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Supplementary Information

An Enzymatic Advance in Nicotine Cessation Therapy

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1. Construction and expression of NicA2 variants and their characterization

Construction and expression of Δ50-NicA2

A 638-bp PCR fragment was amplified using primers NICA2N5 and NICA2ECOR3 (**Table S1**) and pET28b-WT-NicA2 plasmid as a template. The PCR fragment was gel-purified, digested by restriction endonucleases Nco I and EcoR I, and cloned back into the pET28b-WT-NicA2 vector (Nco I and EcoR I digested). The Δ50-NicA2 protein was expressed in BL21(DE3) E. coli cells, purified by IMAC and characterized by SDS-PAGE (see **Fig. S1**).

Construction and expression of ABD035-Δ50-NicA2 (NicA2-J1)

To construct an NicA2-J1 fusion enzyme, a 791-bp PCR fragment was amplified using primers ABDNICA5 and NICA2ECOR3 (**Table S1**) and plasmid pET28b-Δ50-NicA2 as a template. The PCR fragment was gel-purified, digested by restriction endonucleases Nco I and EcoR I, and cloned back into a pET28b-Δ50-NicA2 vector (Nco I and EcoR I digested). The NicA2-J1 protein was expressed in BL21(DE3) E. coli cells, purified by IMAC and characterized by SDS-PAGE (see **Fig. S1**).

Table S1. Primers used for Δ50-NicA2 and NicA2-J1 gene construction.

Primer name	Primer sequence
NICA2N5	5’ ATATACCATGGGTGGCTTCGATTACGATGTGGTAGTAG 3’
NICA2ECOR3	5’ TCGTGCCCCCTTGAATTCTATAATGAGT 3’
ABDNICA5	5’ATATACCATGGATGCCAACAGCCTGGCTGAAGCAAAAGTGTTGGCCAATCGCGA GCTGGATAAATATGGCGTGAGCGACTTCTATAAACGCTTGATCAATAAAGCTAAGA CCGTGGAAGGCGTTGAAGCTCTGAAACTTCATATTTTGGCTGCACTGCCAAGCGGT GGCTTCGATTACGATGTGGTAGTAG 3’

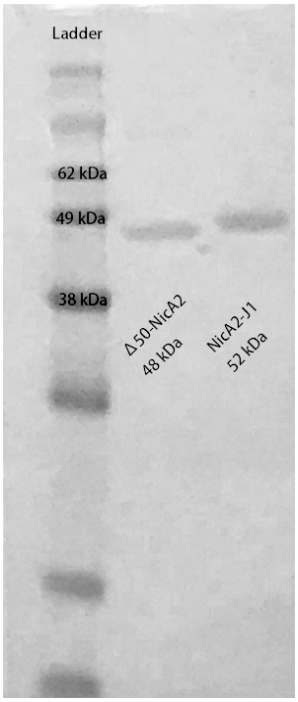

Fig. S1 SDS-PAGE of Δ50-NicA2 and NicA2-J1.

Binding kinetics of NicA2-J1 with various albumins

Binding kinetics for the interaction(s) between NicA2-J1 and mouse, rat or human serum albumin (MSA, RSA, or HAS) were determined on a Biacore 3000 system (GE healthcare) using SPR technology. All experiments were conducted at 25 °C with a flow rate of 30 µL/min using HBS-EP+ buffer as the running buffer. In brief, MSA, RSA, or HSA was immobilized onto a research-grade CM3 sensor chip surface (ligand flow cell) with a ligand density level around 250 RU using NHS/EDC coupling chemistry per manufacturer's instruction. The flow cell preceding the ligand flow cell was activated by NHS/EDC and deactivated by 1.0 M ethanolamine-HCl (pH 8.5), and was served as a reference flow cell in succeeding kinetic analysis. To determine the binding kinetics, various concentrations of NicA2-J1 ranging from 31.25 to 2000.00 nM were injected randomly and individually over both reference and ligand surfaces for 5 min, then dissociated in running buffer for 30 min before the surface was regenerated with 10 mM Glycine-HCl (pH 2.2). All analyses were double referenced and conducted in duplicates. The interaction between NicA2-J1 and immobilized albumin was recorded within the sensorgram. The kinetic data were evaluated via fitting the sensorgram by BIAevaluation software using a 1:1 (Langmuir) binding model. The kinetic constants, including association and dissociation rate constants (ka and kd) and equilibrium dissociation constant (KD), are summarized in **Table 2**.

2. LC-MS assay used for enzyme kinetic determination

NicA2-J1 was purified by FPLC (BIO-RAD) with HiLoad™ 26/600 Superdex 75™ pg size-exclusion column. The concentrations were determined by Nanodrop 2000 with the correlation 1 mg/mL = 1.164 A280.

The kinetic assay was conducted and analyzed as previous reported (with details in below).[1] The Michaelis-Menten curve is shown in **Fig. S2**.

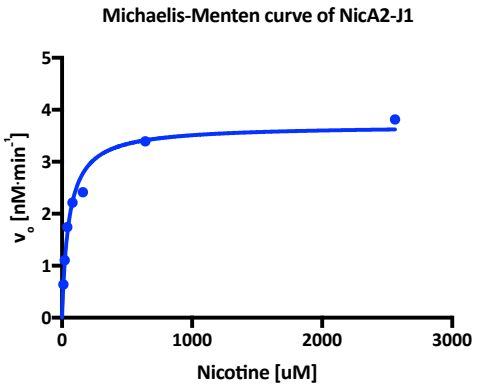

Fig. S2 Michaelis-Menten curves of NicA2-J1.

NicA2-J1 Michaelis-Menten assay

Nicotine was solubilized in ddH_2O to a concentration of 10 mM as stock and diluted with HEPES buffer (50 mM, pH=7.4) in the assay. Then 50 µL nicotine solution was mixed with 50 µL NicA2 solution to obtain final concentrations of 10, 20, 40, 80, 160, 640 and 2560 nM nicotine and 10 nM NicA2. After incubating at room temperature for 20 min, 10 µL nicotine methyl D-3 (2 µM in 20% TFA/H_2O) solution was added to the mixture as an internal standard and to quench the reaction as well. The samples were injected into the LC-MS for analysis.

LC-MS for NicA2-J1 activity assay

NicA2-J1 activity was determined by LC-MS using Agilent 1260 Infinity liquid chromatography system with 6130 quadrupole mass spectrometry. 20 µL of each sample was injected to a Poroshell 120 EC-C8 column (4.6x50 mm, 2.7 µm, Agilent Technologies) subjected to a gradient (A to B where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) of 0% B for 3 min, 0% B to 100% B from 3 to 7 min, and 100% B from 7 to 10 min at a constant flow rate of 0.5 mL/min. A column-solvent equilibration time of 3 min was conducted prior to next sample analysis. MS operational parameters were: API-ES mode, channel 1 (90%) positive single ion monitoring (SIM) of m/z 179 (30%), 161 (30%), 166 (30%) and 163 (10%), corresponding to the M^+ peak of the reaction products, labeled internal standard and substrate respectively and channel 2 (10%) scan for positive ions; nitrogen as a nebulizing and drying gas (35 psi, 12 L/min), HV capillary voltage at 4 kV and the drying gas temperature to 300 ˚C. To protect the detector from salts in the buffer, MS was turned on with a delay 1.4 min after injection.

1. S. Xue, J. E. Schlosburg and K. D. Janda, *Journal of the American Chemical Society*, 2015, **137**, 10136-10139.

3. Pharmacokinetics of WT NicA2 and NicA2-J1

Endotoxin was removed from purified NicA2 (WT and NicA2-J1) through a Pierce High Capacity Endotoxin Removal Resin and Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher) to < 5 EU/mL (determined by PYROGENT™ Gel Clot kit from Lonza).

WT NicA2 and NicA2-J1 were administrated to rats (n=3) intraperitoneally (IP) and after 1, 4, 16, 24, 48 and 72 h; blood was collected from the tail vein and centrifuged. The serum was stored at -20 ˚C. The concentration of NicA2 was determined by ELISA. Serum samples from rats were diluted 10-fold with PBS and then coated in 96-well plate (50 uL/well) by dry method. Various concentrations of pure NicA2-J1 (0, 1, 2, 5, 10, 15, 20 μg/mL, final concentrations, 50 uL/well) in 10% naive rat serum/PBS was coated in the same plate for standard curve. The plate was placed at 37 ˚C for overnight and fixed by methanol, then blocked with blotto (5% nonfat milk in PBS). Polyclonal rabbit anti NicA2 produced by TSRI Center for Antibody Development and Production was used as the primary antibody (rabbit serum, 1:100 dilution) and goat anti rabbit with HRP was used as secondary antibody (1:10000 dilution). TMB Substrate Kit (ThermoFisher) was used for signal development. The enzyme concentrations in blood were calculated based on the standard curve generated by pure NicA2-J1.

The data was analyzed by Prism 7.0a. The concentrations of both enzymes were converted to log units and the elimination phase (16 – 72 h) was fitted with a linear model to afford elimination constants (k, 0.02814 for WT and 0.005399 for NicA2-J1). The half-life was calculated with the equation $t_{1/2}$=0.693/k. The AUC was calculated with build-in function in Prism.

4. Behavioral experiments

For nicotine administration and the PK study, male Wistar rats (*n* = 16; 250-275 g), and female Wistar rats (n=16; 200-250 g Charles River) 2 months old at the beginning of the experiments, were used. The animals were housed in standard cages in a room with artificial lightning (12 h/12 h light/dark cycle, lights off at 8:00 AM) at constant temperature (20-22 °C) and humidity (45-55%) with food and water available *ad libitum*. The rats were handled once daily for 5 min during the first week after arrival to the vivarium. All the procedures were conducted during the dark cycle. The animal procedures met the guidelines of the National Institutes of Health and were approved by The Scripps Research Institute Institutional Animal Care and Use Committee (protocol no. 08-0015). All the surgical procedures were performed under isoflurane anesthesia, and all necessary steps were taken to minimize suffering of the animals.

Nicotine hydrogen tartrate salt was dissolved in 0.9% sterile physiological sodium chloride and the pH was adjusted to 7.3 with NaOH 1 M. The daily dose of nicotine that was delivered by the osmotic minipumps (Alzet, 2ML2, 5 µL/h) was 3.15 mg/kg, based on previous work (Richardson and Tizabi, 1994; Tizabi et al., 1997; Epping-Jordan et al., 1998). Endotoxin-free enzyme was administered intraperitoneally (IP) at the dose 10mg/kg.

Rats (males and females) were divided into 4 groups (8 rats per group). Four males and four females composed each group. Two groups of rats were chronically exposed to nicotine for 7 days. Nicotine (3.15 mg/kg/day) was infused using minipumps (Alzet 2ML2) that releases 5µL of fluid/h implanted in the back underneath the skin of the rats. Two other groups of rats were chronically exposed to saline solution for 7 days that was infused using osmotic minipumps. After 12 hours of osmotic minipumps implantation, rats were daily administered (10:00 AM) with NicA2-J1 (10 mg/mL/kg) or PBS 1% as a control (IP). The groups were divided as above: (1) Saline Minipumps (n=8) + PBS 1%; (2) Saline Minipumps (n=8) + NicA2-J1; (3) Nicotine Minipumps (n=8) + PBS 1%; (4) Nicotine Minipumps (n=8) + NicA2-J1. The body weight of the animals was daily monitored. The eighth day, the minipumps were removed and within the 24-48-hour mark after removal of nicotine a battery of the behavioral withdrawal tests were performed: 1) Withdrawal Score; 2) Mechanical nociceptive hyperalgesia and 3) Irritability score.

5. Nicotine distribution in rat blood and brains

Tissue sample preparation

Nicotine (3.15 mg/kg/day) was infused using osmotic minipumps (Alzet 2ML2) that release 5 µl of fluid/h implanted in the back (underneath the skin) of the rats for 7-days. Endotoxin-free NicA2-J1 was dosed to rats intraperitoneally (IP) at 10 mg/mL/kg daily, with same amount of PBS as control.

After 1 and 5 days, blood was collected and immediately mixed with 4 volume of methanol (with 1 µM of nicotine D3 as internal standard) to quench the enzyme. The samples were centrifuged at 10000 rpm for 30 min and the supernatant was transferred to clean tubes and evaporated in Genevac. The residual was re-dissolved in 5% NH_4OH in water and cleaned up by Oasis HLB 96-well µElution Plate (Waters). The elution was evaporated in Genevac and re-dissolved in HEPES buffer and 2% TFA for LC-MS.

Rats were sacrificed and brains were collected after 7 days and flash-frozen with liquid nitrogen. The brains were cut along commissure and half of each brain was weighted and used for analysis. The brain pieces were homogenized in 1 mL PBS and centrifuged at 10000 rpm for 30 min. The supernatant was mixed with same volume of 5% NH_4OH, nicotine D3 added to final concentration of 0.1 µM as internal standard and then extracted with Oasis HLB 96-well µElution Plate. The elution was evaporated in Genevac and re-dissolved in HEPES buffer and 2% TFA for LC-MS.

Nicotine detection using LC-MS

Nicotine's concentration was determined by LC-MS using Agilent 1260 Infinity liquid chromatography system with 6130 quadrupole mass spectrometry. 20 µL of each sample was injected to a Poroshell 120 EC-C8 column (4.6x50 mm, 2.7 µm, Agilent Technologies) subjected to a gradient (A to B where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) of 0% B for 3 min, 0% B to 100% B from 3 to 7 min, and 100% B from 7 to 10 min at a constant flow rate of 0.5 mL/min. A column-solvent equilibration time of 3 min was conducted prior to next sample analysis. MS operational parameters were: API-ES mode, channel 1 (90%) positive single ion monitoring (SIM) of m/z 166 (50%, nicotine D3) and 163 (50%, nicotine) and channel 2 (10%) scan for positive ions; nitrogen as a nebulizing and drying gas (35 psi, 12 L/min), HV capillary voltage at 4 kV and the drying gas temperature to 300 ˚C. To protect the detector from salts in the buffer, MS was turned on with a delay 1.4 min after injection.