

**Functional presynaptic $\alpha 6$ -containing nicotinic acetylcholine receptors participate
in nicotine reward in the VTA: where and how**

Kechun Yang, Lori Buhlman, Ming Gao, Ghous M. Khan, Robert A. Nichols, Jamie

DeChon, Guozhang Jin, Paul Whiteaker, Ronald J. Lukas and Jie Wu*

Divisions of Neurology (K.Y., M.G., J.D., J.W.) and Neurobiology (L.B., L.L., P.W., R.J.L.), Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013-4496. Department of Physiology & Pharmacology (G.M.K., R.A.N.), Drexel University College of Medicine, Philadelphia, PA 19102, Department of Cell & Molecular Biology (R.A.N.), John A. Burns School of Medicine, University of Hawai'i at Manoa, Honolulu, HI 96813. Department of Pharmacology (K.Y., G.J.), Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China.

*Corresponding author:

Jie Wu, M.D., Ph.D.,

Senior Staff Scientist,

Director of Neural Physiology Laboratory,

Division of Neurology,

Barrow Neurological Institute,

350 West Thomas Road,

Phoenix, AZ 85013-4496.

Voice: (602) 406-6376.

Fax: (602) 406-7172.

E-mail: Jie.Wu@chw.edu

In the ventral tegmental area (VTA), $\alpha 6$ -nAChRs express abundantly, but their location, function, pharmacology, and roles in cholinergic modulation of dopaminergic (DA) neurons remain elusive. Using a VTA neuron-adherent bouton preparation, we report that functional $\alpha 6$ -nAChRs are located on GABAergic presynaptic boutons, where they mediate cholinergic modulation of GABA release onto DA neurons. Smoking-relevant concentrations of nicotine desensitize $\alpha 6$ -nAChRs, cause a disinhibition in DA neurons and consequently mediate nicotine reward.

Dopaminergic (DA) neuronal activity in the ventral tegmental area (VTA) is thought to contribute to reward and drug reinforcement, including nicotine dependence^{1,2}. A variety of nicotinic acetylcholine receptor (nAChR) subunits are expressed in mammals by VTA DA neurons³⁻⁵. In particular, $\alpha 6$ subunit expression is abundant, suggesting possible roles for nAChRs containing $\alpha 6$ subunit ($\alpha 6^*$ -nAChRs) in nicotine-induced reward and dependence in the VTA³⁻⁸. Recent work has focused on $\alpha 6^*$ -nAChRs located on the VTA DA neuronal target area, the nucleus accumbens (NA), where $\alpha 6^*$ -nAChRs participate in nicotinic modulation of DA release⁹⁻¹¹. However, the function, pharmacology, and the roles played in cholinergic modulation of GABA release by $\alpha 6^*$ -nAChRs in the VTA are poorly understood. The objective of this study was to identify functional $\alpha 6^*$ -nAChRs in the VTA, evaluate their roles in cholinergic modulation of GABA release and elucidate how these receptors participate in nicotine reward. We hypothesize that $\alpha 6^*$ -nAChRs are present on GABAergic, pre-synaptic boutons contacting DA neurons and that they play important roles in mediating cholinergic regulation of inhibitory neurotransmission in the VTA.

Initial experiments were designed to determine whether there are functional $\alpha 6^*$ -nAChRs on presynaptic boutons. To achieve this goal, we used a neuron-adherent bouton preparation from the VTA. In this preparation, dissociated single DA neurons contain functional presynaptic boutons¹², which are evident on the neuronal surface upon staining with FM1-43 (Fig. 1a and Supplementary Methods online). We then doubly labeled the bouton-containing neurons using biotinylated- α -conotoxin MII and GAD65/67, and most of the DA neurons exhibited positive, punctate staining for both markers (Fig. 1b), suggesting that there are α -conotoxin MII-sensitive nAChRs natively expressed on the adherent GABAergic presynaptic boutons. In electrophysiologically identified DA neurons (supplementary Fig. 1), spontaneous postsynaptic currents (sPSCs) were evident (Fig. 1).

These sPSCs were completely abolished by a GABA_A receptor-selective antagonist, bicuculline (30 μM, Fig, 1c,f) but were not sensitive to ionotropic glutamate receptor antagonists, CNQX (20 μM) + D-APV (50 μM) (Fig. 1e,f), suggesting that the spontaneous currents were inhibitory sPSCs (sIPSCs). The sIPSCs were enhanced by 4-aminopyridine (4AP, 200 μM, Fig.1d,f), consistent with the currents resulting from neurotransmitter release from the presynaptic boutons.

Fig. 1 near here

Next, we asked whether these presynaptic $\alpha 6^*$ -nAChRs mediate cholinergic modulation of GABA release in the VTA. To address this question, we tested the effects of relatively selective antagonists (α -conotoxin MII or α -conotoxin P1A) for $\alpha 6^*$ -nAChRs on ACh-induced enhancement of GABA release (monitored by changes in sIPSC frequency). Figure 2a (top trace) showed that 1 mM ACh strongly enhanced the frequency but not amplitude of sIPSCs, suggesting that ACh increased presynaptic GABA release in bouton-adherent VTA DA neurons. Pretreatment with 1 nM α -conotoxin P1A (or α -conotoxin MII) for 4 min abolished the ACh-induced increase of sIPSCs (Fig. 2a, middle trace; ACh co-applied with α -conotoxin P1A). After a 20-min washout, the inhibitory effects of α -conotoxin P1A were reversed (Fig. 2a, bottom trace). These results suggest that functional $\alpha 6^*$ -nAChRs mediate an increase of ACh-induced GABA release onto DA neurons in the VTA (Fig. 1b,c). In these α -conotoxin-sensitive neurons, $\alpha 4\beta 2$ -nAChR antagonist (1 μM DH β E) or $\alpha 7$ -nAChR antagonist (10 nM MLA) failed to prevent ACh-induced increase of sIPSCs under the same pretreatment conditions (supplementary Fig. 2). Interestingly, acute exposure to a smoking-relevant concentration of nicotine (500 nM) failed to increase sIPSCs in 30 neurons tested (Fig. 2d-f). However, this nicotine exposure (500 nM for 4 min) abolished ACh-induced increases of sIPSCs (Fig. 2g-i), suggesting that smoking-

relevant levels of nicotine desensitize rather than activate presynaptic $\alpha 6^*$ -nAChRs and eliminate cholinergic enhancement of GABA release. Chronic exposure to nicotine (0.5 mg/kg/day i.p.) for 10 days also eliminated ACh-induced increase of sIPSCs (recorded at the day 11) (Fig. 2i).

Fig. 2 near here

The novel and important finding in the present study is that functional $\alpha 6$ -nAChRs are natively expressed on GABAergic presynaptic boutons where they mediate cholinergic modulation of GABA release onto DA neurons in the VTA. Smoking-relevant concentrations of nicotine desensitize rather than activate these presynaptic $\alpha 6$ -nAChRs and eliminate cholinergic enhancement of GABA release. The nicotine-induced reduction in GABA release will thus increase of DA neuronal activity in the VTA, driving the reward pathway.

Using a fresh neuron-adherent bouton preparation, we have identified functional $\alpha 6$ -nAChRs on GABAergic presynaptic boutons. First, double-labeled DA neurons showed positive reactions to both biotinylated- α -conotoxin MII and GAD65/67, and importantly, both positive reaction dots were co-localized on the surface of labeled neuron, suggesting the α -conotoxin MII-sensitive receptors ($\alpha 6$ -nAChRs) likely localize to the GABAergic boutons. This was further confirmed by pharmacological experiments, in which the block of voltage-gated Na^+ channels (300 nM TTX) did not affect either sIPSCs or ACh-induced increase of sIPSCs (supplementary Fig. 3). We also examined whether ACh-induced Ca^{2+} entry into purified presynaptic boutons (VTA synaptosomes) was sensitive to α -conotoxin P1A, and found that 1 nM α -conotoxin P1A eliminated ACh-induced Ca^{2+} entry (supplementary Fig. 4), confirming the presence of functional $\alpha 6$ nAChRs on presynaptic boutons in the VTA. Although we cannot distinguish between GABAergic and glutamatergic boutons in the preparation of VTA synaptosomes, our data suggest the α -

conotoxin P1A-sensitive portion of ACh-induced intracellular Ca^{2+} elevation is likely mediated through GABAergic boutons as it is well known that the presynaptic glutamatergic terminals/boutons express $\alpha 7$ type nAChRs^{13, 14}. In addition, pharmacological experiments demonstrated that relatively selective $\alpha 6^*$ -nAChR antagonist (1 nM α -conotoxin MII or P1A) abolished ACh-induced increase of sIPSC frequency but not amplitude, suggesting that presynaptic $\alpha 6$ -nAChRs mediated the ACh-induced increase in GABA release.

To determine whether $\alpha 6$ and $\beta 2$ nAChR subunits are assembled together to form presynaptic $\alpha 6$ -nAChRs, we examined ACh-induced increase of sIPSCs using $\beta 2$ subunit knockout (KO) mice and found that the ACh-induced increase of sIPSCs was not detected (supplementary Fig. 5), suggesting that at least the $\beta 2$ subunit is involved in the formation of functional presynaptic $\alpha 6^*$ -nAChRs. Considering that the nAChR $\beta 2$ subunit plays a critical role in nicotine self-administration¹⁵, our data suggest the importance of these presynaptic $\alpha 6\beta 2^*$ -nAChRs in the VTA in nicotine reward and addiction.

Smoking-relevant concentrations of nicotine increase VTA DA neuron firing and DA release to its targets, namely NA and prefrontal cortex; and induce reward through complex mechanisms^{1, 13}. Emerging lines of evidence have indicated that nicotine activates $\alpha 7$ -nAChRs located on glutamatergic presynaptic terminals to increase glutamate release and/or desensitizes $\alpha 4\beta 2$ -nAChRs located on GABAergic neuronal soma to reduce the tonic input from brain stem cholinergic nuclei. This modulation of $\alpha 7$ - and $\alpha 4\beta 2$ -nAChRs by nicotine increases DA neuronal activity and DA release^{2, 14}. Here, we demonstrate an additional mechanism wherein acute or chronic exposure to smoking-relevant concentrations of nicotine does not activate but desensitizes $\alpha 6^*$ -nAChRs located on presynaptic GABAergic boutons in VTA DA neurons.

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AUTHOR CONTRIBUTION:

K. Y. and M. G. Performed patch-clamp experiments and data analysis

L. B. and J. D. Performed immunocytochemistry

G.M.K and R.A.N. Performed Ca²⁺ image experiments

R.J.L., and P. W. and G.J. Designed experiments and wrote the manuscript

J.W. Developed and supervised the overall of experimental design, patch-clamp recording, data analysis and manuscript writing.

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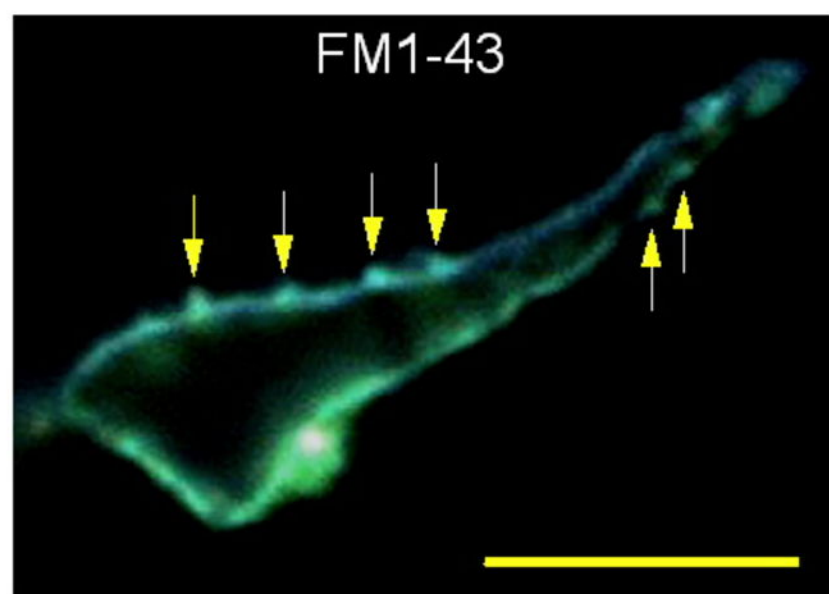
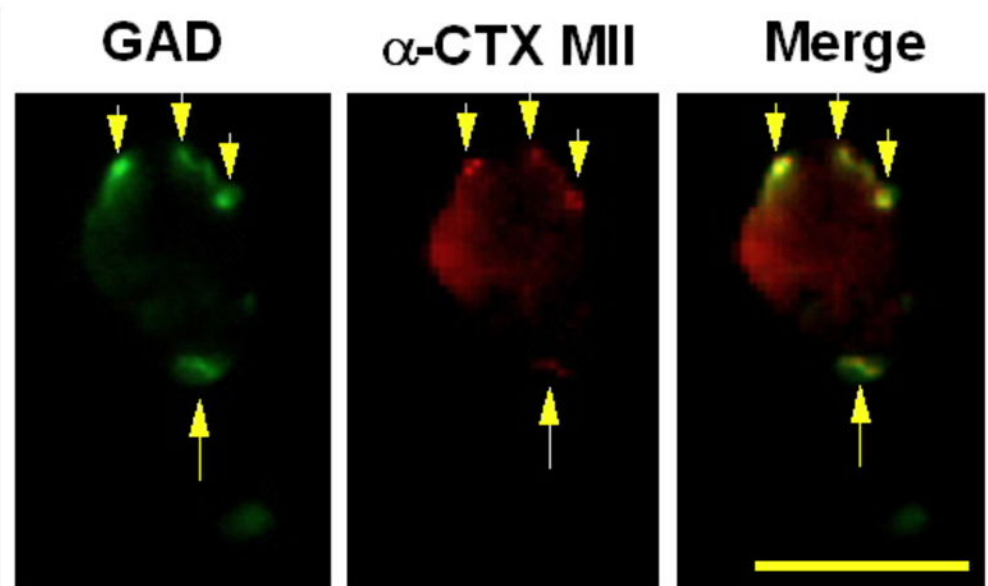
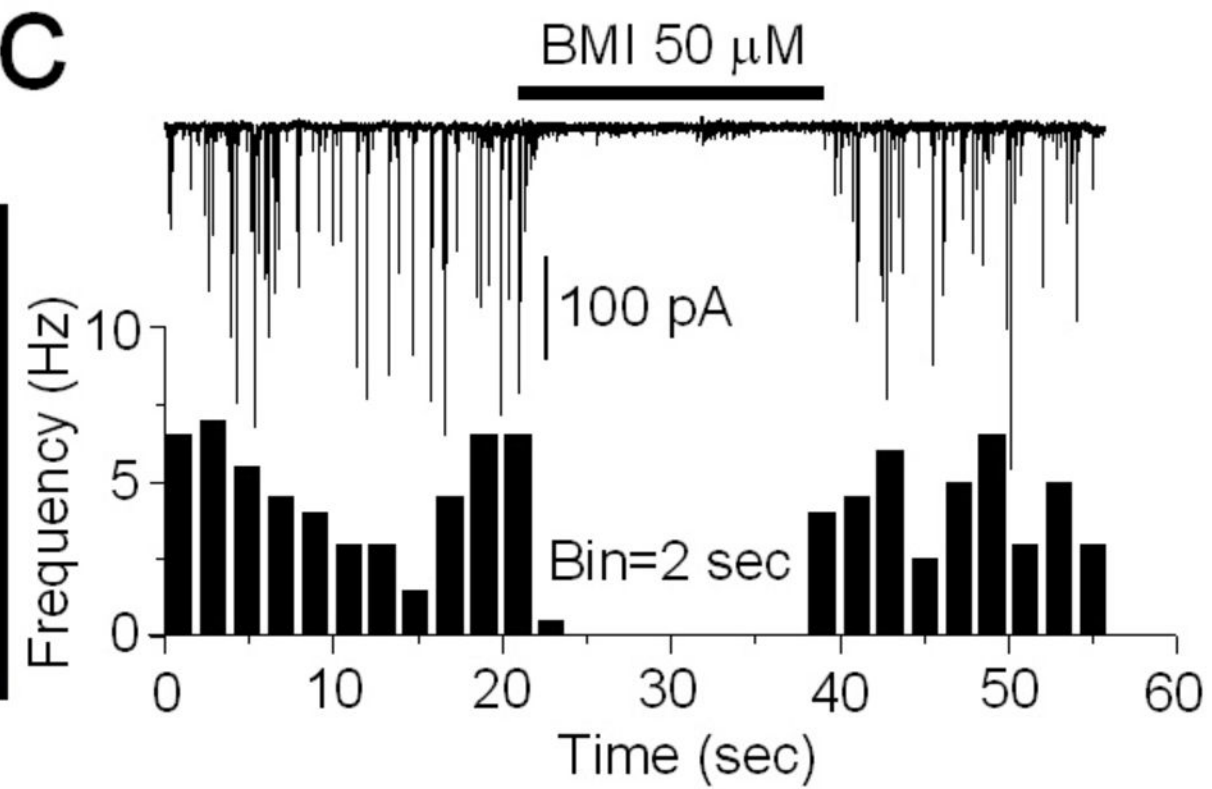
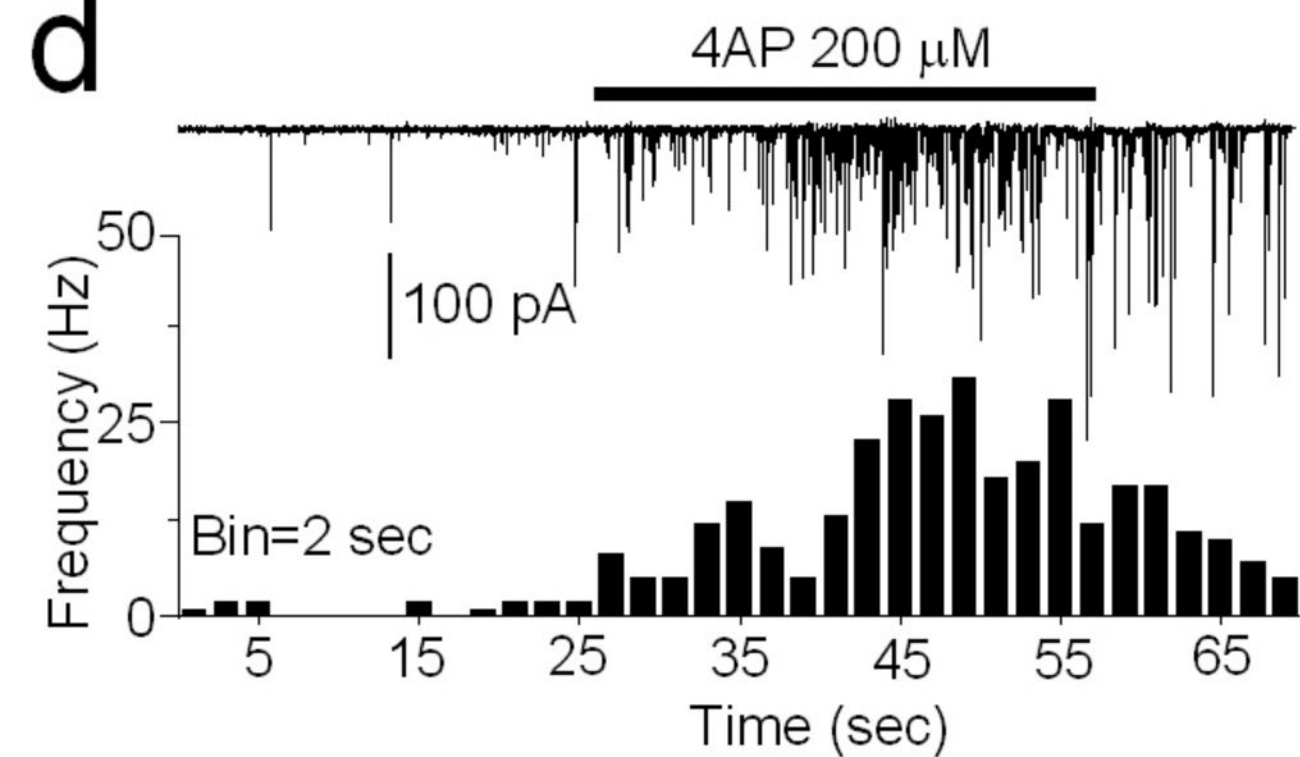
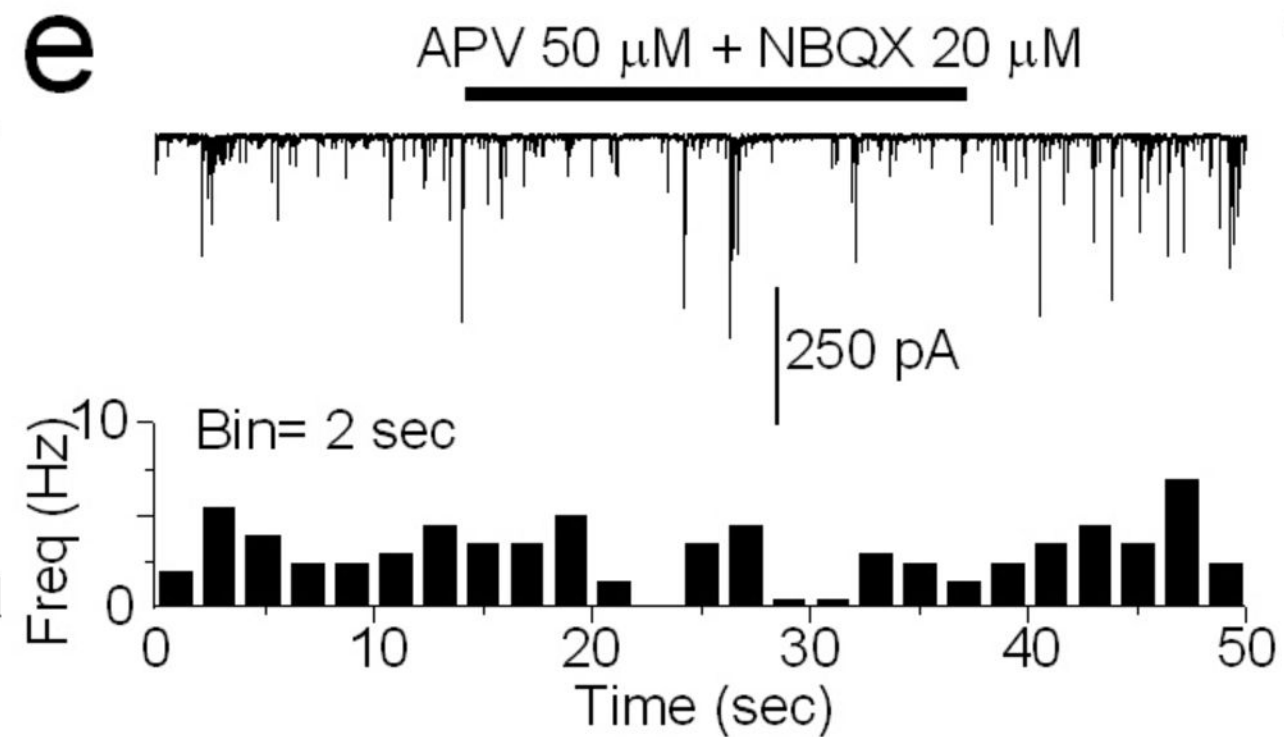
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Figure legends

Fig. 1. Spontaneous postsynaptic currents from GABAergic presynaptic boutons on single DA neurons mechanically dissociated from rat VTA. FM1-43 staining showed that a single neuron maintained presynaptic boutons (indicated by yellow arrows) after pure mechanical dissociation from rat VTA (**a**). GAD (65/67) staining of a dissociated VTA neuron revealed positively stained dots on the surface of the neuron, suggesting the presence of GABAergic presynaptic boutons (**b**, left, pointed by yellow arrows), while cell soma was unstained, suggesting a non-GABAergic neuron. The same neuron also exhibited positive staining with biotinylated α -conotoxin MII labeling (**b**, middle). The surface dots of neuron were double-labeled by GAD and α -conotoxin MII as evident in the merged image (**b**, right), suggesting that there are α -conotoxin MII-sensitive nAChRs located on GABAergic presynaptic boutons. Similar staining results have been obtained in six neurons dissociated from three rats. Under whole-cell patch-clamp recording (in voltage-clamp mode), spontaneous postsynaptic currents (sPSCs) were observed at a holding potential of -60 mV, and these sPSCs were completely abolished by a GABA_A receptor antagonist, bicuculline (BMI, **c**), significantly enhanced by 4-aminopyridine (4AP, **d**), but were not affected by ionotropic glutamate receptor antagonist (D-APV 50 μ M + NBQX 20 μ M, **e**). Fig. 1f summarizes the effects of c-e on the frequency of sIPSCs. Data

are means \pm SEM. The traces c, d and e were recorded from different neurons. Scale bars: 30 μ m.

Fig. 2. Presynaptic $\alpha 6^*$ -nAChR-mediated cholinergic modulation of GABA release onto DA neurons in rat VTA. Under patch-clamp whole-cell recording (in voltage-clamp mode), 1 mM ACh increased the frequency of sIPSCs (**a**, top trace). Pretreatment with the $\alpha 6^*$ -nAChR-selective antagonist 1 nM α -CTX PIA for 4 min reversibly (**a**, bottom trace) abolished (**a**, middle trace) the ACh-induced increase in sIPSC frequency (**b**). Fig. 2c summarizes the α -CTXPIA- and α -CTXMII-sensitive nAChR-mediated cholinergic modulation of presynaptic GABA release. A smoking-relevant concentration (500 nM) of nicotine alone failed to increase sIPSCs (**d-f**), but it desensitized $\alpha 6^*$ -nAChRs and abolished the ACh-induced increase of sIPSCs following chronic treatment at 0.5 mg/kg/day, i.p., for 10 days (**g-i**). These results suggest that $\alpha 6^*$ -nAChRs mainly mediate cholinergic (ACh) modulation of presynaptic GABA release onto VTA DA neurons, and 500 nM nicotine desensitizes rather than activates these receptors, resulting in a disinhibition of VTA DA neurons, which may play important roles in nicotine-induced DA neuron excitation and nicotine reward.

a**b****c****d****e****f**