Xiaohui Wang, Report on ISN CAEN award Cat 1C return home Grant (Dec 2015)

Dr. Xiaohui Wang was a Post-Doc of University of Colorado-Boulder, USA. He moved back to Changchun Institute of Applied Chemistry, Chinese Academy of Sciences and started his independent research career. With the support of ISN CAEN Award Cat 1C return home grant (Dec 2015), he tried to elucidate the methaphatamine induced TLR4 signaling. The progress of the project in 2015-2016 is summarized herein.

Hypothesis: Methamphetamine induces microglia activation and cytokines over-production. However, its molecular and cellular target is not clear. Based on our works on opioids and cocaine, we proposed a XAMP hypothesis. Herein, we speculate that methamphetamine acts as a xenobiotics associated molecular pattern (XAMP), which is sensed by TLR4 accessory protein MD-2 and induces microglia TLR4 activation, therefore contributing to drug addiction.

Methods

MD-2 expression and purification

Insect expression human MD-2-pAcGP67A vector was provided by Dr Jie-Oh Lee (KAIST, Korea). MD-2 baculovirus was prepared by co-transfection of SF-9 insect cells with MD-2-pAcGP67A vector and bright linearized baculovirus DNA as described by the manufacturer's protocol (BD Bioscience, San Diego, CA, USA). After 2-3 rounds of amplification, the MD-2 baculovirus suspension reached a titer of ~10^8/ml virus particles and was used to transfect high 5 insect cells to express MD-2. MD-2 was secreted into the medium. After 3-4 day transfection, the medium was harvested and subjected to IgG sepharose affinity purification.

Fluorescence titrations

Fluorescence measurements were performed on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ, USA). All measurements were carried out under room temperature in a 2×10 mm quartz cell (Starna Cells, Atascadero, CA, USA). 280 nm was chosen as the excitation wavelength of MD-2 intrinsic Tyr and Trp fluorescence and emission at 300- 450 nm was measured. 385 nm was chosen as the excitation wavelength of extrinsic fluorescence probe Bis-ANS and emission at 420-550 nm was recorded. Appropriate controls were subtracted from spectra obtained on the samples. Fluorescence was also corrected by the relation, $F_{corr} = F_{obs}$ anti-log $(OD_{ex} + OD_{em}/2)$ for the inner filter effect when necessary, where OD_{ex} and OD_{em} are the optical densities at excitation and emission wavelengths, respectively.

For methamphetamine quenching MD-2 intrinsic fluorescence assay, 0.5 μM MD-2 was titrated with different concentrations of methamphetamine, and the fluorescence

intensity at 337 nm was plotted against methamphetamine concentration. The raw data was fitted by non-linear least square method using the equation: F=0.5×(2×F_0-F_{PL}× (K_D+[L_T]+[P_T]-((K_D+[L_T]+[P_T])^2-4×[L_T]×[P_T])$^{0.5}$)), where [F], the observed fluorescence; F_0, initial fluorescence of protein in the absence of ligand; F_{RL}, adjustable parameter for protein–ligand complex molar fluorescence; K_D, dissociation constant; [L_T], total concentration of the ligand; [P_T], total protein concentration. The data were also plotted according to Scatchard equation: $\gamma/c=n/K_D - \gamma/K_D$,, where r is the ratio of the concentration of bound ligand to total available binding sites, c is the concentration of free ligand, and n is the number of binding sites per protein molecule and K_D is the dissociation constant.

For displacement assay, different concentrations of methamphetamine were titrated into MD-2 (0.5 μM) and Bis-ANS 0.5 μM) reaction mix. After overnight equilibrium at room temperature, the Bis-ANS fluorescence intensity was measured. The fluorescence intensity at 47nm was plotted against methamphetamine concentration. K_i of methamphetamine was determined using the equation: K_i= K_{app}/(1 + [Bis-ANS]/K_D(Bis-ANS -MD-2)).

Dual luciferase NF- κB activity

NF-κB dual luciferase reporter glial BV-2 cell line was constructed by Cignal Lenti NF-κB Reporter kit (SABiosciences, MD, USA). Firefly luciferase gene was placed under the control the NF-κB transcriptional response element and the constitutively expressing Renilla luciferase was placed under the control of CMV promoter. The internal control Renilla luciferase can overcome technical variability and obtain more reliable data.

NF-κB dual luciferase reporter BV-2 cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and puromycin (4 μg/mL). BV-2 reporter cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After 24 h incubation, medium was changed to Opti-MEM medium supplemented with 0.5% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and 1% of non-essential amino acid (NEAA) and indicated concentration of methamphetamine or methamphetamine and MD-2 antagonists (LPS-RS)/TLR4 signaling antagonists (TAK-242) was added. After further 48 h treatment, the NF-κB activity was analyzed by Dual-Glo Luciferase Assay System (Promega, Madison, MI, USA) according to manufacturer's instructions. The ratio of Firefly luciferase activity to Renilla luciferase activity represents the NF-κB activity. It should be noted that NF-κB activity of the untreated control group was set as 100%.

In vivo Microdialysis

Microdialysis guide cannulae (CMA Microdialysis) were surgically implanted, aimed at the right or left NAc shell using stereotaxic coordinates relative to bregma: anterior/posterior=+1.7 mm; medial/lateral=+/-0.8 mm; relative to dura: dorsal/ventral = -5.6 mm, bite bar = 0 in a counterbalanced fashion.

Rats were placed in separate Plexiglas bowls with *ad libitum* food and water in the microdialysis testing room. Microdialysis probes were inserted through each guide cannula and artificial CSF perfused through the probes using a CMA infusion pump at a rate of 0.2 µl/min overnight. The next morning, the flow rate was increased to 1.5 µl/min for the duration of the experiment. Two hours later, 3 baseline samples were collected and then drug treatments were administered. The sample tubes were changed every 20 min for a total of 4 h (12 samples total) and stored at -80° C until high performance liquid chromatography (HPLC) analysis.

All rats received two subcutaneous injections, of either 2.5 mg/kg TLR4 antagonist (+)-naloxone, for a total of 5 mg/kg (+)-naloxone, 7.5 mg/kg (+)-naloxone for a total of 15 mg/kg, or equivolume saline. 10 min following the first subcutaneous injection, rats received the second identical subcutaneous injection along with an intraperitoneal injection of either 1 mg/kg methamphetamine or saline

Results

As shown in the Figure 1a, methamphetamine binds to MD-2 and causes the quenching of MD-2 intrinsic fluorescence. By fitting the curves by nonlinear least-square regression method, a K_D of 6.7 ± 0. 6 µM was obtained. A stoichiometry of 0.89 ± 0.0 8 was obtained for the methamphetamine binding to MD-2 (Figure 1b). As a comparison, roxithromycin, a compound used as a negative control, shows no MD-2 binding activity. Bis-ANS, a MD-2 molecular probe, has been shown to bind to the LPS binding pocket of MD-2 and its fluorescence intensity enhances when binding to MD-2. As shown in Figure 1c, methamphetamine caused the decrease of Bis-ANS fluorescence from the Bis-ANS-MD-2 complex with a Ki of 16.0 ± 5.7 µM, suggesting methamphetamine replaces Bis-ANS binding to MD-2. Furthermore, methamphetamine shows negligible binding to Protein A (Figure 1d). Taken together, these data suggest methamphetamine specifically binds to the LPS binding pocket of MD-2.

NF-κB activation and up-regulation of proinflammatory cytokines were used to monitor TLR4 activation. As show in Figure 2, methamphetamine induced NF-κB activation in BV-2 microglia, macrophage-like immunocompetent cells in the CNS that express TLR4. It should be noted that no cell proliferation inhibition was observed at the concentrations of methamphetamine used here. The competitive inhibitor LPS-RS suppressed methamphetamine -induced NF-κB activation. TAK-242, an intracellular TLR4 antagonist acting on Cys747 of TLR4, also significantly attenuated methamphetamine induced NF-κB activation.

Our in vitro data indicate that methamphetamine binds to MD-2 and thereby activates TLR4 signaling. Our previous findings with morphine and cocaine indicate that drug-induced TLR4 activation is a critical component of the ability of these drugs to elicit increased concentration of dopamine within the nucleus accumbens (NAc).

Using in vivo micro-dialysis, we tested the effects of TLR4 antagonism on methamphetamine induced dopamine levels with the NAc. We have recently characterized the non-opioid, (+)-isomer of naloxone as a selective, competitive TLR4 antagonist. It is readily blood-brain-barrier permeable and thus far demonstrated a notable lack of side-effect or off-target actions. As shown in Figure 3, methamphetamine (1mg/kg) produced robust increase in extracellular NAc dopamine compared to saline control ($p<0.0001$) that were dose-dependently attenuated with (+)-naloxone (15mg/kg:$p<0.01$; 5 mg/kg: $p>0.05$). Two-way ANOVA revealed a main effect of treatment ($F(_{4,17})=17.57$, $p<0.0001$) and time ($F(_{8,136})=20.82$, $p<0.0001$) and was followed by bonferroni post-hocs. Importantly, (+)-naloxone treatment alone did not alter basal dopamine levels ($p>0.05$) suggesting that (+)-naloxone did not independently produce effects on dopamine signaling. We have previously established that (+)-naloxone treatment does not interfere with dopaminergic cell functioning, and that non-TLR4 stimulation of increased DA within the NAc is preserved in the presence of (+)-naloxone.

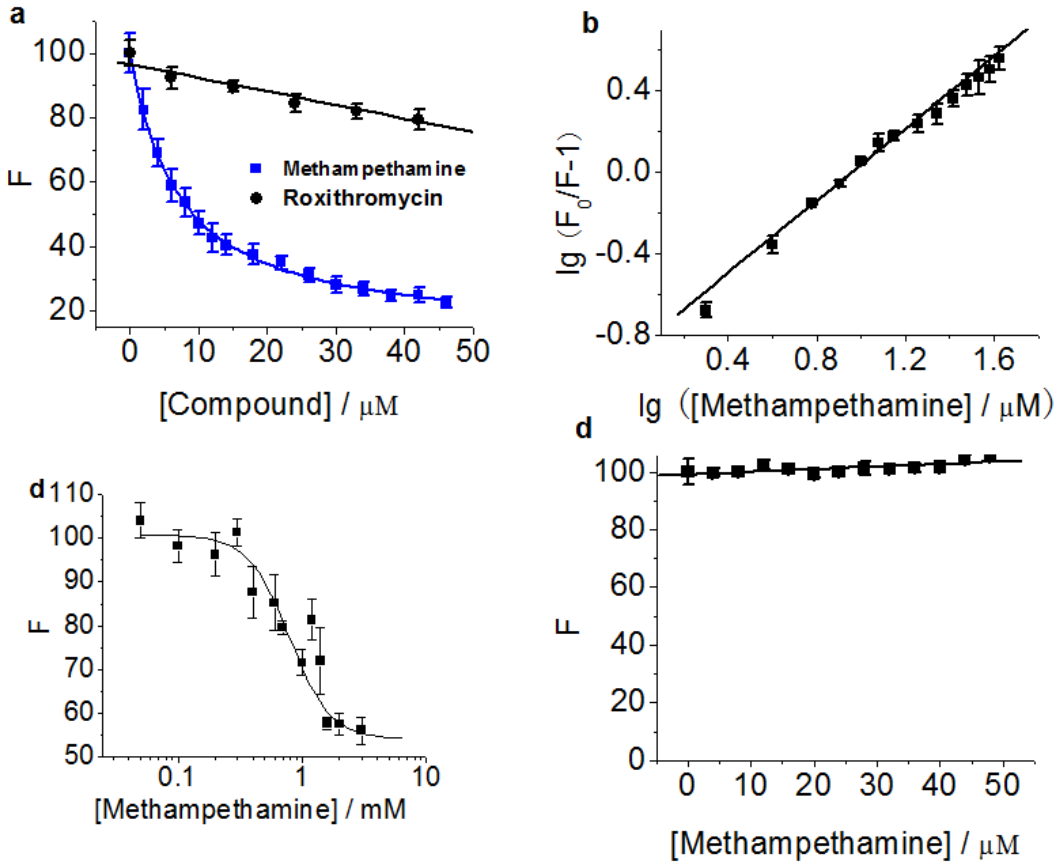

Figure 1. Methamphetamine binds to MD-2. (a), Titration curves of methamphetamine binding to MD-2. 280 nm was chosen as the excitation and emission at 337 nm (peak position) was plotted against the titrated naloxone concentration. Roxithromycin, showing no apparent binding to MD-2, was served as the negative control compound. By fitting the curves by nonlinear least-square regression, the K_D of 6.7 ± 0. 6 μM was obtained. (b), the methamphetamine binding curve shown in (a) was plotted according to the equation: lg (F_0/F-1) = -lgK_D + n×lg ([Naloxone]). K_D = 7.6 ± 0. 9 μM and n =0.89 ± 0.0 8 were derived for the methamphetamine binding to MD-2. (c), methamphetamine displaces Bis-ANS binding to MD-2. Different concentrations of methamphetamine were titrated into MD-2 (0.5 μM) and Bis-ANS (0.5 μM) mixture. 385 nm was chosen as the excitation wavelength for Bis-ANS and the fluorescence signal at 478 nm was plotted against the titrated methamphetamine concentration. Data fitting to a one-site competitive model gives a K_i of 16.0 ± 5.7 μM μM. (d), methamphetamine demonstrates negligible binding to Protein A. 280 nm was chosen as the excitation and emission at 308 nm (peak position) was plotted against the titrated methamphetamine concentration.

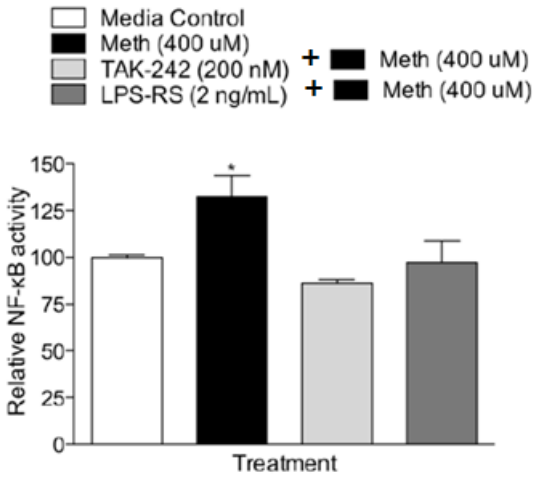

Figure 2. MD-2 antagonists (LPS-RS) and TLR4 signaling inhibitor (TAK-242) suppress methamphetamine induced NF-κB activation. BV-2 dual luciferase NF-κB reporter cells were stimulated with methamphetamine (400 μM) , methamphetamine (400 μM) and LPS-RS (2 ng/ml), or methamphetamine (400 μM) and TAK-242 (0.2 μM) for 48 h. NF-κB activity was analyzed by Dual-Glo luciferase assay. The ratio of Firefly luciferase activity to Renilla luciferase activity represents the NF-κB activity. NF-κB activity of the untreated control group was set as 100%.*$p<0.05$ compared to untreated control.

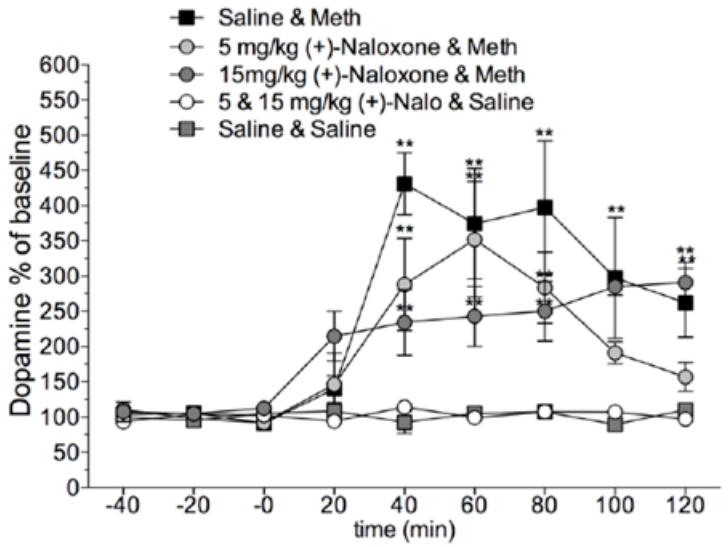

Figure 3. TLR4 antagonist (+)-naloxone attentuates methamphetamine induced increases of extracellular dopamine with the NAc shell. methamphetamine (1mg/kg) produced robust increase in extracellular NAc dopamine compared to saline control ($p<0.0001$) that were dose-dependently attenuated with (+)-naloxone (15mg/kg:$p<0.01$; 5 mg/kg: $p>0.05$). Two-way ANOVA revealed a main effect of treatment ($F(_{4,17})=17.57$, $p<0.0001$) and time ($F(_{8,136})=20.82$, $p<0.0001$) and was followed by bonferroni post-hocs. Prior to drug treatment there were no differences in the extracellular dopamine concentrations in the Nac shell across all groups ($p>0.05$). There were no differences between this group and the saline or (+)-nalooxne treated rats ($p>0.05$). Data are means ± SEMs, n=4/group.

Materials & Supplies Summary ($ 4 000):

Fetal bovine serum (FBS, Life-science, Cat.No. 10437-028), $620/each (including shipment fee), 2 bottles; cost = $620×2= $1, 240

DMEM cell culture medium (FBS, Life-science, Cat. No. 10566-024), $284/each (including shipment fee); cost=$284×5= $1, 420

MAPK and Phospho-MAPK Family Antibody Sampler Kit: $740

SD rats (60): $10×100= $600