Meng Xiao[3], Zhi-Yong Zhang[3], Chuan Gao [1,] and Guo-Jun Zhang[1,*]*

[1]School of Laboratory Medicine, Hubei University of Chinese Medicine, 1 Huangjia Lake West Road, Wuhan 430065, P.R. China

[2]Clinical School of Traditional Chinese Medicine, Hubei University of Chinese Medicine, 1 Huangjia Lake West Road, Wuhan 430065, P.R. China

[3]Key Laboratory for the Physics and Chemistry of Nanodevices, Department of Electronics, Peking University, 5 Yiheyuan Road, Beijing 100871, P.R. China

[§]These authors contributed equally to this work

[*]Corresponding author: Tel: +86-27-68890259, Fax: +86-27-68890259

E-mail: zhanggj@hbtcm.edu.cn; cgao@hbtcm.edu.cn

Abstract

Glutamate, as one of the most important central excitatory neurotransmitters, plays crucial roles in nerve signal transduction and is implicate in several neurological disorders. However, no effective means has been developed for specific detection of glutamate released from primary cultured neurons. Here we present a reduced graphene oxide (RGO)-based field effect transistor （FET） biosensor functionalized with synthesized glutamate receptor for real time monitoring of glutamate release from primary cultured rat hippocampus neurons. Metabotropic glutamate receptors (mGluR) was specifically synthesized and then immobilized on the RGO surface by 1-Pyrenebutanoic acid succinimidyl ester (PASE) linker, after which target glutamate (pI=3.22) could specifically bind to the synthesized mGluR in the neutral buffer, causing the charge density change. After the neurons were cultured on the sensing channel with a self-made liquid reservoir, the FET biosensor could discriminate glutamate in fM range in complete cell culture medium and generate encouraging results in real time monitoring of glutamate release from primary rat hippocampus neurons. This work is the first report of specific and direct detection of glutamate molecules released from primary culture of differentiated central neurons, which may further help understand nature of neuronal communications. Moreover, this work paves a way for the detection of electrochemically inactive small molecules released by cells.

Keywords: Receptor-mediated; Field effect transistor; Real-time; Hippocampus neurons; Glutamate

Introduction

Glutamate is among the most important neurotransmitter in the mammalian central nervous system (CNS), which plays a vital role in nerve signal transduction and is implicated in several neurological disorders, such as stroke, schizophrenia, Alzheimer's and Parkinson's disease. Developing effective methods for real time detection of glutamate is essential for understanding neuronal activities behind physiological processes.[1-4]

Existing analytical techniques for quantitative measurement of glutamate release from cultured neuron such as patch clamp,[5] microdialysis[6] etc. exhibit poor signal-to-noise ratio (SNR) and target-specificity. High performance liquid chromatography[7] is just off-line analysis, which is lack of kinetics and real-time information. Recently, developed fluorescent methods, generate low signal change and are proof-of-principle experiments.[8-9] Although a glutamate-sensing fluorescent reporter has been reported to visualize glutamate neurotransmission with the advantage of rapid and direct access to the synaptic cleft,[10] expensive imaging equipment and complex transfection processes limit the use of the method in conventional laboratories. What is more, the fluorescent bleaching is always an unavoidable problem for all fluorescent methods. Electrochemical sensing of glutamate has been developed over the years using glutamate oxidase or glutamate dehydrogenase as the mediator, in which it converts an inert substrate to an electroactive product by a specific enzymatic reaction using an analyte-dependent step.[11-13] Under optimal conditions, their detection limits can reach nanomolar levels, which can basically meet the detection

requirements. However, enzyme-based glutamate sensors generally involve complicated enzyme immobilization procedure and are prone to be influenced by environmental factors. An ambient glutamate concentration in vivo was reported to be at the low micromolar range[14,15] by microdialysis studies. Moreover, a lower concentration down to 2 nM extracellular glutamate was predicted by the other method[16].

Over the past decade, FET biosensors have attracted great attentions for applications in ultrasensitively detecting biomolecules like nucleic acids, proteins and cells.[17-19] The FET biosensor has intrinsic signal amplification capability with high signal-to-noise ratio. They operate at relatively low detection potential (V_{ds}=0.1 V), and the impact on the organism is minimal. What' more, they can be used to detect biological molecules without labeling. Therefore, the FET biosensors have great potential for real-time detection of molecules release during cell-cell signaling in physiological conditions.[20] Several enzyme-mediated FET technologies have been reported for the measurement of glutamate. Braekenet al. presented a floating-gate FET device coated with glutamate oxidase layer to detect glutamate with the detection limit of 100 nM.[21] Kergoat et al. introduced a method of detecting glutamate and acetylcholine with organic electrochemical transistors based on conducting polymer/platinum nanoparticle composites, in which the detection limit was 5 μM.[22] Lee et al. reported a probe-type carbon nanotube (CNT) transistor by immobilizing L-glutamate oxidase on CNT to real-time detect glutamate in a standard solution in vitro and in the 11 vessel occlusion rat model in vivo, in which the sensitivity was up to 200 μM. [23]

Essentially, there have been some recent reports about monitoring of nerve cell signaling using the FET biosensor. Fromherz et al. constructed a cell-semiconductor transistor for monitoring of vesicle release in chromaffin cells.[24] Pan et al. reported an aptamer-modified nanowire transistor for detection of dopamine and neuropeptide Y from PC12 cell.[25] Delacour et al. recorded spikes activity in cultured hippocampal neurons using transparent graphene transistors.[26] Xu et al. described a new material based on ultrathin MXene-Micropattern FET for probing hippocampal neuronal activity.[27] However, none of them has been designed for direct and specific detection of glutamate released by neurons, probably owing to the technical challenge for FET-based biosensors to recognize weakly charged small molecules or glutamate receptors are currently not commercially available.

In this work, we synthesize a metabotropic glutamate receptor (mGluR) capable of specifically binding with glutamate, and modify the receptor on a RGO based FET by a PASE linker. After that, primary isolated hippocampal neurons are cultured on the sensing channel for several days, until they fully differentiate. Finally, the mGluR modified RGO FET biosensor is used for real time recording of glutamate released from neuron upon addition of high K^+ stimulus, as shown in Schematic 1. Key novel advantages of this system include: (i) Specific identification of glutamate because of synthesized specific binding receptor; (ii) Real time monitoring of neuron in situ with high temporal resolution; (iii) Successful detection of non-electroactive signal molecules released by neurons; (iv) The pattern of the array sensors

allowing for parallel monitoring of multiple neuronal signals. The work may help provide new insights into the nature of neuronal communications.

Experimental Section

Materials. Graphite powder (99.99995%, -325 mesh) was purchased from Alfa Aesar (Tianjin, China). Sodium dodecyl sulfate (SDS) and hydrazine (98%) were purchased from Generay Biotech Co. Ltd. (Shanghai, China). 1-Pyrenebutanoic acid succinimidyl ester (PASE), Histamine(HI), Norepinephrine(NE), Acetylcholine (Ach), Laminin, Poly-L-lysineand, Neuron grown factor (NGF) were purchased from Sigma-Aldrich (St.Louis, MO, U.S.A.). Paraformaldehyde (PFA) and Glycine (Gly) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Cy5 NHS ester was purchased from Nanjing Bioorth Biotech Co. Ltd. (Nanjing, China). Rabbit Polyclonal anti-TAU antibody (Catalog number: 10274-1-AP) and FITC-conjugated goat anti-Rabbit IgG antibody (Catalog number: 10285-1-AP) were purchased from Proteintech (Wuhan, China). The glutamate receptor used in this work (mGluR) was a peptide corresponding to amino acid 18-592 of human metabotropic glutamate receptor 1 alpha (Gen Bank: AAA87843.1), which was kindly synthesized by Proteintech (Wuhan, China). The isoelectric point of synthesized mGluR is 6.5.4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Sangon Biotech (Shanghai, China). TritonX-100 was purchased from Solarbio (Beijing, China). L-Glutamic acid (Glu) was purchased from REGAL Biotech (Shanghai, China). Bovine serum albumin (BSA) was purchased from Thalys (Wuhan, China).

Fluo-4-AM was purchased from DOJINDO chemical technology Co. Ltd. (Shanghai, China). DMEM/F12 medium and B-27 supplement were purchased from Thermo Fisher (Waltham, MA, USA). Ultrapure water was produced from Milli-Q Direct 8 water purification system (Millipore, Burlington, MA, US).

SDS-PAGE and Western blot. The purified proteins were analyzed by 12% SDS-PAGE under reducing conditions, followed by Coomassie Brilliant Blue staining. For Western blot analysis, proteins on gel were electrophoretically transferred onto polyvinylidinedifluoride membranes (Merck, Kenilworth, U.S.A.). After that, the membranes were blocked in 5% skim milk dissolved in TBST buffer (mixture of tris-buffered saline and Polysorbate 20) followed by incubating them with HRP-conjugated mouse anti-6 His antibody (Proteintech, Wuhan, China) diluted in blocking buffer. Bands were visualized using 3, 3'-diaminobenzidine (Sigma, St. Louis, MO, U.S.A.).

Binding Test Using Flow Cytometry. The aldehyde latex beads were incubated with the mGluRs at 4 °C for 30 min, then the receptor-immobilized beads were treated with 10% BSA for 1 h with continuous rotation to prevent the nonspecific adsorption of biomolecules, and finally the n-terminal FITC-Ahx-Glu were mixed with the modified-latex beads for 5 min at room temperature. The aldehyde latex beads were incubated without mGluRs but treated with 10% BSA for 1 h as the control. The fluorescence intensities of the beads were determined with Accuri C6 flow cytometery. Data were analyzed with Flowjo software.

Fabrication of RGO FET Devices. RGO was produced using chemical reduction method as described in our previous work.[28-31] In brief, 2 mg GO in 1mL of DI water was

homogenized by sonication for 15 min and mixed with 1mL 98% hydrazine. The misture was allowed to restore in the 4°C refrigerator for a week. The resulting 1 mg/mL RGO stock was stable for months without aggregation.

The diluted RGO (0.2 mg/mL) suspension was drop-casted onto the channel between two Au bands on the chip array, which contained 6 pairs of electrodes (Figure S3). Then the chip was thermally annealed at 80 °C in a vacuum oven for 2 h, followed by 5 s sonication in Piranha solution (7:3 v/v concd H_2SO_4/35% H_2O_2). The RGO FET device was finalized by rinsing sequentially using ethanol and ultrapure water and drying under nitrogen.

Immobilization of mGluR on the FET Devices. mGluR was immobilized onto the RGO surface using PASE as the cross-linker. Firstly, 10 μL of 5 mM PASE in dimethyl-sulfoxide (DMSO) was dropped on the RGO of the chip channel at room temperature. 1 h later, the chip was washed sequentially in DMSO, ethanol and DI water. Then the chip was incubated with 10 μL of 100 μg/mL mGluR in 1× PBS for 2 h. Unbound mGluR was removed by sequential wash in 0.2% SDS, 1×PBS and DI water. Lastly, the chip was blocked in 1 mg/mL BSA solution for 1 h, rinsed with DI water and dried by N_2.

Electrical Measurements. All the electrical measurements were performed on a Keithley 4200 semiconductor characterization system combined with an EverBeingBD-6 probe station as previously described.[28-31]A silver wire immersed in 1 x PBS solution was used as the liquid gate. For electrical transfer curve measurements of the FET biosensor, the constant bias was set V_{ds} = 0.1 V (the drain source voltage). 10 μL Glu solution was dropped onto the receptor modified channel and incubated for 20 min at 25°C for immunoreactions. Then the chip was

rinsed with DI water to remove the unbound biomolecule and dried with N_2. Electrical detection of Glu was monitored in real time under a constant bias voltage of 10 mV and liquid gate of 0.1 V, and a self-made reservoir was installed on the chip. Glu was manually added to self-made liquid reservoir with a gradually increasing concentration ranging from 0.1 fM to 100 pM for the sensitivity experiment. The electrical output curves measurement was at a designated Vg value.

Cell Culture. Primary embryonic rat hippocampal neurons were obtained through dissection and enzymatic dissociation of E18 wistar rat tissue as previously described.[32, 33] Neurons were planted at a density of 1×10^5 cells/cm^2 on poly-L-lysine coated glass coverslips and maintained in D-MEM/F-12 supplemented with B-27 and NGF at 37°C in 5% CO_2. For electrical detection experiments, hippocampal cells were trypsinazed and seeded onto the mGluR functionalized FET chip, which was previously sterilized for 30 min under UV in a biosafety cabinet. The volume of medium used for culturing the neurons was 100 μL and the medium was concentrated in a cell reservoir mountained on the chip (Figure 5a). The original density of 1×10^4 cells/cm^2 were added on the sensor chip for culture. The electrically real time detection experiments were performed after neurons were cultured for 3-7 days. To carry out detection, culture medium was replaced with 1×PBS solution. 10 mM high potassium stimulating fluid was injected into the system after the sensor reached a steady-state baseline. Glu released from cells upon stimulation was reflected by the current change.

Immunohistochemistry. Cultured hippocampal neurons were rinsed with 1×PBS and fixed for 30 min in 4% paraformaldehyde in 1×PBS at room temperature. After 3 times washes with 10 mM glycine, cells were permeabilized in 0.2% Triton X-100 in 1×PBS for 30 min and

blocked in 10% BSA for 60 min. To label the axons, cells were incubated with TAU-1 primary antibody overnight at 4°C followed by washing in 1×PBS and incubation with a FITC conjucated secondary antibody for 1.5 h at 37°C. DAPI was used to stain the nucleus. Pictures were acquired using a Zeiss Axiovert microscope with appropriate fluorescent filters.

Results and Discussion

Characterization of Receptor. The glutamate receptor used in this work is a peptide corresponding to amino acid 18-592 of human metabotropic glutamate receptor 1 alpha (mGluR), which was kindly synthesized by Proteintech (Wuhan, China). In brief, a fragment corresponding to amino acid 19-592 of human metabotropic glutamate receptor 1 isoform alpha precursor (NP_001264993.1) was cloned into pET28a vector and transformed into DE3 competent cells to express. The polyhistidine-tagged mGluR in crude extract was affinity purified using Ni-NTA agarose (Figure 1a, S1). As shown in Figure 1a and SI, the molecular weight of mGluR is 65 kDa.

It has been reported that mGluR has good affinity and specificity with glutamate.[34, 35] In order to test the binding affinity between synthesized mGluR and glutamate, we synthesized 6-aminohexanoic acid fluorescein isothiocyanate labeled glutamate (FITC-Ahx-Glu), and the corresponding synthetic characterization data are shown in Figure S2. 6-aminohexanoic acid was specifically designed to bridge FITC and glutamate, with an attempt to reduce steric hindrance of glutamate binding to the mGluR (Figure 1b). The corresponding fluorescence signal was then confirmed by flow cytometry. The specific experimental steps were as follows: mGluRs were

immobilized on aldehyde-modified beads (4 μm in diameter), then the receptor-immobilized beads were treated with 10% BSA to prevent the nonspecific adsorption, and finally the FITC-Ahx-Glu were mixed with the modified-latex beads. Data were analyzed with Flowjo software. As shown in Figure 1b, the specific interaction-based signal intensity (Bead+mGluR+FITC-Ahx-Glu) was significantly different from that of the control tests, such as Bead+ FITC-Ahx-Glu (Figure 1c), Bead+mGluR and Bead (data not shown). Above results demonstrate that the synthesized mGluR could specificly bind with glutamate.

Characterization of RGO FET biosensor. The RGO FET device was fabricated as described by our previously papers.[28-31] The functionalization process was validated in a stepwise manner. Firstly, in order to confirm the successful surface modification of mGluR on the RGO surface, target mGluR labeled with fluorescent dye (Cy5) was applied to conjugate with the RGO surface through PASE and the resulting fluorescence signal was characterized by fluorescence microscope. As shown in Figure 2a, an obvious red fluorescence was observed when Cy5-labeled mGluR was bound to the RGO surface. As a control, a negligible signal was seen when Cy5-labeled mGluR was dropped on RGO surface without PASE (Figure 2a, Inset). These results demonstrate that mGluR was successfully immobilized on the RGO surface. Then the stepwise modification process on the FET device was characterized by the transfer curves as illustrated in Figure 2b. In all steps we observed the similar ambipolar nature. The Dirac point of pristine RGO was found to be 0.25 V. After droping PASE on the RGO surface, the V_{CNP} shifted to the positive gate voltage direction (0.3 V), suggesting that PASE introduced strong p-doping in graphene. When mGluR (pI=6.5) was immobilized on the RGO surface through PASE

coupling agent, left-shifted Dirac point (0.19 V) was observed. This is because negatively charged mGluR increased negative charge density on the RGO. After introduction of 10 pM Glutamate (pI=3.22), Dirac point further shifted from 0.19 to 0.08 V due to contribution of negatively charged Glutamate bond to mGluR immobilized on the FET device. All above electronic results suggest that the stepwise functionalization process was successful.

Affinity study. To further demonstrate the affinity between the newly synthesized mGluR and glutamate, the mGluR modified RGO-FET sensor tested for a series of concentrations of glutamate from 0.1 fM to 10 nM was performed. As shown in Figure 3a, in the range between 0.1 fM and 10 nM, the I_{ds} of the device decreased with increased concentrations of the glutamate (C_{Glu}) and the V_{CNP} of the device shifted toward the left with increased concentration of the glutamate, indicating the p-type characteristics of the mGluR/RGO-FET device. For C_{Glu}>100 pM, V_{dirac} didn't shift any more, indicating that all the immobilized receptors had theoretically reacted with glutamate and the signal reached saturated. In order to effectively analyze the data, a quantification method was chosen as reported in the literature.[36] $\Delta V_{g,Glu}^{cal}$ is defined as the change of the Dirac point between the concentration of the Glu and the mGluR/RGO-FET sensor device, $\Delta V_{g,Glu}^{cal.max}$ is defined as the maximal change of the Dirac point of mGluR/RGO-FET transfer cuver (ΔV_g of C_{Glu}=10 nM). Through normalization, the $\Delta V_{g,Glu}^{cal}/\Delta V_{g,Glu}^{cal.max}$ was acquired. Figure 3b plots the calibrated response of $\Delta V_{g,Glu}^{cal}$ as a function of C_{Glu}, of which the data were converted from Figure 3a. To calculate the binding affinity dissociation constant (K_d) of forming the mGluR-Glu complex, we plotted the $C_{Glu}/V_{g,Glu}^{cal}$ against various C_{Glu} as shown in the Inset of Figure 3b. The K_d of the mGluR-Glu

complex was found to be 3.86 ± 2.68 pM, which was calculated from the least-squares fit to the Langmuir adsorption isotherm model, indicating an extremely high affinity between mGluR and glutamate. Compared to the currently reported K_d values (nanomolar or subnanomolar level) of aptamer-based and protein-based immunosensors,[37,38] the K_d value of mGluR-Glu complex generated by the RGO FET device was much lower than that of other sensors. It shows that the high affinity between mGluR and glutamate has been achieved.

Stability and Selectivity. To test the stability of the fabricated mGluR/RGO-FET device in working conditions, we firstly immersed the chip in complete culture medium for 7 days. As shown in Figure S4a, b, the current signal was just slightly reduced after the sensor device was kept in culture medium. The Dirac voltage barely shifted and retained more than 93.62% of its original value after 7 days, demonstrating that non-specific binding was negligible. We also did the stability test on sensors before and after they were used for culturing hippocampal neurons for 7 days. As shown in Figure S4c, d, electrical properties of the mGluR/RGO-FET device were comparable with those before cell culture, indicating that the sensor was stable even if it worked with cultured cells.

The selectivity of the mGluR/RGO-FET device was further investigated by involving various interferents. Some possible interfering neurotransmitters such as glycine (Gly), acetylcholine (Ach), norepinephrine (NE), histamine (HI), and dopamine (DA) were selected. In the meanwhile, high potassium stimulant (K^+) was also investigated. As indicated in Figure 4c, negligible changes in current were observed even when 100 pM of interferences were introduced. On the other hand, dramatic decrease of current was evident when low concentration of Glu was

injected. The results demonstrate that the mGluR/RGO-FET device has an excellent selectivity to glutamate. The drain current change rate versus concentrations of Glu and the interferents are summarized in Figure 4d. The drain current change rate of Gly, Ach, NE, HI, DA, high K^+ were about 0.16%, 0.16%, 0.08%, 0.04%, 0.04%, -0.24%, respectively. All interferences were injected into the FET sensor at a relative high concentration, no significant current changes of interferences were observed upon injection. However, a large drain current change rate approximately 0.41% was obtained with 10 pM of Glu, and 0.70% with 100 pM Glu, indicating that a high selectivity to Glu molecules by the FET sensor was realized. All above results indicate that the mGluR/RGO-FET biosensor has a satisfactory selectivity.

Real time electrical measurement. The glutamate detection was performed by using the mGluR/RGO-FET sensor in real-time manner. We examined the sensitivity of the mGluR/RGO-FET by applying different concentrations of Glu to the sensing channel immobilized with mGluR. As shown in Figure 4a, the higher the concentration of Glu, the larger the decrease of I_{sd}. The baseline was the reponse of the biosensor by injecting PBS into the liquid reservoir without adding Glu. After 0.1 fM Glu was introduced, a slight current change was observed. When 1 fM Glu was applied, a significant current change was obtained. As the concentrations of Glu increased, the current decreased gradually. Based on the signal that exceeds the baseline by 3-fold, we roughly calculated the limit of detection as 1 fM. As above-mentioned, we normalized the data. The Inset of Figure 4a shows the linear relationship of current change (ΔI) versus different concentrations of Glu, which is represented by ΔI (μA) = $0.0823 \lg C_{Glu}(M) + 1.248$. To verify that the electric signals observed were indeed mediated by

mGluR, control experiments using a bare RGO FET without immobilizing the mGluR on the RGO surface were performed. As shown in Figure S5, no significant change in drain current was evident upon addition of series concentration of glutamate, suggesting that signal generated by non-specific binding of glutamate to RGO is neglectable.

To further mimick the physiological condition, the real time measurement in complete cell culture medium (DMEM/F12 with 10% FBS) was also conducted. A real-time electrical response to different concentrations of Glu from 1 fM to 100 pM in the cell medium is shown in Figure 4b. It is clearly observed that the current reponses were consistent with the above-mentioned PBS experimental results. Similarly, the detection limit was found to be 10 fM. The Inset of Figure 4b shows the linear relationship of the current change (ΔI) versus different concentrations of Glu, represented by $\Delta I\ (\mu A) = 0.0392 \lg C_{Glu}(M) + 0.6028$. The above results reveal the sensor's excellent performance in a more complex cell culture medium.

The performance characterization of the developed sensor compared to traditional methods has been shown in Table S1 in Supporting Information. It is clear that the developed FET sensor shows the lowest detection limit, high selectivity, and relative fast response time. In addition, the noise level of the method was estimated to be about 10 nA. Up to now, several researches have been devoted to quantitative analysis of glutamate in terms of tissue or bulk release from cell population.[11,13,23] The reported glutamate concentration detected by these methods is relatively high. Very recently, Qiu et al. reported[12] a GluOx/Pt/CFMDE sensor with a detection limit down to 0.87 μM. In addition, they realized real-time monitoring of glutamate exocytosis from single hippocampal varicosities. On the basis of the low detection limit achieved

by the presented sensor, it is hopeful that the FET sensor is adequate for the study of glutamatergic neurotransmission, especially for low density neuron study. Moreover, it is also adequate for the analysis of glutamate release at single cell level.

Real time monitoring of glutamate release from primary hippocampal neurons. The experimental setup of the mGluR/RGO-FET sensor for real-time monitoring of glutamate released from primary hippocampal neurons is illustrated in Figure 5a. A cell reservoir was mountained on the sensing channel for culturing hippocampal neurons in situ and an Ag/AgCl liquid gate was mounted in the reservoir for electrical measurement. The separation and culture of the primary hippocampal neurons was referred to literatures.[33,34] Fluorescence image of neurons immunostained with TAU-1 primary antibody and merged with the bright-field channels in Figure 5b clearly demonstrates that the hippocampal neurons were well differentiated and grown well on the sensor surface. These features guaranteed good interfacing of neuron with the electronic components in the system, enabling realization of real-time monitoring of neuronal activities.

As reported, neurons released neurotransmitters through exocytosis are largely calcium dependent, and neurons can be depolarization by elevating the external potassium concentration.[39,40] When high K^+ solution is applied on neuron, it causes the cell membrane depolarization and opens the Ca^{2+} channels, causing a remarkable release of glutamate. Primary isolated neurons were cultured on surface of sensors for 7 days until the maturation of the network was complete. Before detection, the culture medium was replaced with 1×PBS solution. As shown in Figure 5c, when 10 mM high K^+ was added into the cell reservoir, the current

declined immediately and then slowly stabilized in a short time (red line). For control experiments, high K^+ was added to the RGO surface without culturing the hippocampal neurons. On this occasion, a slight increase in current was obtained (black line). Furthermore, we also used calcium channel inhibitor (1 mM Cd^{2+}) to down-regulate glutamate release.[41,42] After 30 min incubation, we basically didn't see the current response of released glutamate from neurons (blue line) when high K^+ was applied. The above-mentioned results prove that the observed current response indeed came from glutamate release from the hippocampal neurons. The corresponding current response of the sensor to glutamate release from different neuron sensing channel and their corresponding control experiment are summarized in Figure 5d. It was found that the similar obvious responses from the 6 different recording channels were observed. Based on the current change generated in Figure 5c, we could semi-quantify the released glutamate from hippocampal neurons by the working curve in Figure 4c. The glutamate concentration was then calculated to be about 100 fM, and the average concentration of glutamate calculated from 6 times results was about 230 fM.

Conclusion

In summary, a receptor functionalized RGO-FET sensor has successfully been developed for the first time to real time monitor glutamate release from neurons with reliable performance. Moreover, the sensor chip possesses the advantages of ultra-high sensitivity and specificity, and can be used in real-time monitoring glutamate release from culture hippocampus neurons in situ. The ultrasensitive mGluR/RGO-FET sensor, capable of probing Glu down to

1fM, has a strong binding affinity for Glu over the other catecholamines (such as DA, NE, Ach, HI, and etc.). Furthermore, the sensor chip has good biological compatibility and can be used for long time neurons culturing with neglectable impact on the sensor's detection performance. Using this mGluR/RGO-FET sensor, we have successfully monitored glutamate release from culture hippocampus neurons with satisfactory results. This novel electronic detection device may have significant impact on future studies of neuron activity and could provide new insights into understanding functions of the nervous system. The major limitations of the deveioped approach lie in the following aspects: 1) The RGO assembly on the sensing channel is completed by the drop-casting method, so the small chip-to-chip variation exists; 2) This FET biosensor can, for the time being, detect one type of target molecule, because only one probe molecule is modified on the channel. However, if the biosensor is integrated with the multi-channel microfluidic system, multiple signal molecules can be detected simultaneously.

Corresponding Author

*G.-J. Zhang. Tel: +86-27-68890259. Fax: +86-27-68890259.

E-mail: zhanggj@hbtcm.edu.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Proteintech Group Inc. Co. Ltd. (Wuhan, China) for kindly synthesising mGluR. This work was supported by the National Natural Science Foundation of China (Nos. 21505037, 21675041).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at -- Additional information about Coomassie Brilliant Blue staining of affinity purified mGluRs, FITC-Ahx-Glu synthetic characterization data, Stability data and a Table comparing performance of various glutamate sensors (PDF).

References:

1. Nedergaard, M.; Takano, T.; Hansen, A. J. Beyond the Role of Glutamate as a Neurotransmitter. *Nat. Rev. Neurosci*. **2002**, 3, 748-755.

2. Takano, T.; Lin, J. H.; Arcuino, G.; Gao, Q.; Yang, J.; Nedergaard, M. Glutamate Release Promotes Growth of Malignant Gliomas. *Nat. Med*. **2001**, 7, 1010-1015.

3. Hamdan, S. K.; Mohd, Zain A. In vivo Electrochemical Biosensor for Brain Glutamate Detection: A Mini Review. *Malays. J. Med. Sci.* **2014**, 21, 12-26.

4. Hoeben, Bart. Gunneweg, Freddy. A critical review on glutamate sensing. 2015 DOI:10.13140/RG.2.1.2450.7360 (book).

5. Copenhagen, D. R.; Jahr, C. E. Release of Endogenous Excitatory Amino Acids from Turtle Photoreceptors. *Nature.* **1989**, 341, 536-539.

6. Watson, C. J.; Venton, B. J.; Kennedy, R.T. In vivo Measurements of Neurotransmitters by Microdialysis Sampling. *Anal. Chem*. **2006**, 78, 1391-1399.

7. Jedlicka, S. S.; Dadarlat, M.; Hassell, T.; Lin, Y.; Young, A.; Zhang, M.; Irazoqui, P.; Rickus, J. L. Calibration of Neurotransmitter Release from Neural Cells for Therapeutic Implants. *Int. J. Neural. Syst.* **2009**, 19, 197-212.

8. Okumoto, S.; Looger, L. L.; Micheva, K. D.; Reimer, R. J.; Smith, S. J.; Frommer, W. B. Detection of Glutamate Release from Neurons by Genetically Encoded Surface-Displayed FRET Nanosensors. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, 102, 8740-8745.

9. Okubo, Y.; Sekiya, H.; Namiki, S.; Sakamoto, H.; Iinuma, S.; Yamasaki, M.; Watanabe, M.; Hirose, K.; Iino, M. Imaging Extrasynaptic Glutamate Dynamics in the Brain. *Proc. Natl. Acad. Sci. U. S. A*. **2010**, 107, 6526-6531.

10. Marvin, J. S.; Borghuis, B. G.; Tian, L.; Cichon, J.; Harnett, M. T.; Akerboom, J.; Gordus, A.; Renninger, S. L.; Chen, T. W.; Bargmann, C. I.; Orger, M. B.; Schreiter, E. R.; Demb, J. B.; Gan, W. B.; Hires, S. A.; Looger, L. L. An Optimized Fluorescent Probe for Visualizing Glutamate Neurotransmission. *Nat. Methods*. **2013**, 10, 162-170.

11. Day, B. K.; Pomerleau, F.; Burmeister, J. J.; Huett, P.; Gerhardt, G. A. Microelectrode Array Studies of Basal and Potassium-Evoked Release of L-glutamate in the Anesthetized Rat Brain. *J. Neurochem*. **2006**, 96, 1626-35.

12. Qiu, Q.; Zhang, F.; Tang, Y.; Zhang, X.; Jiang, H.; Liu, Y.; Huang, W. Real-time Monitoring of Exocytotic Glutamate Release from Single Neuron by Amperometry at an Enzymatic. Biosensor. *Electroanal*. **2018**, 30, 1054-1059.

13. Castillo, J.; Blöchl, A.; Dennison, S.; Schuhmann, W.; Csöregi, E. Glutamate Detection from Nerve Cells using a Planar Electrodes Array Integrated in a Microtiter Plate. *Biosens. Bioelectron.* **2005**, 20, 2116-2119.

14. Baker, D. A.; Xi, Z. X.;Shen, H.; Swanson, C. J.;Kalivas, P. W. The Origin and Neuronal Function of in vivo Nonsynaptic Glutamate. *J. Neurosci.* **2002**, 22, 9134-9141.

15. Herman, M. A.;Jahr, C. E. Extracellular Glutamate Concentration in Hippocampal Slice. *J. Neurosci.* **2007**, 27, 9736-9741.

16. Zerangue, N.; Kavanaugh, M. Flux Coupling in a Neuronal Glutamate Transporter. *Nature***1996**, 383, 634-637.

17. Dong, X.; Shi, Y.; Huang, W.; Chen, P.; Li, L. Electrical Detection of DNA Hybridization with Single-Base Specificity using Transistors Based on CVD-Grown Graphene Sheets. *Adv. Mate*r. **2010**, 22, 1649-1653.

18. Ohno, Y.; Maehashi, K.; Yamashiro, Y.; Matsumoto, K. Electrolyte-Gated Graphene Field-Effect Transistors for Detecting pH and Protein Adsorption. *Nano. Lett*. **2009**, 9, 3318-3322.

19. Peitz, I.; Voelker, M.; Fromherz, P. Recombinant Serotonin Receptor on a Transistor as a Prototype for Cell-Based Biosensors. *Angew. Chem. Int. Ed. Engl.* **2007**, 46, 5787-5790.

20. Kaisti， M. Detection Principles of Biological and Chemical FET sensors. *Biosens. Bioelectron.* **2017**, 98, 437-448.

21. Braeken, D.; Rand, D. R.; Andrei, A.; Huys, R.; Bartic, C. Glutamate Sensing with Enzyme-modified Floating-gate Field Effect Transistors. *Biosens. Bioelectron*. **2009**, 24, 2384-2389.

22. Kergoat, L.;Piro, B.; Simon, D. T.; Pham, M. C.;Noël, V.; Berggren, M. Detection of Glutamate and Acetylcholine with Organic Electrochemical Transistors Based on Conducting Polymer/Platinum Nanoparticle Composites. *Adv. Mater.* **2014**, 26, 5658-5664.

23. Lee, G.; Lim, J.; Park, J.; Choi, S.; Hong, S.; Park, H. Neurotransmitter Detection by Enzyme-immobilized CNT-FET. *Curr. Appl. Phys.* **2009**, 9, S25-S28.

24. Lichtenberger, J.; Fromherz, P. A Cell-Semiconductor Synapse: Transistor Recording of Vesicle Release in Chromaffin Cells. *Biophys. J.* **2007**, 92, 2262-2268.

25. Banerjee, S.; Hsieh, Y. J.; Liu, C. R.; Yeh, N.; Hung, H.; Lai, Y.; Chou, A.; Chen, Y.; Pan, C. Differential Releases of Dopamine and Neuropeptide Y from Histamine-Stimulated PC12 Cells Detected by an Aptamer-Modified Nanowire Transistor. *Small*. **2016**, 12, 5524-5529.

26. Farida, V.; Zheng, H.; Dipankar, K.; Anne. B. M.; Vincent, B.; Cécile Delacour. Recording Spikes Activity in Cultured Hippocampal Neurons Using Flexible or Transparent Graphene Transistors. *Front. Neurosci*. **2017**, 11, 466.

27. Xu, B.; Zhu, M.; Zhang, W.; Zhen, X.; Pei, Z.; Xue, Q.; Zhi, C.; Shi, P. Ultrathin MXene-Micropattern-Based Field-Effect Transistor for Probing Neural Activity. *Adv. Mater.* **2016**, 28, 3333-3339.

28. Cai, B.; Wang, S.; Huang, L.; Ning, Y.; Zhang, Z.; Zhang, G. J. Ultrasensitive Label-Free Detection of PNA-DNA Hybridization by Reduced Graphene Oxide Field-Effect Transistor Biosensor. *ACS. Nano.* **2014**, 8, 2632-2638.

29. Xie, H.; Li, Y.; Lei, Y.; Liu, Y.; Xiao, M.; Gao, C.; Pang, D.; Huang, W.; Zhang, Z.; Zhang, G. J. Real-Time Monitoring of Nitric Oxide at Single-Cell Level with Porphyrin-Functionalized Graphene Field-Effect Transistor Biosensor. *Anal. Chem*. **2016**, 88, 11115-11122.

30. Zhang, C.; Xu, J.; Li, Y.; Huang, L.; Pang, D.; Ning, Y.; Huang, W.; Zhang, Z.; Zhang, G. J. Photocatalysis-Induced Renewable Field-Effect Transistor for Protein Detection. *Anal. Chem*. **2016**, 88, 4048-4054.

31. Jin, X.; Zhang, H.; Li, Y. T.; Xiao, M. M.; Zhang, Z. L.; Pang, D. W.; Wong, G.; Zhang, Z. Y.; Zhang, G. J. A Field Effect Transistor Modified with Reduced Graphene Oxide for Immunodetection of Ebola Virus. *Microchim. Acta* **2019**, 186, 223. https://doi.org/10.1007/s00604-019-3256-5.

32. Banker, G. A.; Cowan, W. M. Rat Hippocampal Neurons in Dispersed Cell Culture. *Brain Res*. **1977**, 126, 397-342.

33. Kaech, S.; Banker, G. Culturing Hippocampal Neurons. *Nat. Protoc*. **2006**, 1, 2406-2415.

34. Desai, M. A.; Burnett, J. P.; Mayne, N. G.; Schoepp, D. D. Cloning and Expression of a Human Metabotropic Glutamate Receptor 1 Alpha: Enhanced Coupling on Co-transfection with a Glutamate Transporter. *Mol. Pharmacol*. **1995**, 48, 648-657.

35. Namkoong, J.; Shin, S. S.; Lee, H. J.; Marin, Y. E.; Wall, B. A.; Goydos, J. S.; Chen, S. Metabotropic Glutamate Receptor 1 and Glutamate Signaling in Human Melanoma. *Cancer Res*. **2007**, 67*,* 2298-2305.

36. Li, B.; Hsieh, Y. J.; Chen Y.; Chung, Y.; Pan, C. Y.; Chen, Y. T. An Ultrasensitive Nanowire-Transistor Biosensor for Detecting Dopamine Release from Living PC12 Cells under Hypoxic Stimulation J. *Am. Chem. Soc.* **2013**, 135, 16034-16037.

37. Ohno, Y.; Maehashi, K.; Matsumoto, K. Label-Free Biosensors Based on Aptamer-Modified Graphene Field-Effect Transistors. *J. Am. Chem. Soc*. **2010**, 132, 18012-18013.

38. Kim, D. J.; Sohn, I. Y.; Jung, J. H.; Yoon, O. J.; Lee, N. E.; Park, J. S. Reduced Graphene Oxide Field-Effect Transistor for Label-free Femtomolar Protein Detection. *Biosens. Bioelectron*. **2013**, 41, 621-626.

39. Sudhof, T. C. Neurotransmitter Release: The Last Millisecond in the Life of a Synaptic Vesicle. *Neuron.* **2013**, 80, 675-690.

40. Robinson, D. L.; Hermans, A.; Seipel, A.T.; Wightman, R. M. Monitoring Rapid Chemical Communication in the Brain. *Chem. Rev.* **2008**, 108, 2554-2584.

41. Lansman, J. B,; Hess, P.; Tsien, R. W. Blockade of Current through Single Calcium Channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}. Voltage and Concentration Dependence of Calcium Entry into the Pore. *J. Gen Physiol.***1986**, 88, 321-347.

42. Wang, Y-W.; Liu, Y-L.; Xu, J.; Qin, Y.; Huang, W-H. A Stretchable and Photocatalytically Renewable Electrochemical Sensor Based on Sandwich Nanonetworks for Real-time Monitoring of Cells. *Anal. Chem.***2018**, *90*, 5977-5981.

Figure captions:

Schematic 1. Schematic diagram of the mGluR/RGO-FET biosensor for real-time monitoring of glutamate release from primary cultured hippocampal neurons.

Figure 1. (a) Polyhistidine-tagged GRM1 in DE3 extract was detected using an Anti-His antibody (Proteintech, Cat# 66005-1-Ig). (b) Schematic diagram of specially designed FITC-Ahx-Glu binding with mGluR and fluorescent receptor-glutamate complex-conjugated microspheres (Bead+mGluR+FITC-Ahx-Glu). (c) The analysis of fluorescent receptor-glutamate complex-conjugated microspheres and the controls by flow cytometry.

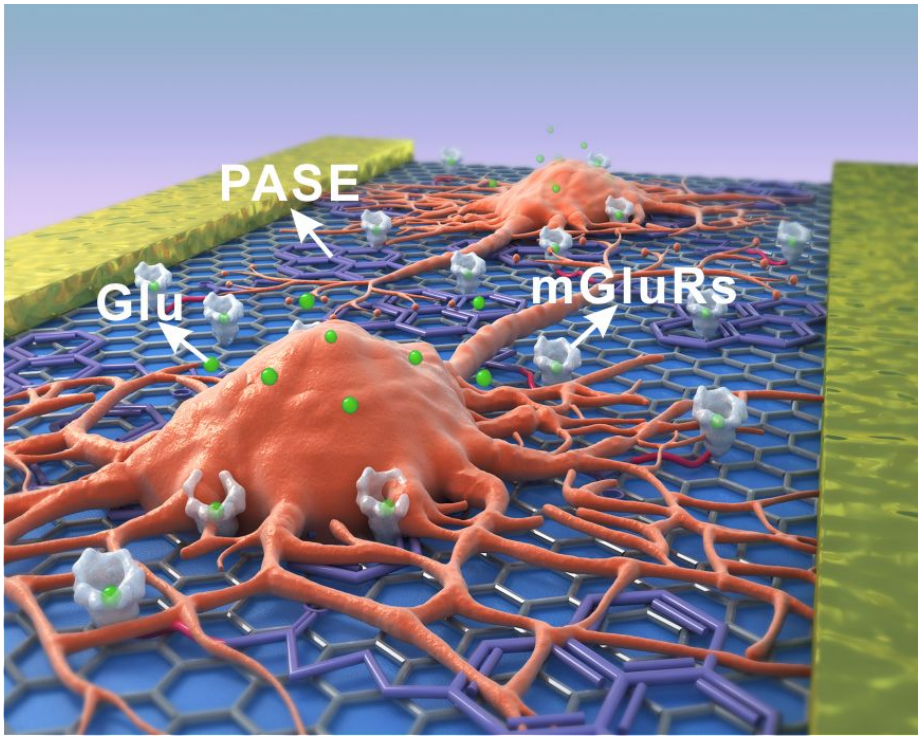
Figure 2. (a) The fluorescence microscope image of the nanochannels observed with and without (inset) immobilization with Cy5 fluorophore labeled mGluR. (b) Plots of transfer curves at V_{ds}=100 mV for the RGO FET biosensor in the process of RGO formation, PASE modification, mGluR immobilization and glutamate binding.

Figure 3. (a) Characterristic transfer curves for mGluR/RGO-FET sensor in response to different concentrations of glutamate solution in PBS solution. (b) The normalized $\Delta Vg,Glu^{cal}$ /$\Delta Vg,Glu^{cal.max}$ as a function of C_{Glu}. The Inset shows a least-aquares fit of the measured data points to the Langmuir adsorption isotherm model to yield a dissociation constant of forming mGluR-Glu complex, n=3 measurements.

Figure 4. Real-time electrical measurement of different concentrations (1, 10, 100 fM and 1, 10, and 100 pM) of glutamate solution in PBS solution (a) and cell medium (b). Inset: The calibration curve of mGluR/RGO-FET to a series of glutamate concentrations in PBS. Error bars

represent standard deviations of measurements (n = 3). (c) Selectivity measurement with the addition of a series of interferents (all chemicals have the same concentration of 100 pM; Gly, Ach, NE, HI, DA.10 mM high K^+) followed by glutamate solutions (10 pM, 100 pM). (d) Histogram of the current change of the mGluR/RGO-FET to Gly, Ach, NE, HI, DA, high K^+, glutamate， respectively. Signal is defined by $\Delta I/I_0$, where ΔI is the current change [drain current (I_{ds}) − initial drain current (I_0)] and I_0 is the initial drain current.

Figure 5. (a) Photograph of mGluR/RGO-FET device coupled with a cell reservoir for real-time electrical measurement of glutamate. (b) Fluorescence image of neurons immunostained with TAU-1 primary antibody and merged with the bright-field channels to show good compatibility between the neuron cells and the mGluR/RGO-FET device. (c) Real-time monitoring of glutamate released from hippocampal neurons. Red line: response of the mGluR/RGO-FET stimulated by 10 mM K^+ with neurons. Black line: response of the mGluR/RGO-FET stimulated by 10 mM K^+ without neurons. Blue line: response of the mGluR/RGO-FET stimulated by 10 mM K^+ with neurons after 30 min incubation with 1 mM Cd^{2+}. (d) The normalized recording channels results of mGluR/RGO-FET to the response of hippocampal neurons. Error bar: six time results.



Schematic 1

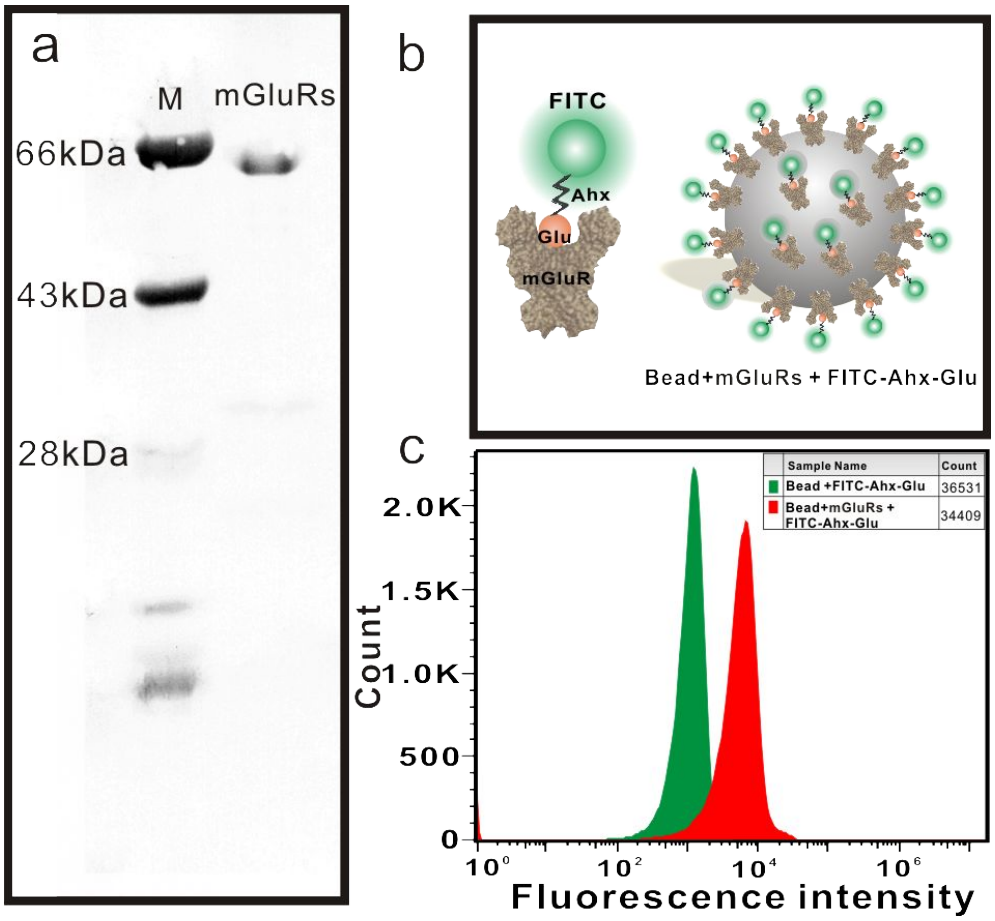

a
M
mGluRs
66kDa
43kDa
28kDa
b
FITC
Ahx
Glu
mGluR
Bead+mGluRs + FITC-Ahx-Glu
c
Sample Name
Count
Bead +FITC-Ahx-Glu
36531
Bead+mGluRs + FITC-Ahx-Glu
34409
2.0K
1.5K
1.0K
500
0
Count
10^0
10^2
10^4
10^6
Fluorescence intensity

Figure 1

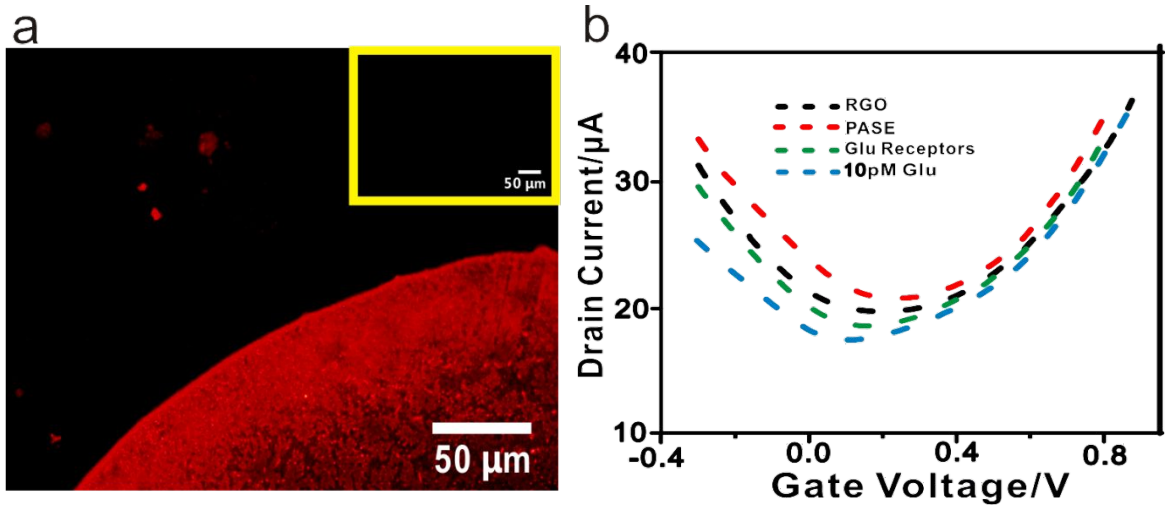

a
50 μm
50 μm
b
40
30
20
10
Drain Current/μA
RGO
PASE
Glu Receptors
10pM Glu
-0.4
0.0
0.4
0.8
Gate Voltage/V

Figure 2

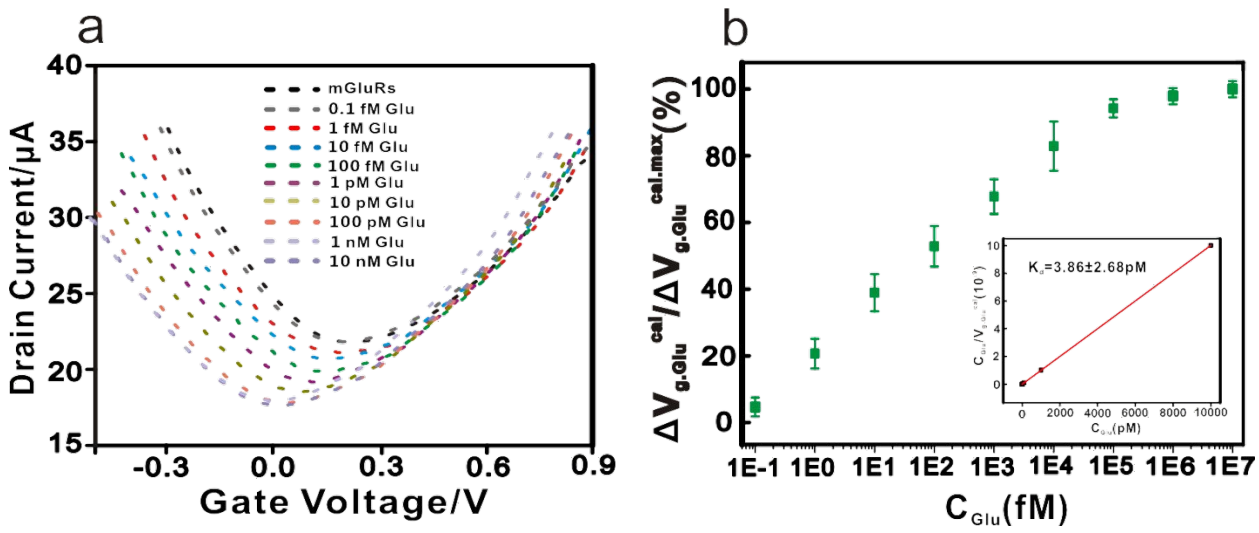

a
Drain Current/μA
Gate Voltage/V
mGluRs
0.1 fM Glu
1 fM Glu
10 fM Glu
100 fM Glu
1 pM Glu
10 pM Glu
100 pM Glu
1 nM Glu
10 nM Glu
b
$\Delta V_{g.Glu}^{cal}/\Delta V_{g.Glu}^{cal.max}$(%)
C_{Glu}(fM)
K_d=3.86±2.68pM
C_{Glu}(pM)

Figure 3

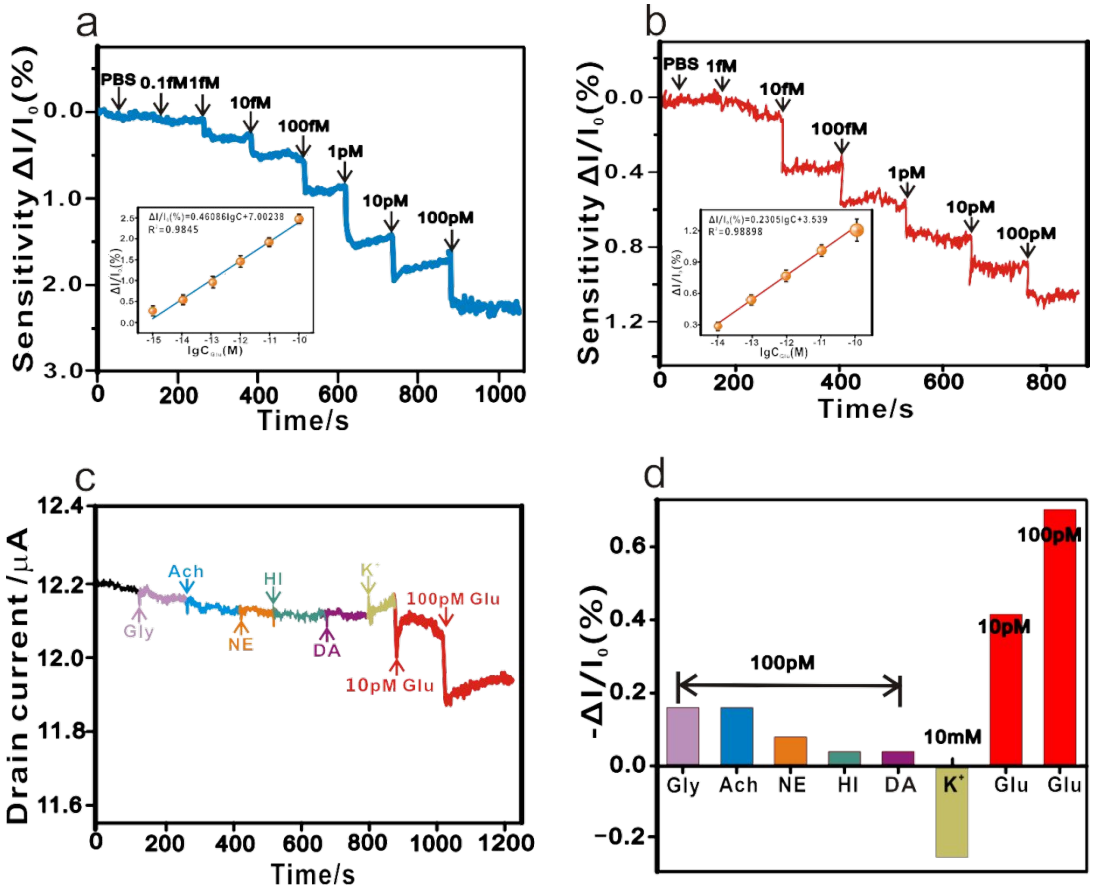

a
PBS
0.1fM
1fM
10fM
100fM
1pM
10pM
100pM
Sensitivity ΔI/I0(%)
Time/s
b
PBS
1fM
10fM
100fM
1pM
10pM
100pM
Sensitivity ΔI/I0(%)
Time/s
c
Drain current /μA
Ach
HI
K+
Gly
NE
DA
100pM Glu
10pM Glu
Time/s
d
-ΔI/I0(%)
100pM
10mM
10pM
100pM
Gly
Ach
NE
HI
DA
K+
Glu
Glu

Figure 4

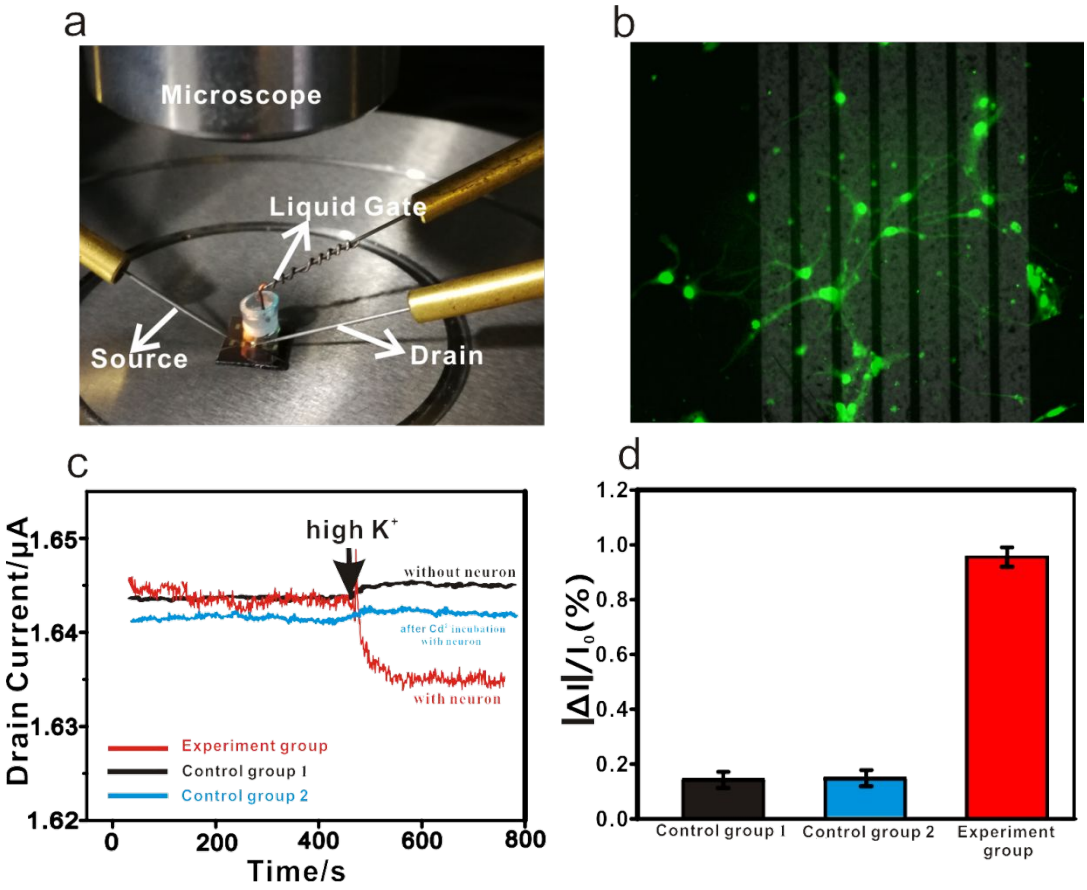

a
Microscope
Liquid Gate
Source
Drain
b
c
high K+
without neuron
after Cd2+ incubation with neuron
with neuron
Experiment group
Control group 1
Control group 2
Drain Current/μA
1.65
1.64
1.63
1.62
0
200
400
600
800
Time/s
d
|ΔI|/I0(%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control group 1
Control group 2
Experiment group

Figure 5

For TOC only