

**Kainate receptors mediate regulated exocytosis of secretory phospholipase A<sub>2</sub> in SH-SY5Y neuroblastoma cells**

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**Running head:**

Kainate receptors mediate exocytosis of sPLA<sub>2</sub>-IIA

**Key words:**

Secretory phospholipase A<sub>2</sub>, sPLA<sub>2</sub>-IIA, kainate receptors, PKC, GluR5, exocytosis, neurotransmission, synaptic plasticity, nociception, pain

## **Abstract**

Secretory phospholipase A<sub>2</sub> isoforms are widely expressed in the brain and spinal cord. Group IIA secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-IIA) has been shown to stimulate exocytosis and release of neurotransmitters in neuroendocrine PC12 cells and neurons, suggesting a role of the enzyme in synaptic transmission. However, the mechanisms by which sPLA<sub>2</sub> is itself released, and a possible relation between glutamate receptors and sPLA<sub>2</sub> exocytosis is unknown. This study was carried out to elucidate the effects of glutamate receptor agonists on exocytosis of sPLA<sub>2</sub>-IIA in transfected SH-SY5Y neuroblastoma cells. sPLA<sub>2</sub>-IIA enzyme was found to be packaged in fusion-competent vesicles and released constitutively or upon stimulation, suggesting regulated secretion. The signal peptide of sPLA<sub>2</sub>-IIA is required for its vesicular localization and exocytosis. External application of AMPA and kainate, but not NMDA, induced vesicular exocytosis and release of sPLA<sub>2</sub>-IIA. UBP 302, a GluR5 specific kainate receptor antagonist, abolished the effect of kainate, confirming the role of kainate receptors in mediating sPLA<sub>2</sub>-IIA secretion. Moreover, kainate-induced sPLA<sub>2</sub>-IIA secretion is dependent on Ca<sup>2+</sup> and protein kinase C. Together, these findings provide evidence of a link between glutamate receptors and regulated sPLA<sub>2</sub> secretion in neurons, that may play an important role in synaptic plasticity, pain transmission, and neurodegenerative diseases.

## Introduction

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>) are lipolytic enzymes that cleave the *sn*-2-acyl ester linkage of glycerophospholipid to liberate a free fatty acid and lysophospholipid [1]. The secretory type of mammalian PLA<sub>2</sub> (sPLA<sub>2</sub>) has low molecular weight of 13-19 kDa and is divided into several subtypes, sPLA<sub>2</sub>-IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, -XIIA and -XIIB based mainly on the position of cysteine residues [2,3]. Many sPLA<sub>2</sub> isoforms contain a signal peptide essential for their secretion. sPLA<sub>2</sub> type -IIA, -IIC, -IID, -IIE, -IIF and -V are clustered on the same chromosome locus (1p34-p36) [4]. sPLA<sub>2</sub>-IB, -IIA, -IIC, -IIE, -III, -V and -X are found in the central nervous system (CNS) [5-7]. Relatively high levels of sPLA<sub>2</sub> activity are detected in the hippocampus, medulla oblongata and pons [8]. PLA<sub>2</sub> and their enzymatic products are involved in neurotransmission and synaptic plasticity [9,10]. Stimulation of glutamate receptors not only results in Ca<sup>2+</sup> influx essential for long-term potentiation (LTP) [11] but also activates PLA<sub>2</sub>. This leads to formation of arachidonic acid and lysophospholipids [12,13] which increase vesicle exocytosis [14,15]. PLA<sub>2</sub> facilitates neurotransmission in hippocampal neurons [16] whereas inhibition of PLA<sub>2</sub> decreases LTP [17,18].

sPLA<sub>2</sub>-IIA is localized in the CNS [6,19]. Dense immunolabeling of sPLA<sub>2</sub>-IIA is observed in the rat brainstem and spinal cord, where the enzyme is present in a postsynaptic location in neurons [6]. sPLA<sub>2</sub>-IIA plays a role in nociception from the paw and orofacial region [20,21], and may contribute to neuronal injury in acute and chronic neurodegenerative diseases. sPLA<sub>2</sub>-IIA activity and expression are upregulated after cerebral ischemia [22,23] and spinal cord injury [24]. Enzyme expression is also increased in Alzheimer's disease, especially in regions containing amyloid β plaques

[25]. The latter induces the expression of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  which in turn upregulate sPLA<sub>2</sub>-IIA [26,27]. Treatment of cortical neurons with sPLA<sub>2</sub>-IIA induces the release of arachidonic acid and apoptosis [28]. sPLA<sub>2</sub> could mediate neuronal death through glutamate ionotropic receptors [29]. The enzyme also increases Ca<sup>2+</sup> influx through L-type voltage gated calcium channels [30].

$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors are classified as ionotropic glutamate receptors [31]. AMPA receptors are composed of GluR1-4 subunits, whilst KA receptors consist of GluR5-7 or KA1-2 subunits [32-34]. Many studies show that glutamate receptors mediate synaptic transmission and plasticity in the brain [33,35,36]. In addition, glutamate receptors regulate secretion of neuromodulators such as substance P from synaptosomes [37,38]. There is also evidence that KA receptors can have metabotropic actions via G-protein signaling cascades linked to protein kinase C (PKC) [33,36,39]. sPLA<sub>2</sub>-IIA stimulates exocytosis and release of neurotransmitters from neurons [40], and could itself be released from synaptosomes upon high potassium induced depolarization [41]. However, till date, a possible relation between glutamate receptor activation and sPLA<sub>2</sub>-IIA exocytosis is unknown. In this study we elucidated the differential effects of glutamate receptor agonists on vesicular exocytosis and release of sPLA<sub>2</sub>-IIA in human neuroblastoma SH-SY5Y cells.

## **Materials and Methods**

Construction of the rat spinal sPLA<sub>2</sub>-IIA cDNAs and expression vectors

Total RNAs from the rat spinal cord was extracted and isolated using Trizol reagent (Invitrogen, CA, USA), and purified using the RNeasy Mini Kit (Qiagen Inc., CA, USA). The spinal cord was chosen as the tissue source for sPLA<sub>2</sub>-IIA mRNA, since our recent study showed high relative expression of the enzyme in this organ [6]. The RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) with the following conditions: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds. The sPLA<sub>2</sub>-IIA enzyme includes a signal peptide [6,42]. By using the SignalP 3.0 Server, the sequence of sPLA<sub>2</sub>-IIA without N-terminal signal peptide was determined to be from the 121<sup>st</sup> to 441<sup>st</sup> base pairs of sPLA<sub>2</sub>-IIA (NM\_031598.3).

Two sets of primers were designed based on the sequence of the rat sPLA<sub>2</sub>-IIA gene: 1) F1 5'-TGGATGAAGGTCCTC CTGTTGCT-3' and B1 5'-TCAGCAACTGGGCGTCTTCCCTT-3' for insertion into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen), and 2) F2 5'-CAGTCTCGAGCGGA TGAAGGTCCTCCTGTTGCTA-3' and B2 5'-GTACGAATTCCTATCA GCAACTGGCGT-3' for insertion into the pIRES2-EGFP vector (BD Biosciences Clontech, Mountain View, USA). The primers for sPLA<sub>2</sub>-IIA without signal peptide sequence for insertion into the pcDNA3.1/CT-GFP-TOPO vector are: F1 5'-TGGATGAGCCTTCTGGAGTTTGG-3' and B1 5'-TCAGCAACTGGGCGTCTTCCCTT-3'. The PCR temperature profile was 95 °C for 30 seconds followed by 33 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min. PCR products were separated on agarose gel, and the fragments containing the cDNA of either full-length sPLA<sub>2</sub>-IIA or sPLA<sub>2</sub>-IIA without signal

peptide sequence were extracted using the QIAquick Gel Extraction protocol (Qiagen). To obtain sPLA<sub>2</sub>-IIA-GFP fusion protein (sPLA<sub>2</sub>-IIA with or without signal peptide), cDNA inserts of sPLA<sub>2</sub>-IIA (sPLA<sub>2</sub>-IIA with or without signal peptide sequence) were cloned into pcDNA3.1/CT-GFP-TOPO using the CT-GFP Fusion TOPO Expression kit (Invitrogen). Likewise, recombinant plasmid sPLA<sub>2</sub>-IIA-IRES-EGFP (full length sPLA<sub>2</sub>-IIA) was produced by cloning the cDNA insert for sPLA<sub>2</sub>-IIA with signal peptide, into pIRES2-EGFP at the sites of XhoI and EcoRI. The plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and verified by DNA sequencing to confirm integrity and orientation.

#### Cell culture and generation of stable cell lines

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (Gibco). Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO<sub>2</sub>. In order to establish stable cell lines, FuGENE Transfection Reagent (Roche, Indianapolis, IN, USA) was used. Specifically, after 70-80% of confluence, cells were transfected with 1-2 µg of recombinant expression vectors, sPLA<sub>2</sub>-IIA-GFP (sPLA<sub>2</sub>-IIA with or without signal peptide sequence), or sPLA<sub>2</sub>-IIA-IRES-EGFP (sPLA<sub>2</sub>-IIA with signal peptide sequence), or empty vector, followed by incubation of cells at 37 °C for 2 days in culture medium. The transient cell lines were subcultured and grown in culture medium containing selection reagent (G418 sulfate, 500 µg/ml; Invitrogen) for 10 days, with a

change of medium every 2 days. Subsequently, transfected cells were transferred into a 96-well plate ensuring that only a single colony is present in each well, grown for another 2 weeks, and subcultured into larger wells to obtain stable cell lines.

#### Total internal reflection fluorescence microscopy (TIRFM) analysis

TIRFM experiments were performed at 37 °C using a Zeiss Axiovert-200M inverted microscope system (Carl Zeiss, Göttingen, Germany) equipped with a 100x 1.45 NA TIRF objective lens. Glass coverslips (refractive index=1.52) with SH-SY5Y cells were mounted in an imaging chamber containing bath solution (150mM NaCl, 5.4mM KCL, 2mM MgCl<sub>2</sub>, 2mM CaCl<sub>2</sub>, 5mM glucose, 10mM HEPES, pH 7.4). The laser beam of 488 nm was incident on the coverslip at 68-70° from normal. Penetration depth of the evanescent field was calculated to be around 100 nm. The emission of green fluorescence protein (GFP) was collected at 520 nm. Using MetaMorph 6.3 software (Molecular Devices, Downingtown, PA, USA), real-time motions of GFP-labelled sPLA<sub>2</sub>-IIA vesicles, as time-lapse digital images, were acquired from a single cell by a CCD camera for 2 min, with exposure times of 25 ms and 2 Hz sampling frequency, and with time intervals of 0.3 or 0.5 second. Fusion events of sPLA<sub>2</sub>-IIA vesicles in SH-SY5Y cells stably expressed with sPLA<sub>2</sub>-IIA-GFP were analyzed by ImageJ software (National institute of Health, [http://rsb/info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). These are characterized by rapid increase of fluorescence signal followed by diffusion to the peripheral region. The number of events was counted in randomly chosen cells during a 2 min time interval, and the mean and standard error calculated.

### Immunocytochemical analyses

SH-SY5Y cells stably expressing sPLA<sub>2</sub>-IIA-GFP (full length sPLA<sub>2</sub>-IIA) were double labeled with synaptotagmin-1 or NPY to determine possible co-localization of the enzyme with other vesicles markers. The cells were plated on glass cover slips and fixed with 4% formaldehyde for 10 min. They were then permeabilized with 0.1% TritonX-100 and blocked with 1.5% bovine albumin before overnight incubation with a mouse monoclonal antibody to synaptotagmin-1 (Synaptic Systems GmbH, Göttingen, Germany, diluted 1:100) or a rabbit polyclonal antibody to neuropeptide Y (NPY, Santa Cruz Biotech, CA, USA, diluted 1:100). The cells were washed with PBS and incubated with donkey anti-mouse secondary antibody conjugated with AlexaFluor 555 (Invitrogen) or goat anti-rabbit secondary antibody conjugated with Atto 647 NHS (Sigma-Aldrich, St Louis, USA) for 1 hr at room temperature. Cells were then washed with PBS and viewed using a LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany).

### Western blot analysis

SH-SY5Y cells stably expressing sPLA<sub>2</sub>-IIA were seeded in 75-cm<sup>2</sup> flasks (4x10<sup>5</sup> cells/ flask) and grown till confluence. Cells (5x10<sup>6</sup> cells/ flask) were then washed three times with PBS and incubated in serum free DMEM medium with or without test chemicals for 120 minutes at 37 °C. The medium was then harvested and stored at -20 °C. After washing the cells three times with ice-cold phosphate buffer saline (PBS), cells were lysed using RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotech, Santa Cruz, CA, USA). Protein concentrations in the collected medium or the cell lysates

were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, San Diego, CA, USA). Protein samples were separated on the 12% SDS polyacrylamide gels under reducing conditions and then blotted onto nitrocellulose membranes using a Mini-Transblot apparatus (Bio-Rad). The membranes were blocked for 2 hr with 5% non-fat milk in Tris-buffered saline-Tween (TBST) buffer (10mM Tris, 150mM NaCl and 0.1% Tween-20, pH7.4), and incubated overnight with rabbit primary antibodies to GFP, GAPDH (1: 200-400; Biovision, Mountain View, CA, USA), or sPLA<sub>2</sub>-IIA (1:5000) in TBST, followed by washing 3 times with TBST buffer. The anti-sPLA<sub>2</sub>-IIA antibody was prepared by immunizing a rabbit with purified recombinant rat sPLA<sub>2</sub>-IIA as described earlier [43]. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:5000; Thermo Scientific, Rockford, IL, USA) for 6 hr at room temperature. The enzyme protein was visualized with optiCN detection kit and quantified with QualityOne software (Bio-Rad). Immunoblots of secreted proteins and GAPDH were obtained using the same sample sets. GAPDH served as an internal control. Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). UBP302 and AMPA were purchased from Tocris (Tocris Bioscience, Ellisville, MO, USA).

#### Statistical analysis

Results are reported as mean  $\pm$  SEM, and the statistical significance was analyzed by 1 way ANOVA with Bonferroni's post-hoc test.  $p < 0.05$  was considered significant.

## Results

sPLA<sub>2</sub>-IIA proteins are packed into fusion-competent vesicles

Individual secretory vesicles in the subplasmalemmal region and their fusion with the plasma membrane were visualized using the technique of total internal reflection fluorescence microscopy (TIRFM), which evanescently illuminates the thin region (<200 nm) immediately adjacent to the cell membrane [44]. sPLA<sub>2</sub>-IIA proteins (GFP-tagged full length sPLA<sub>2</sub>-IIA) were found to be segregated into vesicle-like structures in human neuroblastoma SH-SY5Y cells stably expressing sPLA<sub>2</sub>-IIA-GFP (Fig. 1A). The vesicles undergo constant lateral (parallel to the plasma membrane) and vertical trafficking (vesicle transit between the inner cytosol to the subplasmalemmal region) (Fig. 1C). Spontaneous fusion events of sPLA<sub>2</sub>-IIA vesicles were frequently observed by TIRFM similar to that observed for secretory vesicles in neuroendocrine cells and synaptic vesicles in neurons [44-46]. They appeared as an abrupt increase of fluorescence intensity due to rapid release of fluorescent molecules from a vesicle, followed by broadening and disappearance of fluorescence as the molecules diffuse away [47] (Fig. 1D). These observations suggest that sPLA<sub>2</sub>-IIA molecules are contained in fusion-competent vesicles which are released constitutively or upon stimulation. Confocal microscopy of SH-SY5Y cells expressing GFP-tagged full length sPLA<sub>2</sub>-IIA revealed a punctuate fluorescence pattern characteristic of secretory vesicles (Fig.2). We also examined possible co-localization of sPLA<sub>2</sub>-IIA with synaptotagmin-1, a Ca<sup>2+</sup> sensor localized in secretory vesicles [48] or NPY, a marker of large dense core vesicles [49]. sPLA<sub>2</sub>-IIA containing vesicles were found to be colocalized with synaptotagmin-1 (Fig. 2A), but not NPY (Fig. 2B).

KA induces vesicular release of sPLA<sub>2</sub>-IIA

The effects of glutamate and its analogs on the exocytosis of sPLA<sub>2</sub>-IIA from SH-SY5Y cells stably transfected with sPLA<sub>2</sub>-IIA-GFP was examined by TIRFM. Increased number of fusion events of sPLA<sub>2</sub>-IIA vesicles was observed 30 min after application of 10 μM KA (Fig. 3A). This is corroborated by the detection of increased sPLA<sub>2</sub>-IIA release (sPLA<sub>2</sub>-IIA-GFP, ~ 42 kDa) ( $p < 0.05$ ) after 2 hr incubation with 10 μM of KA (Fig. 3B). This effect was dose-dependent, and was observed after treatment with 100 nM to 10 μM KA (Fig. 3C). Addition of AMPA (10 μM, 2h incubation) also induced sPLA<sub>2</sub>-IIA secretion. However, no effect on sPLA<sub>2</sub>-IIA secretion or fusion frequency was detected after addition of 10 μM of NMDA or glutamate (Fig. 3A and 3B).

Effect of CNQX on sPLA<sub>2</sub>-IIA secretion

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) is an inhibitor of AMPA receptors. As shown in Fig. 4A, 20 μM of CNQX did not significantly suppress KA-induced vesicular exocytosis of sPLA<sub>2</sub>-IIA. CNQX however, significantly inhibited AMPA-induced sPLA<sub>2</sub> secretion ( $p < 0.05$ ) (Fig. 4B).

KA-induced sPLA<sub>2</sub>-IIA secretion occurs through the GluR5 containing KA receptor

KA receptors include GluR5 or GluR6 subunits which have been localized pre- or post- synaptically and involved in synaptic transmission and plasticity [36]. A GluR5-specific KA receptor antagonist UBP302 [S-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl) pyrimidine-2,4-dione] [50] or a GluR6 specific antagonist NS 102 NS

102 (6,7,8,9-Tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione 3-oxime) [51] was used to determine whether the effect of KA on sPLA<sub>2</sub>-IIA secretion was mediated through the GluR5 or GluR6 -containing receptors. 20 μM of UBP302 abolished the KA-induced vesicular exocytosis and release of sPLA<sub>2</sub>-IIA ( $p < 0.05$ ) (Fig. 5A, B). On the other hand, NS 102 did not significantly suppress sPLA<sub>2</sub>-IIA secretion. These results indicate that KA-induced exocytosis and release of sPLA<sub>2</sub>-IIA in SH-SY5Y cells is mediated by receptors containing the GluR5 subunit.

#### KA-induced sPLA<sub>2</sub>-IIA secretion is Ca<sup>2+</sup> dependent

The number of spontaneous fusion events was significantly reduced when extracellular Ca<sup>2+</sup> was depleted by the calcium chelator EGTA (2 mM), or when the intracellular Ca<sup>2+</sup> pool was depleted by thapsigargin (1 μM, 15 min pretreatment) [52] (Fig. 6A). In addition, 10 μM KA induced sPLA<sub>2</sub>-IIA secretion was suppressed when external or internal Ca<sup>2+</sup> was depleted (Fig. 6A and B). The results indicate that Ca<sup>2+</sup> is required for exocytosis of sPLA<sub>2</sub>-IIA in SH-SY5Y cells under basal condition or after KA stimulation.

#### KA-induced sPLA<sub>2</sub>-IIA secretion is PKC dependent

KA receptors especially those containing GluR5 could sometimes signal through a metabotropic action involving phospholipase C and activation of PKC [33,36]. The latter is known to regulate exocytosis via phosphorylation of key proteins in the exocytotic cascade [53]. Bisindolylmaleimides (BIS, selective and potent inhibitors of PKC) were used to block PKC activity [54,55] in SH-SY5Y cells to determine a possible

role of PKC in spontaneous and KA-induced sPLA<sub>2</sub>-IIA secretion. Inhibition of PKC by either of the BIS compounds Ro-32-0432 (BIS-XI) [56] or Ro-31-8220 (BIS-IX) [57,58] reduced the number of fusion events under basal and KA stimulated conditions (Fig. 7A).

BIS compounds did not affect basal sPLA<sub>2</sub>-IIA release as detected by western blots (Fig. 7B) but significantly inhibited KA-induced sPLA<sub>2</sub>-IIA release. In addition, 100 nM of phorbol-12-myristate-13-acetate (PMA), a potent diacylglycerol (DAG) analog that activates PKC, induced the exocytosis and release of sPLA<sub>2</sub>-IIA while this effect was largely blunted in the presence of PKC inhibitor (Fig. 7). These findings indicate a role of PKC in KA induced exocytosis of sPLA<sub>2</sub>-IIA.

Signal peptide is required for vesicular localization and exocytosis of sPLA<sub>2</sub>-IIA

We next examined the role of signal peptide of sPLA<sub>2</sub>-IIA in vesicular localization and exocytosis using TIRFM. In contrast to the numerous vesicle-like structures in SH-SY5Y cells stably cloned with sPLA<sub>2</sub>-IIA-GFP (full length sPLA<sub>2</sub>-IIA) (Fig. 1A), cells that stably expressed sPLA<sub>2</sub>-IIA without signaling peptide showed homogenous green fluorescence without any visible vesicle-like structures (Fig. 8A).

Exocytosis was not observed in cells that stably expressed sPLA<sub>2</sub>-IIA without signal peptide (Fig. 6B). There was also very little sPLA<sub>2</sub>-IIA in the culture medium of cells expressing sPLA<sub>2</sub>-IIA-GFP without signal peptide. Moreover, 10 μM of KA could no longer induce sPLA<sub>2</sub>-IIA secretion from these cells (Fig. 6C). These findings indicate that the signal sequence of sPLA<sub>2</sub>-IIA is essential for its vesicular localization and exocytosis.

### KA induced sPLA<sub>2</sub>-IIA secretion

The effects of glutamate and its analogs on the secretion of sPLA<sub>2</sub>-IIA from SH-SY5Y cells stably transfected with sPLA<sub>2</sub>-IIA-IRES-EGFP vector were analyzed by western blots. Cells were transfected with sPLA<sub>2</sub>-IIA-IRES-EGFP vector which produces separate expression of sPLA<sub>2</sub>-IIA and EGFP, and sPLA<sub>2</sub>-IIA released into the medium was directly detected with a specific antibody to the enzyme. As shown in Fig. 9A, 10 μM of KA stimulated sPLA<sub>2</sub>-IIA secretion (sPLA<sub>2</sub>-IIA, ~15 kDa) ( $p < 0.05$ ). However, no significant enhancement of sPLA<sub>2</sub>-IIA secretion was found after addition of NMDA, AMPA or low doses of glutamate itself. Very low levels of EGFP (EGFP, about 27 kDa) were detected in the culture medium and no increase in EGFP was detected after addition of glutamate analogs (Fig. 9B), consistent with regulated secretion of sPLA<sub>2</sub>-IIA but not EGFP upon stimulation by KA.

### Discussion

The present study was carried out to elucidate the factors that result in sPLA<sub>2</sub> exocytosis in neurons. sPLA<sub>2</sub>-IIA was stably expressed as an GFP fusion protein in a neuroblastoma cell line, SH-SY5Y cells, which has morphological and biochemical characteristics and proteomic expression of mature neurons [59,60]. sPLA<sub>2</sub>-IIA was found to be contained in fusion-competent vesicles in these cells and released through exocytosis, even at basal conditions. In contrast, cells that stably express sPLA<sub>2</sub>-IIA without signal peptide, showed homogenous green fluorescence without visible vesicle-like structures and exocytosis. These results are striking, as they demonstrate that sPLA<sub>2</sub>-IIA is packaged in secretory vesicles and released in a similar manner as several of the

neuropeptide modulators such as substance P and NPY, which are contained in granules at nerve endings [61,62]. In addition, immunocytochemical analyses showed that sPLA<sub>2</sub>-IIA is colocalized with a marker of secretory vesicles, synaptotagmin-1 [48]. The latter is a calcium sensor important for synaptic vesicle release in axons, and vesicular exocytosis in dendrites [63,64].

Several studies have reported sPLA<sub>2</sub> in neurons of the CNS [5,6]. We therefore sought to determine whether sPLA<sub>2</sub> could be released from cells in response to excitatory, glutamatergic stimulation. This was carried out on SH-SY5Y cells, which have been shown to express the NMDA, AMPA, and KA receptors [65-67] including the GluR5 subunit [59] (see online supplementary figure 1, [www.karger.com/doi/10.1159/000330414](http://www.karger.com/doi/10.1159/000330414)). Besides direct visualization and quantitation of exocytotic events by TIRFM (Fig.2B), western blot analyses were carried out to detect secretion of sPLA<sub>2</sub>-IIA-EGFP fusion protein into the culture media. The dose of AMPA, KA, NMDA or glutamate used in these experiments was 10 µM, which is less than the toxic doses of 40 µM for AMPA [68]; 25-200 µM for KA [67,69]; 0.1-5 mM for NMDA [69,70]; and 2-100 mM for glutamate [71,72] for human neuroblastoma SH-SY5Y cells. External application of KA or AMPA resulted in increased exocytosis and the release of sPLA<sub>2</sub>-IIA, whereas NMDA did not show any significant effect (Fig.2). In contrast, no exocytosis was observed after AMPA or KA stimulation in cells expressing sPLA<sub>2</sub>-IIA without signal peptide (Fig.6 A and B). These observations confirm the requirement of this peptide in sPLA<sub>2</sub> vesicular localization and exocytosis, and importantly, demonstrate that AMPA or KA induced exocytosis of sPLA<sub>2</sub>-IIA was not the result of general toxicity or lysis of cells, due to excessive glutamatergic stimulation under our experimental conditions (Fig.9A).

Together, the results indicate that AMPA and KA receptors are important for regulated secretion of sPLA<sub>2</sub>-IIA from neuron-like cells.

The relative contributions of AMPA and KA receptors to sPLA<sub>2</sub>-IIA exocytosis were verified using antagonists to these receptors. Cells that express sPLA<sub>2</sub>-IIA-GFP fusion protein were treated with AMPA or KA, in the presence of receptor antagonists. AMPA receptors are composed of GluR1-4 subunits, whilst KA receptors consist of GluR5-7 and KA1-2 subunits [32-34]. These subunits likely exist as heteromers in vivo [73]. KA can activate AMPA receptors and AMPA can bind to KA receptors suggesting overlapping binding affinity for AMPA and KA receptors [74]. CNQX is an antagonist at AMPA receptors but has some activity against KA receptors. 10-20  $\mu$ M of CNQX blocks KA-receptor mediated excitatory post-synaptic currents by 40-80% [75]. Addition of CNQX partially blocked AMPA- but not KA-induced sPLA<sub>2</sub>-IIA exocytosis. This is expected, since CNQX acts mainly at AMPA receptors. UBP 302 and NS102 are selective antagonists of KA receptor subunits GluR5 and GluR6 respectively at 10  $\mu$ M [76,77]. Application of UBP 302 (a GluR5-subunit specific KA receptor antagonist) abolished the KA-induced sPLA<sub>2</sub>-IIA secretion, whereas addition of NS102 (GluR6-subunit specific KA receptor antagonist) did not show such inhibition. These observations indicate that both AMPA and KA receptors are important for stimulating exocytosis of sPLA<sub>2</sub>-IIA, although the majority of the effect appears to be mediated by the KA GluR5 receptor.

The above experiments were carried out on SH-SY5Y cells that stably express the sPLA<sub>2</sub>-IIA-GFP fusion protein. As controls against any unforeseen effects of the GFP tag on sPLA<sub>2</sub>-IIA enzyme function, we conducted further studies using cell lines that express

full length sPLA<sub>2</sub>-IIA, independent of the GFP tag. This was carried out using the sPLA<sub>2</sub>-IIA-IRES-EGFP vector, i.e. sPLA<sub>2</sub>-IIA and EGFP were co-transfected in same cell using a single vector, but not as a fusion protein. Stable cell lines expressing sPLA<sub>2</sub>-IIA were selected based on the EGFP fluorescence label, and enzyme release from these cells directly detected using an antibody to sPLA<sub>2</sub>-IIA. The findings confirm that sPLA<sub>2</sub>-IIA protein is released from the SH-SY5Y cells by KA stimulation.

Possible mechanisms of KA action on sPLA<sub>2</sub>-IIA release were also elucidated. AMPA, KA and NMDA receptors are typically described as ligand-gated ionotropic glutamate receptors [31]. Activation of these receptors had been shown to increase free intracellular Ca<sup>2+</sup> in neurons including SH-SY5Y cells [65]. Significantly reduced spontaneous or KA induced fusion events and release of sPLA<sub>2</sub>-IIA was detected in cells treated with the Ca<sup>2+</sup> chelator EGTA or after depletion of intracellular calcium by thapsigargin, consistent with a requirement for Ca<sup>2+</sup> in vesicular exocytosis [78]. Exocytosis was also induced by high potassium depolarization of cells (online suppl. Fig. 2). In several CNS regions including the hippocampus and spinal cord, reports have indicated that KA receptors could be metabotropically coupled with G-protein signaling cascades that involves the activation of phospholipase C generation of diacylglycerol and subsequent activation of protein kinase C (PKC) [33,36,39]. We observed that KA induced sPLA<sub>2</sub>-IIA exocytosis was inhibited by a PKC inhibitor, whereas activation of PKC by PMA triggered vesicular secretion of sPLA<sub>2</sub>-IIA. This may involve several proteins involved in exocytosis which are PKC substrates, including SNAP-25 (synaptosome-associated protein) and Munc-18 (essential component of the synaptic vesicle fusion protein complex) which potentiate vesicle recruitment and sensitize vesicle

release [53]. Moreover, GAP-43, a specific PKC substrate which is important in priming of exocytosis [79] is upregulated in hippocampal granule neurons treated with KA [80,81]. PKC may in turn, phosphorylate KA receptors leading to changes in channel conductance [82,83]. Together, the results indicate PKC-dependent KA-induced vesicular exocytosis and release of sPLA<sub>2</sub>-IIA.

The link between glutamate receptors and sPLA<sub>2</sub>-IIA exocytosis may be functionally important. Since sPLA<sub>2</sub> is present in the brain under basal conditions, this could implicate a link between glutamate signaling, sPLA<sub>2</sub> secretion, and neurotransmitter exocytosis. sPLA<sub>2</sub> is detected in different parts of the brain including the hippocampus [6,7] and is involved in synaptic transmission and LTP [84,85]. KA receptor subunits GluR5 and KA2 also form the predominant heteromeric pair in postsynaptic KA receptors of the hippocampus [33]. KA-mediated excitatory postsynaptic currents in hippocampus are generated mainly by GluR5 containing KA receptors [77]. In addition, a postsynaptic KA-mediated excitatory post-synaptic current onto second order neurons in spinal cord has been reported [86]. Electron microscopy reveals that the majority of KA receptors GluR5,6,7, are primarily postsynaptic to substance P containing axon terminals in the trigeminal dorsal horn [87]. Together with electron microscopic observations that sPLA<sub>2</sub>-IIA is localized in a postsynaptic location in the spinal cord [6], we hypothesize that the release of glutamate at a glutamatergic synapse may activate GluR5 containing KA receptors, to induce sPLA<sub>2</sub>-IIA exocytosis from storage vesicles in dendrites. Previous electrophysiological study indicates that sPLA<sub>2</sub>-IIA could stimulate release of neurotransmitters from PC12 neuroendocrine cells and cultured neurons [40]; hence, it is conceivable that sPLA<sub>2</sub>-IIA could diffuse across

the synaptic cleft to induce glutamate release from axon terminals. In this scenario, sPLA<sub>2</sub>-IIA is released from dendrites as a result of AMPA or KA receptor activation, and acts as a retrograde signal to amplify neurotransmission.

Our findings of high level of expression of sPLA<sub>2</sub>-IIA in the dorsal horn and spinal trigeminal nucleus [6] and the present link with KA receptors are consistent with a role of these receptors and sPLA<sub>2</sub> in nociceptive transmission. Intraperitoneal injection of the KA receptor antagonist SYM 2081 reduced mechanical allodynia and thermal hyperalgesia after chronic constriction nerve injury [88] or freeze injury [89]. The GluR5 antagonist LY 382884 produced antinociceptive responses to formalin in rats [90]. Moreover, responses to capsaicin or inflammatory pain were significantly reduced in mice lacking GluR5 but not GluR6 subunits [91]. Similarly, intrathecal administration of the sPLA<sub>2</sub> inhibitor, LY311727 reduced inflammatory hyperalgesia in rats [20], and intracerebroventricular injection of the sPLA<sub>2</sub> inhibitor, 12-episcalaradial decreased nociceptive responses after facial carrageenan injection in mice [21].

In conclusion, the present findings show that sPLA<sub>2</sub>-IIA enzymes are packed into fusion-competent vesicles in human neuroblastoma SH-SY5Y cells and are released through exocytosis by glutamate analogs. Together, these findings provide evidence of a link between glutamate receptors and sPLA<sub>2</sub> regulated secretion in neurons that may play an important role in the normal physiology of the CNS, nociception, and neurodegenerative diseases.

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### Figure Legends

**Fig. 1.** sPLA<sub>2</sub>-IIA enzymes are packed into fusion-competent vesicles. **[A]** Typical TIRFM image and **[B]** bright field image of SH-SY5Y cell stably expressed with GFP-tagged sPLA<sub>2</sub>-IIA fusion protein (full-length sPLA<sub>2</sub>-IIA). Individual sPLA<sub>2</sub>-IIA vesicles can be resolved as bright dots **[A]**. Scale = 5  $\mu$ m. **[C]** Typical motion trajectory of a subplasmalemmal sPLA<sub>2</sub>-IIA vesicle. Scale = 200 nm. **[D]** Sequential images of fusion event (top) of a sPLA<sub>2</sub>-IIA vesicle. The curves in the bottom panel represent the change of the mean fluorescence intensity over time within the region <0.5  $\mu$ m away from the vesicle center (solid line) and the change of fluorescence intensity within the annular region >0.5 and <0.65  $\mu$ m away from the vesicle center (dotted line). Full fusion event of vesicle is characterized by simultaneous transient increase in these two curves due to rapid release of the fluorescent molecules and their diffusion to the peripheral region. The vertical dashed line indicates the onset of fusion event. Scale = 1  $\mu$ m.

**Fig. 2.** sPLA<sub>2</sub>-IIA enzymes are colocalized with synaptotagmin-1. Distribution of GFP-tagged sPLA<sub>2</sub>-IIA molecules (full-length sPLA<sub>2</sub>-IIA) (left lane) in SH-SY5Y cells, with **[A, middle]** immunostained synaptotagmin-1 (AlexaFluor 555 secondary antibody) or **[B, middle]** immunostained NPY (Atto 647 secondary antibody), were analyzed using confocal microscopy. Respective co-localization (merge of left and middle) are shown at the right lane. Scale = 5  $\mu$ m.

**Fig. 3.** Glutamate analogs differentially modulate exocytosis and release of sPLA<sub>2</sub>-IIA from SH-SY5Y cells stably expressed sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA). **[A]** Cells were incubated for 30 min without (control, Con) or with 10  $\mu$ M each of glutamate, KA,

NMDA or AMPA, followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean  $\pm$  SEM (5-6 cells from 3-4 separate experiments). **[B and C]** Top panels show the representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released from cells ( $5 \times 10^6$  cells/ flask) into the serum-free DMEM media during 2 hr incubation at 37 °C, **[B]** without (control, Con) or with 10  $\mu$ M each of glutamate, KA, NMDA or AMPA, or **[C]** without (control, Con) or with KA (10 nM to 100  $\mu$ M). The lower panels show the statistics (mean  $\pm$  SEM, n = 6) of optical density of the sPLA<sub>2</sub>-IIA-GFP blots (arbitrary unit; a.u.) under different conditions, normalized by optical density of GAPDH blots, from 3-4 separate experiments of each group. \*  $p < 0.05$  vs. control, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

**Fig. 4.** CNQX fails to block the KA-induced sPLA<sub>2</sub>-IIA secretion from SH-SY5Y cells stably cloned with sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA). **[A]** Cells were 30 min incubated without (control, Con), or with KA (10  $\mu$ M), or KA plus CNQX (20  $\mu$ M), or NMDA (10  $\mu$ M), or NMDA plus CNQX, or AMPA (10  $\mu$ M), or AMPA plus CNQX, followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean  $\pm$  SEM (5-6 cells from 3-4 separate experiments). **[B]** Top panel shows the representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released into the serum-free media during 2 hr incubation, without (control, Con), or with KA (10  $\mu$ M), or KA plus CNQX (20  $\mu$ M), or NMDA (10  $\mu$ M), or NMDA plus CNQX, or AMPA (10  $\mu$ M), or AMPA plus CNQX. The lower panel shows the statistics (mean  $\pm$  SEM, n = 6) of optical density of the sPLA<sub>2</sub>-IIA-GFP blots (a.u.), normalized by optical density of GAPDH blots, from 3-4 separate experiments. \*  $p < 0.05$  vs. control; #

$p < 0.05$  vs. AMPA treatment alone, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

**Fig. 5.** KA-induced sPLA<sub>2</sub>-IIA secretion is through the GluR5 containing receptors. **[A]** SH-SY5Y cells stably expressed sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA) were 30 min incubated without (control, Con) or with KA (10  $\mu$ M), KA plus UBP 302 (UBP 302, GluR5-containing KA receptor antagonist, 20  $\mu$ M), or KA plus NS 102 (NS 102, GluR6-containing KA receptor antagonist, 20  $\mu$ M), followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean  $\pm$  SEM (5-6 cells from 3-4 separate experiments). **[B]** Top panel shows the representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released into the serum-free medium during 2 hr incubation, without (control, Con) or with KA (10  $\mu$ M), KA plus UBP 302 (20  $\mu$ M), or KA plus NS 102 (20  $\mu$ M). The lower panel shows the statistics (mean  $\pm$  SEM, n = 6) of optical density of the sPLA<sub>2</sub>-IIA-GFP blots (a.u.), normalized by optical density of GAPDH blots, from 3-4 separate experiments. \*  $p < 0.05$  vs. control; #  $p < 0.05$  vs. KA treatment alone, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

**Fig. 6.** KA-induced sPLA<sub>2</sub>-IIA secretion is Ca<sup>2+</sup> dependent. **[A]** SH-SY5Y cells stably expressed with sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA) were incubated 30 min in bath solution, without (control, Con) or with KA (10  $\mu$ M), EGTA (2 mM), EGTA plus KA, thapsigargin (1  $\mu$ M), or thapsigargin plus KA, followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean  $\pm$  SEM (6-7 cells from 3-4 separate experiments). **[B]** Top panel shows the

representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released into the serum-free medium during 2 hr incubation, without (control, Con) or with KA (10 μM), EGTA (2 mM), EGTA plus KA, thapsigargin (1 μM, 15 min pretreatment), or thapsigargin plus KA. The lower panel shows the statistics (mean ± SEM, n = 6) of optical density of the sPLA<sub>2</sub>-IIA-GFP blots (a.u.), normalized by optical density of GAPDH blots, from 3-4 separate experiments. \*  $p < 0.05$  vs. control; #  $p < 0.05$  vs. KA treatment alone, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

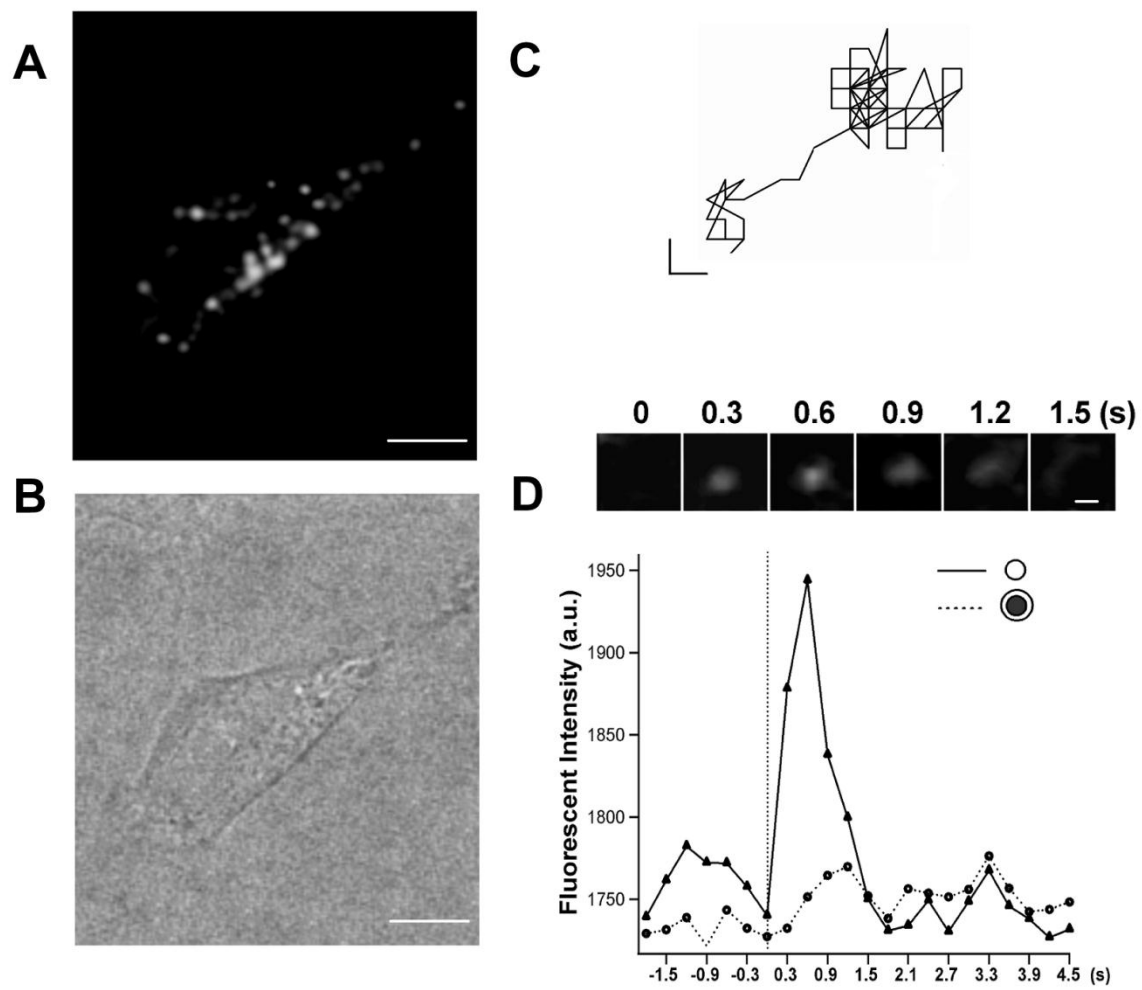
**Fig. 7.** KA-induced sPLA<sub>2</sub>-IIA secretion is PKC dependent. [A] SH-SY5Y cells stably expressed with sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA) were incubated 30 min without (control, Con) or with KA (10 μM), KA plus BIS-XI (bisindolylmaleimide XI, Ro32-0432, 2.5 μM), BIS-XI, BIS-IX (Ro31-8220, 2.5 μM), PMA (phorbol-12-myristate-13-acetate, 100 nM), or PMA plus BIS-XI, followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean ± SEM (6-7 cells from 3-4 separate experiments). [B] Top panel shows the representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released, from, into the serum-free medium during 2 hr incubation, without (control, Con) or with KA (10 μM), KA plus BIS-XI (2.5 μM, 30 min pretreatment), BIS-XI, BIS-IX (2.5 μM), PMA (100 nM), or PMA plus BIS-XI. The lower panel shows the statistics (mean ± SEM, n = 7) of optical density of the sPLA<sub>2</sub>-IIA-GFP blots (a.u.), normalized by optical density of GAPDH blots, from 3-4 separate experiments. \*  $p < 0.05$  vs. control; #  $p < 0.05$  vs. KA or PMA treatment alone, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

**Fig. 8.** Signal peptide of sPLA<sub>2</sub>-IIA is required for vesicular localization and exocytosis of sPLA<sub>2</sub>-IIA. **[A]** Typical TIRFM image and **[B]** bright field image of SH-SY5Y cell stably expressed GFP-tagged sPLA<sub>2</sub>-IIA (sPLA<sub>2</sub>-IIA with no signal peptide) showing the homogenous green fluorescence without any visible vesicle-like structures **[A]**. Scale = 5  $\mu$ m. **[C]** TIRFM analysis of the vesicular exocytosis (fusion events) from the two cloned cells stably expressed with either GFP tagged- full-length sPLA<sub>2</sub>-IIA (FL, full-length sPLA<sub>2</sub>-IIA) or GFP-tagged sPLA<sub>2</sub>-IIA without signal peptide (NSP, sPLA<sub>2</sub>-IIA with no signal peptide). The cloned cells were incubated without or with KA (10  $\mu$ M) for 30min, followed by imaging the live cells. The number of fusion events during a 2 min time interval was counted. Values represent mean  $\pm$  SEM (5-6 cells from 3 separate experiments). **[D]** Top panel shows the representative immunoblots of sPLA<sub>2</sub>-IIA fusion protein released from the respective cloned cells into the serum-free medium during 2 hr incubation, without or with KA (10  $\mu$ M). The lower panel shows the statistics (mean  $\pm$  SEM, n = 6) of optical density of the blots in arbitrary unit (a.u.), normalized by optical density of GAPDH, from 3-4 separate experiments. Negative control used the same serum-free medium before 2 hr incubation. \*  $p < 0.05$  vs. NSP (without KA); #  $p < 0.05$  FL (without KA) vs. FL (treated with KA), analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

**Fig. 9.** KA stimulates release of sPLA<sub>2</sub>-IIA (not GFP tagged fusion protein) from SH-SY5Y cells stably cloned with sPLA<sub>2</sub>-IIA-IRES-EGFP (full-length sPLA<sub>2</sub>-IIA). Top panels show the representative immunoblots of **[A]** sPLA<sub>2</sub>-IIA or **[B]** EGFP released from the cloned cells, which stably and separately expressed both full-length sPLA<sub>2</sub>-IIA

and EGFP, into the serum-free medium during 2 hr incubation, without (control, Con) or with 10  $\mu$ M each of glutamate, KA, NMDA, or AMPA. The lower panel shows the statistics (mean  $\pm$  SEM, n = 6) of optical density of the blots (a.u.), normalized by optical density of GAPDH, from 3-4 separate experiments. Immunoblots of sPLA<sub>2</sub>-IIA and EGFP were obtained from the same sample sets. \*  $p < 0.05$  vs. control, analyzed by 1 way ANOVA and Bonferroni's post-hoc test.

Fig. 1



**Fig. 2**

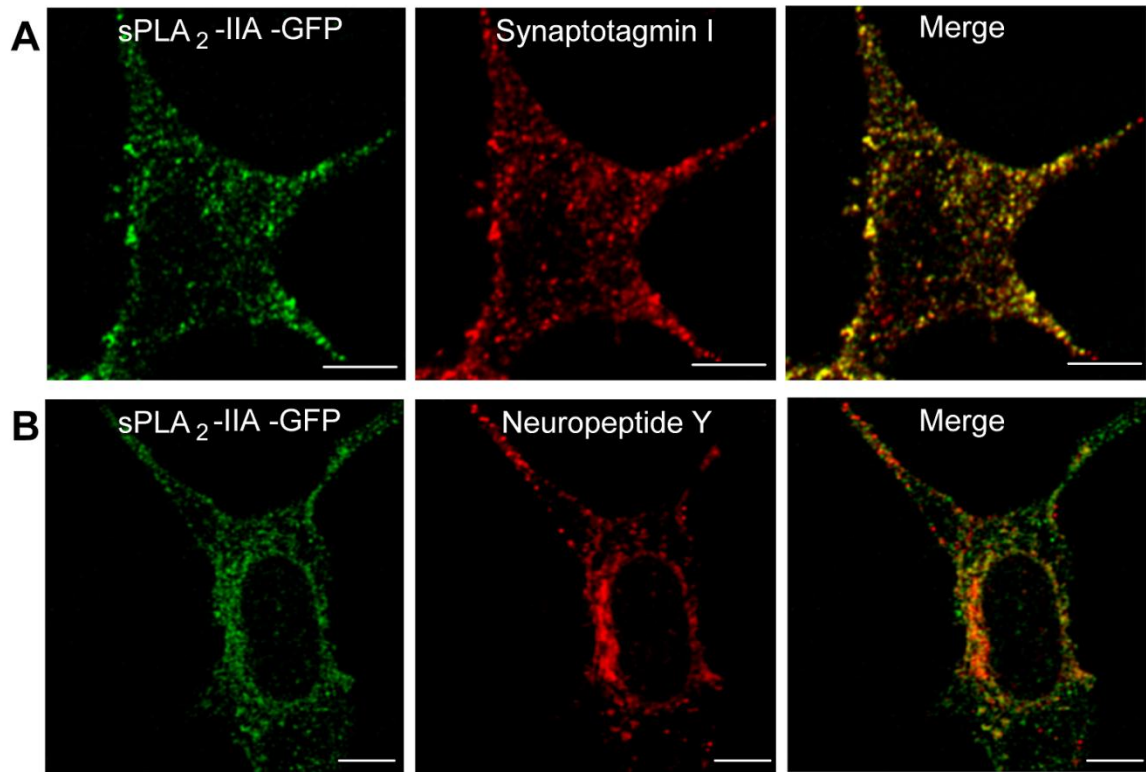


Fig. 3

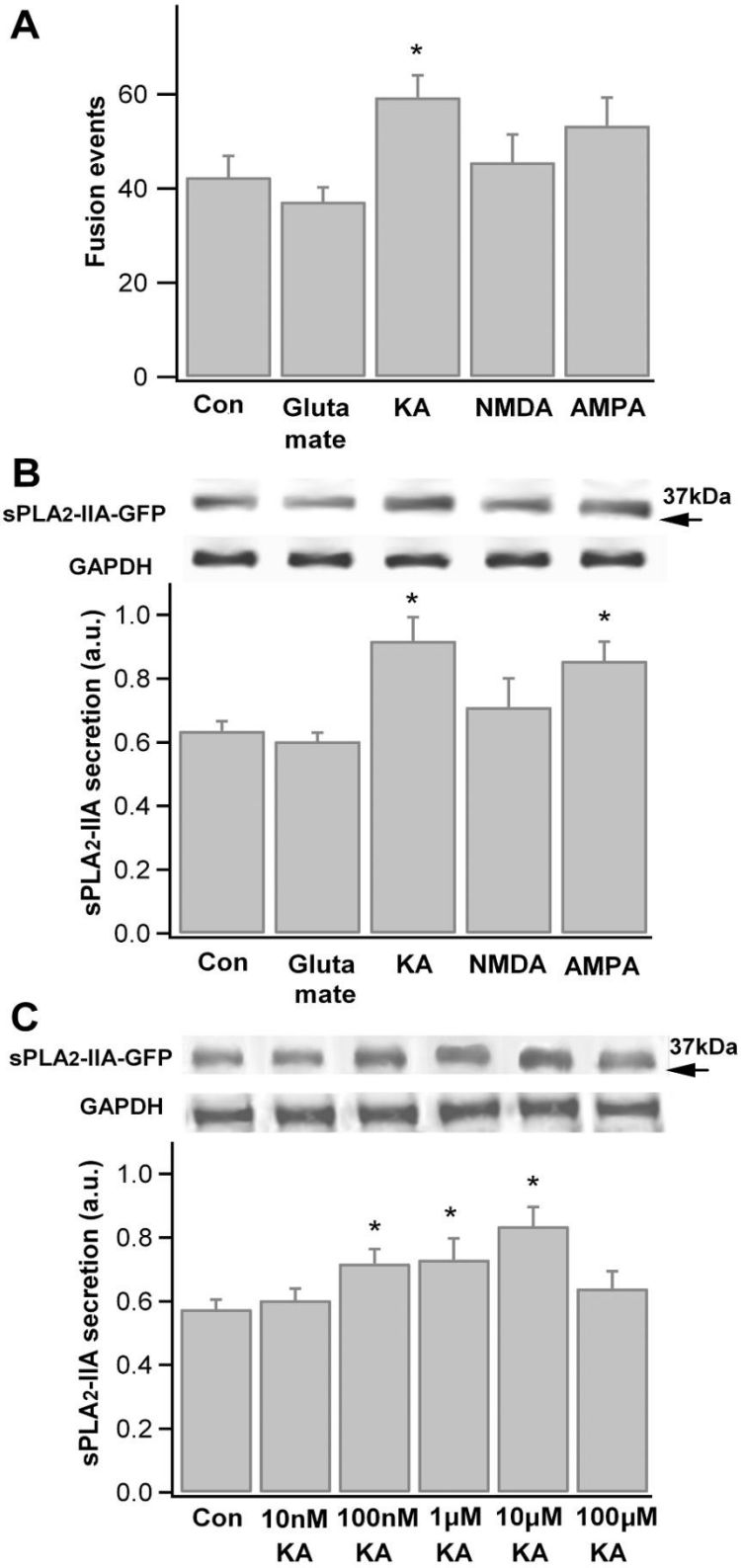
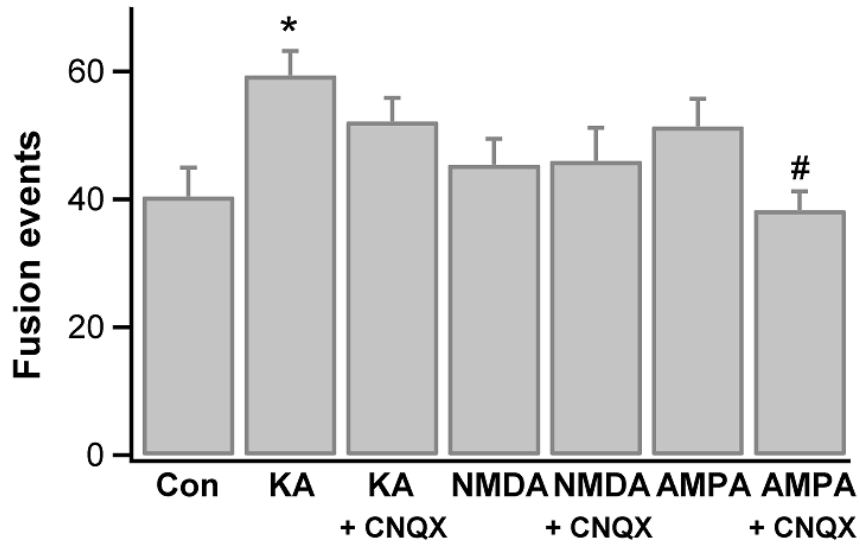


Fig. 4

**A**



**B**

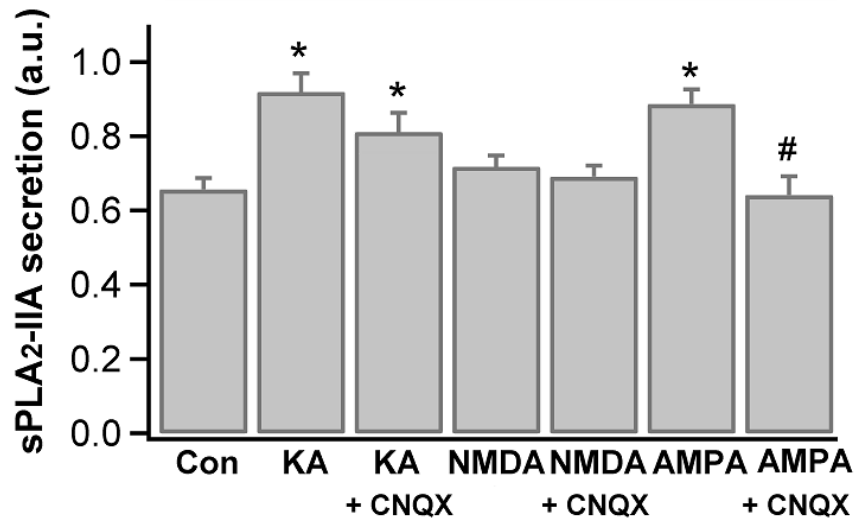


Fig. 5

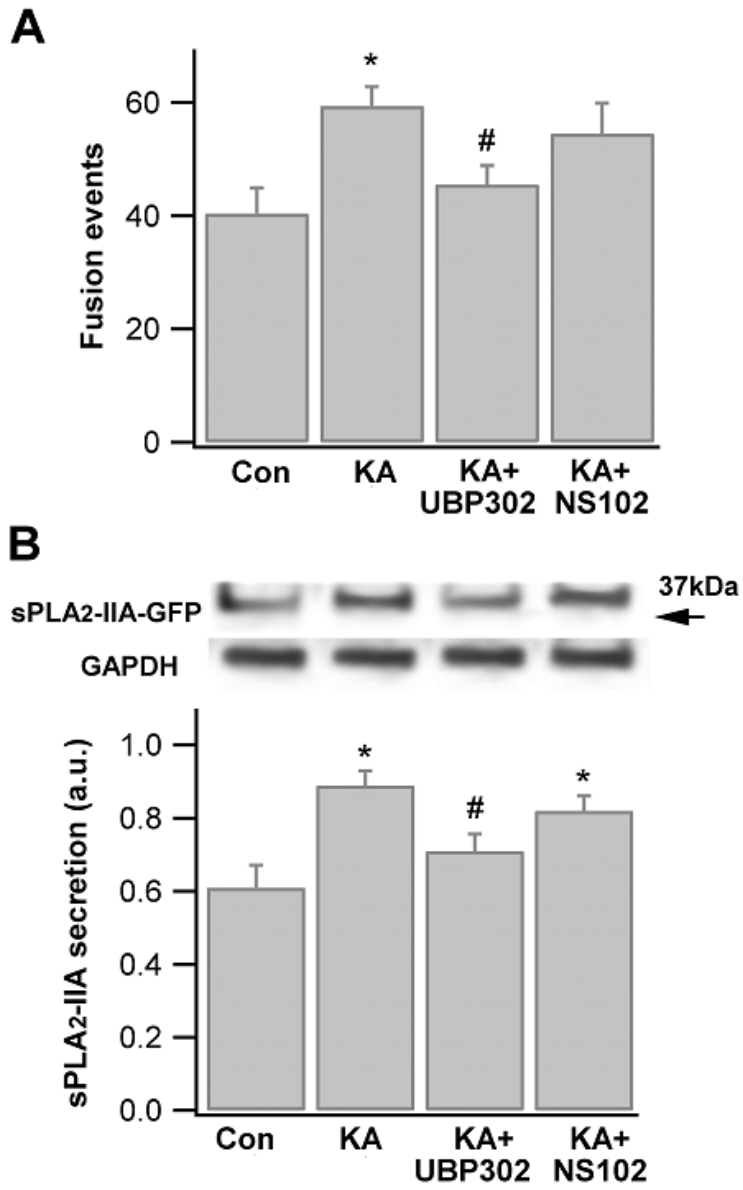


Fig. 6

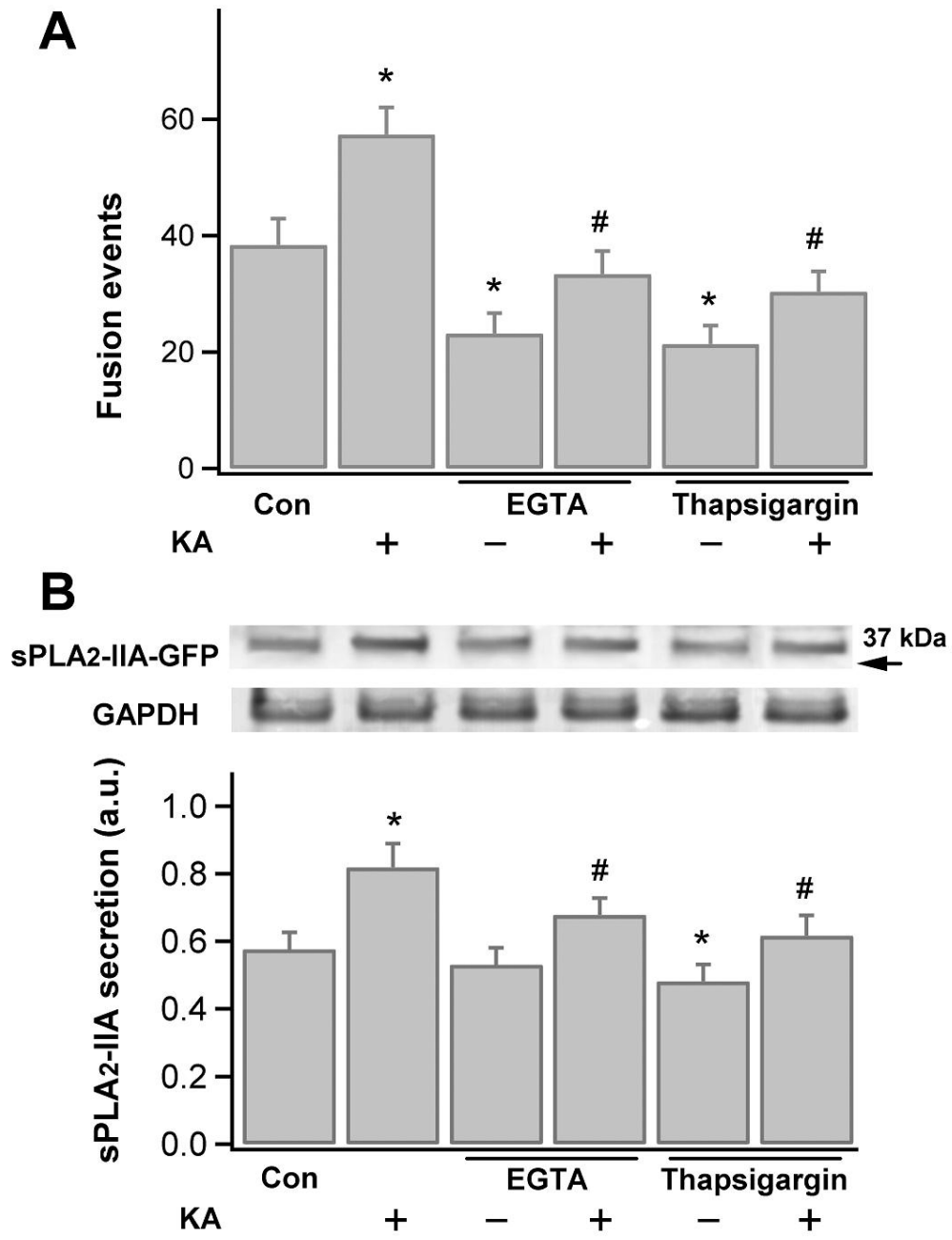


Fig. 7

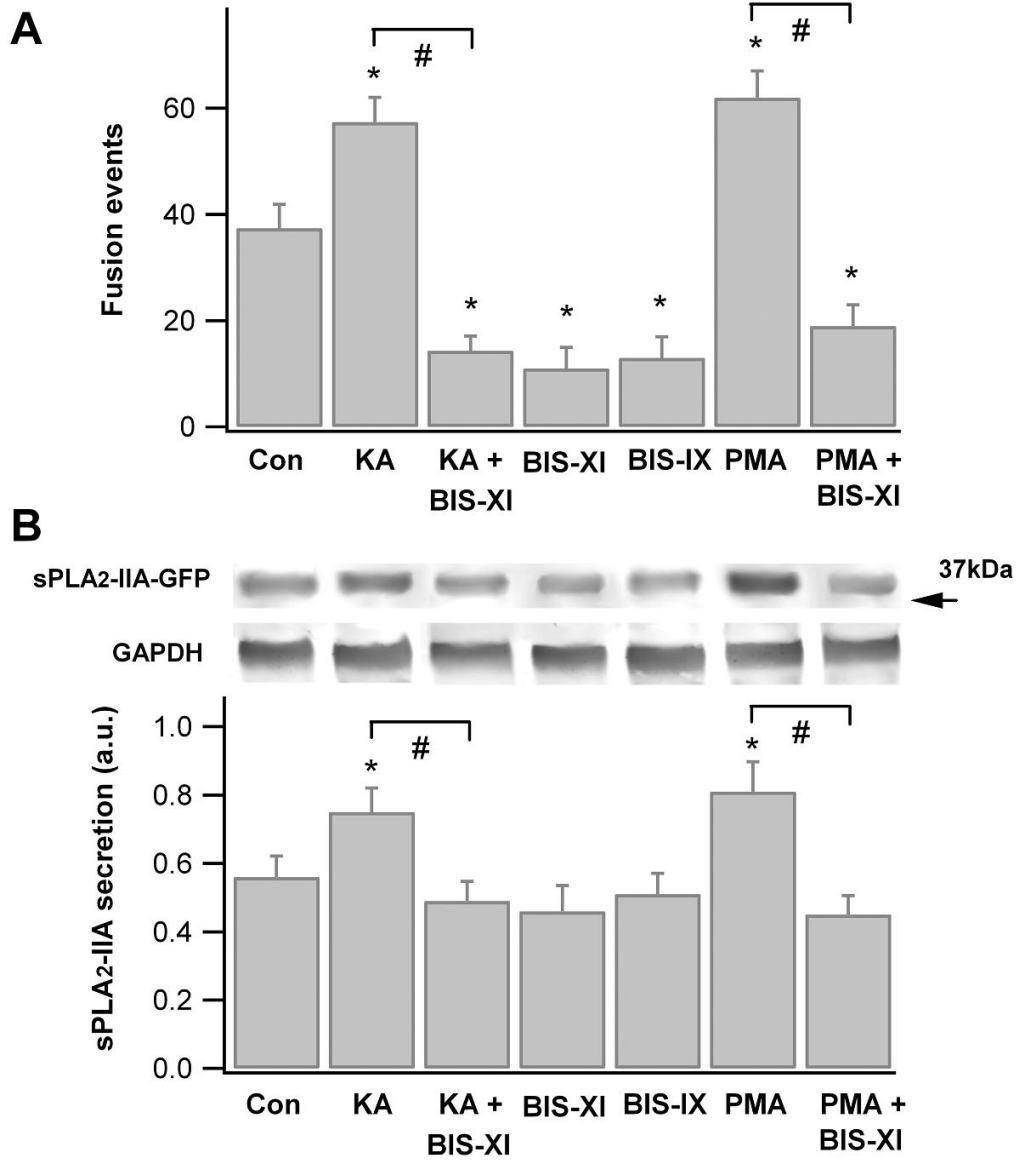


Fig. 8

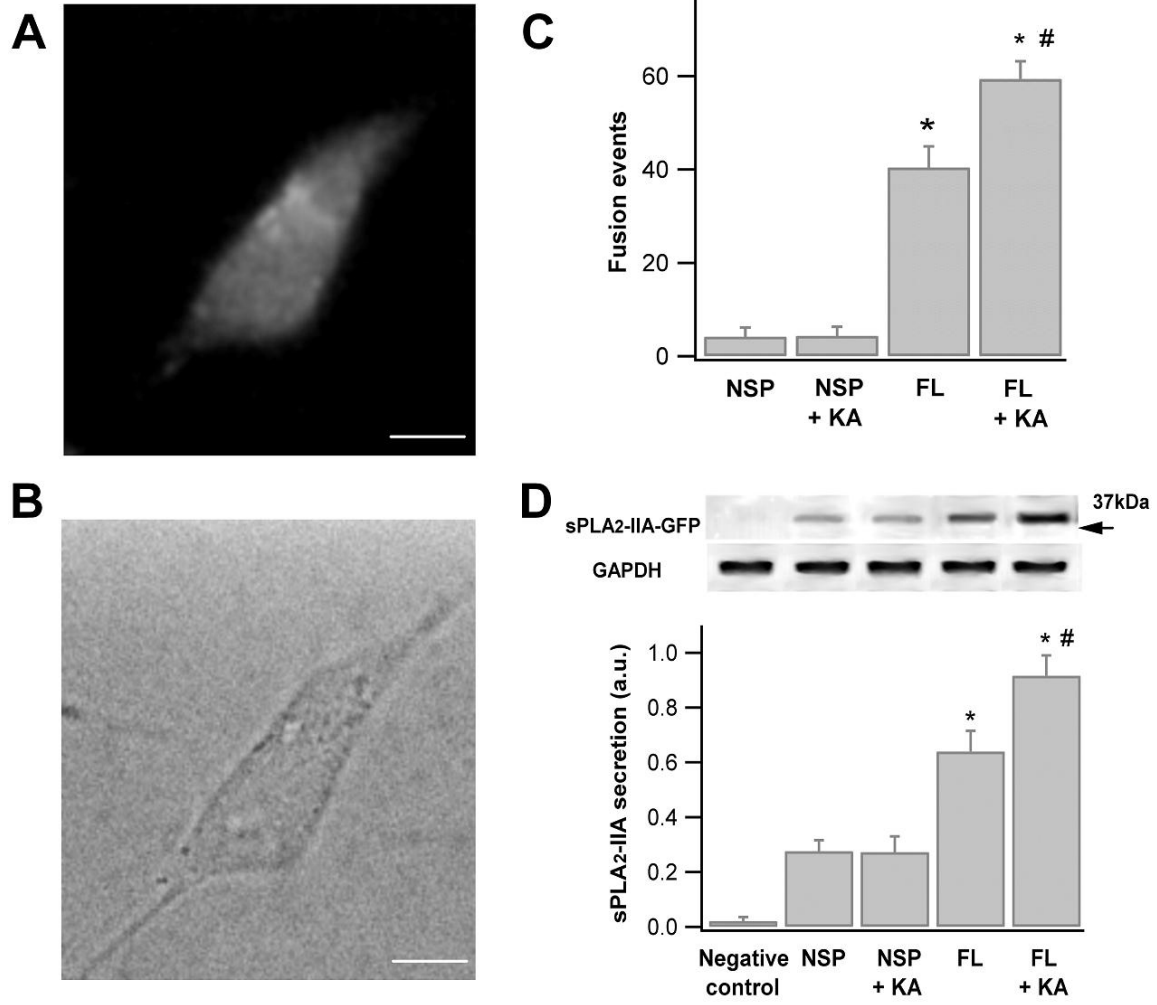
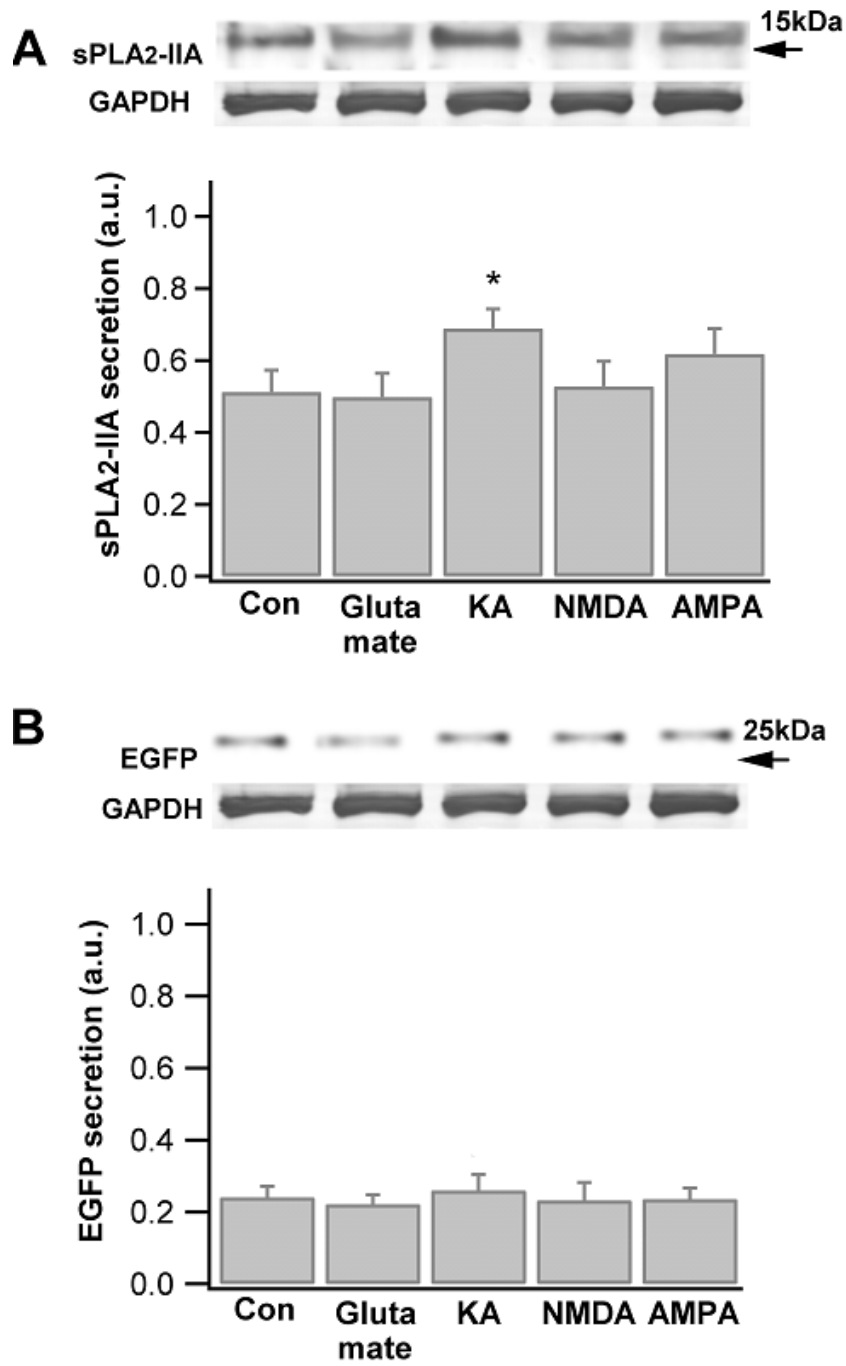
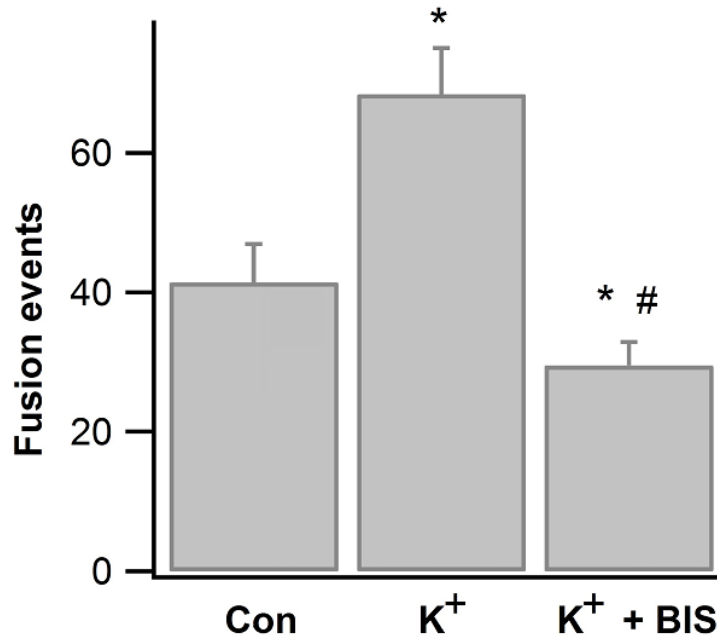


Fig. 9

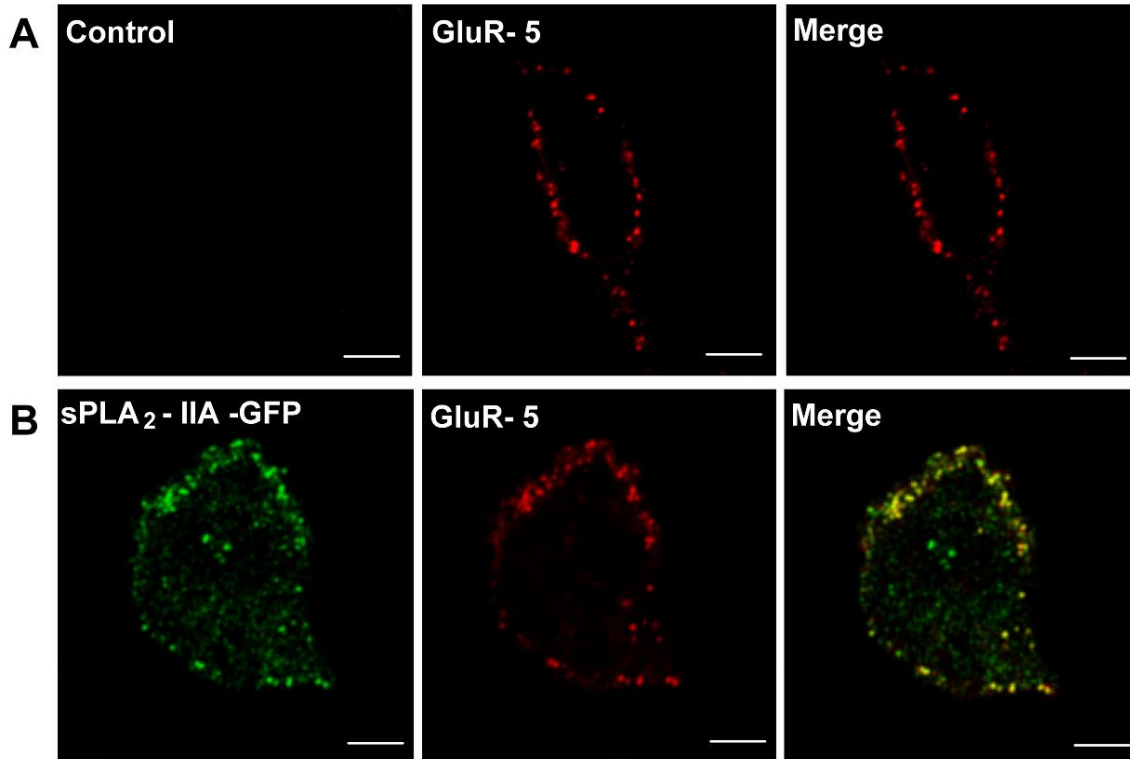


Supplementary Document



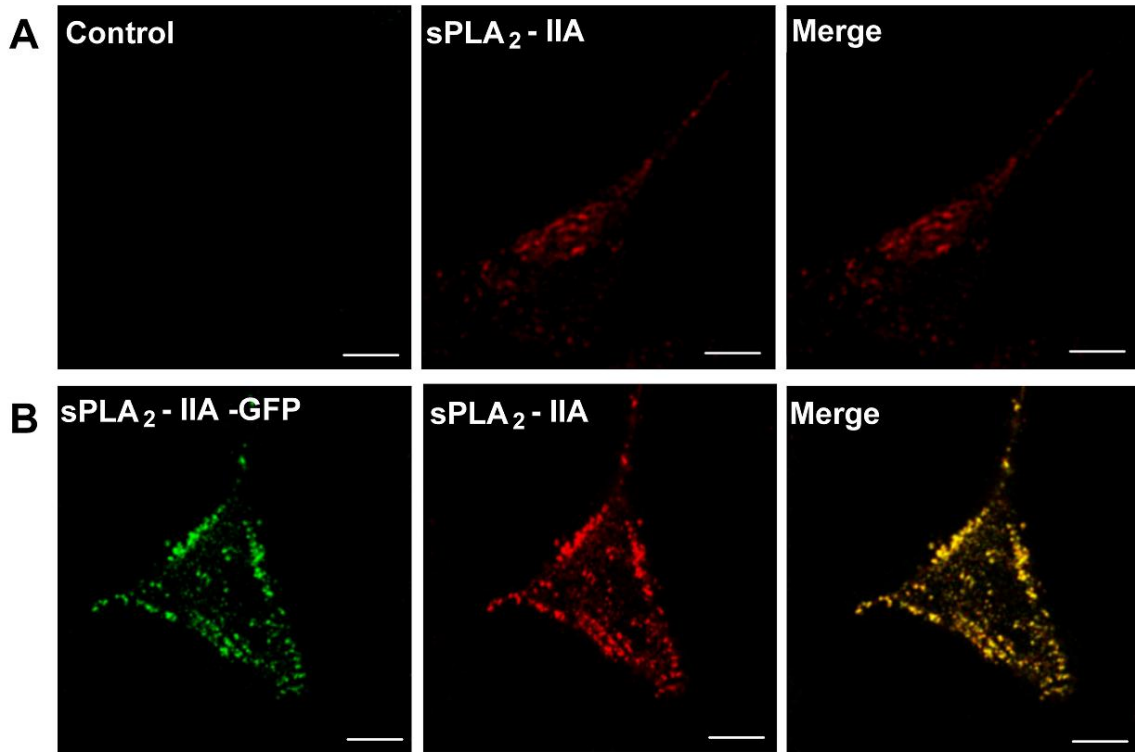
Supplementary Fig. 1.

SH-SY5Y cells stably expressed with sPLA<sub>2</sub>-IIA-GFP (full-length sPLA<sub>2</sub>-IIA) were incubated in bath solution (150mM NaCl, 5.4mM KCL, 2mM MgCl<sub>2</sub>, 2mM CaCl<sub>2</sub>, 5mM glucose, 10mM HEPES, pH 7.4) (control, Con), or in high K<sup>+</sup> solution (37mM NaCl, 105mM KCL, 2mM MgCl<sub>2</sub>, 5mM CaCl<sub>2</sub>, 5mM glucose, 10mM HEPES, pH 7.4) without or with BIS (2.5 μM, 30 min pretreatment), followed by imaging of cells by TIRFM. The number of fusion events during a 2 min time interval was counted. Values represent mean ± SEM (5-6 cells from 3 separate experiments).



**Supplementary Fig. 2.**

Immunocytochemical analysis of GluR-5 expression in SH-SY5Y cells [A] before (as control) or [B] after transfection with sPLA<sub>2</sub>-IIA-GFP (full length sPLA<sub>2</sub>-IIA). Cells were fixed with 4% formaldehyde, blocked with 1.5% bovine albumin and incubated overnight with a goat polyclonal antibody to GluR-5 (Santa Cruz Biotech, diluted 1:100), followed by incubation for 1 hr with donkey anti-goat secondary antibody conjugated with AlexaFluor 555 (Invitrogen). Cells were washed with PBS and viewed using a LSM 510 Meta confocal laser scanning microscope. GFP-tagged sPLA<sub>2</sub>-IIA molecules (full-length sPLA<sub>2</sub>-IIA) can be seen as green dots (left lane), while red color represents the immunostaining of GluR5 (middle lane), and merge of left and middle are shown at the right lane. Scale = 5  $\mu$ m.



**Supplementary Fig. 3.**

Immunocytochemical analysis of sPLA<sub>2</sub>-IIA expression in SH-SY5Y cells [A] before (as control) or [B] after transfected with sPLA<sub>2</sub>-IIA-GFP (full length sPLA<sub>2</sub>-IIA). Cells were fixed with 4% formaldehyde, permeabilized with 0.1% TritonX-100, blocked with 1.5% bovine albumin and incubated overnight with a rabbit primary antibody to sPLA<sub>2</sub>-IIA (diluted 1:5000), followed by incubation for 1 hr with goat anti-rabbit secondary antibody conjugated with Atto 647 NHS (Sigma). Cells were washed with PBS and viewed using a LSM 510 Meta confocal laser scanning microscope. GFP-tagged sPLA<sub>2</sub>-IIA molecules (full-length sPLA<sub>2</sub>-IIA) can be seen as green dots (left lane), while red color represents the immunostaining of sPLA<sub>2</sub>-IIA (middle lane), and merge of left and middle are shown at the right lane. Scale = 5  $\mu$ m.