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nAChR-Mediated Calcium Responses and Plasticity in *Drosophila* Kenyon Cells

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ABSTRACT: **In *Drosophila*, nicotinic acetylcholine receptors (nAChRs) mediate fast excitatory synaptic transmission in mushroom body Kenyon cells, a neuronal population involved in generation of complex behaviors, including responses to drugs of abuse. To determine whether activation of nAChRs can induce cellular changes that contribute to functional plasticity in these neurons, we examined nicotine-evoked responses in cells cultured from brains of late stage OK107-GAL4 pupae. Kenyon cells can be identified by expression of green fluorescent protein (GFP+). Nicotine activates α-bungarotoxin-sensitive nAChRs, causing a rapid increase in intracellular calcium levels in over 95% of the Kenyon cells. The nicotine-evoked calcium increase has a voltage-gated calcium channel (VGCC) dependent component and a VGCC-independent component that involves calcium influx directly through nAChRs. Thapsigargin treatment reduces the nicotine response consistent with amplification by calcium release from intracellular stores. The response to nicotine is experience-dependent: a short conditioning pulse of nicotine causes a transient 50% reduction in the magnitude of the response to a test pulse of nicotine when the interpulse interval is 4 h. This cellular plasticity is dependent on activation of the VGCC-component of the nicotine response and on cAMP-signaling, but not on protein synthesis. These data demonstrate that activation of nAChRs induces a calcium-dependent plasticity in Kenyon cells that could contribute to adult behaviors involving information processing in the mushroom bodies including responses to nicotine.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 1520–1532, 2007

Keywords: **calcium imaging; mushroom bodies; nicotine; Kenyon cells; nAChR**

INTRODUCTION

Behavioral plasticity relies on the ability of experience to cause persistent alterations in central neural circuits in all animals. In the mammalian brain, where glutamate is the major excitatory neurotransmitter, activation of calcium permeable glutamate receptors is important in mediating long-lasting changes in synaptic efficacy that contribute to memory formation (Malenka and Bear, 2004; Cull-Candy et al., 2006). Like mammals, insects are capable of learning and remembering information but acetylcholine as opposed to glutamate is the primary excitatory eurotransmitter in the central nervous system (CNS) (Breer and Sattelle, 1987; Gundelfinger, 1992). While previous studies have demonstrated that nicotinic acetylcholine receptors (nAChRs) in some insect neurons are calcium permeable (Goldberg et al., 1999; Oertner et al., 1999, 2001; Single and Borst, 2002), it

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is not known whether activation of nAChRs can induce persistent changes in neuronal activity that could contribute to behavioral plasticity.

Mushroom bodies are association areas involved in generation of complex behaviors, including memory formation, in the insect brain (Heisenberg, 2003). In *Drosophila*, each mushroom body contains about 2500 Kenyon cells that receive olfactory information directly from cholinergic projection neurons in the antennal lobe (Bicker, 1999; Oleskevich, 1999; Perez-Orive et al., 2002; Yasuyama et al., 2002). Reversible blockade of synaptic transmission in Kenyon cells during olfactory associative conditioning suggests that plasticity contributing to memory formation and storage occurs at synapses on these neurons (Dubnau et al., 2001; McGuire et al., 2001). Studies in our laboratory show that nAChRs mediate fast excitatory synaptic transmission in Kenyon cells *in vitro* (Su and O'Dowd, 2003) and in the adult brain (Gu and O'Dowd, 2006). Nicotine, a specific nAChR agonist, causes a rapid increase in cytoplasmic calcium in mushroom body Kenyon cells cultured from honeybee, cricket, and *Drosophila* (Bicker and Kreissl, 1994; Bicker, 1996; Cayre et al., 1999; Jiang et al., 2005). Thus, nAChR-mediated calcium-dependent signaling in Kenyon cells could initiate cellular events involved in regulating functional plasticity.

In addition to its ability to induce cellular responses, nicotine can evoke a range of behaviors in adult *Drosophila*, from hyperkinesis at low doses to hypokinesis at higher doses (Bainton et al., 2000). A recent study also indicates that repetitive exposure to nicotine induces a transient increase in nicotine-dependent inhibition of a startle response in adult flies. Analysis of genetic mutants suggests that this nicotine-induced hypersensitivity involves activation of cAMP signaling and CREB-mediated gene transcription (Hou et al., 2004). The behavioral changes are centrally mediated since in *Drosophila*, as in other insects, nAChRs are confined to the CNS (Breer and Sattelle, 1987). These data suggest that activation of nAChRs could mediate cellular changes that contribute to behavioral plasticity.

To determine whether activation of nAChRs can induce changes that contribute to neuronal plasticity, we examined the properties and regulation of nicotine-evoked calcium responses in cultured *Drosophila* Kenyon cells. Our data demonstrate that brief exposure to nicotine causes a rapid and reversible increase in intracellular calcium that depends on calcium flux through voltage-gated calcium channels (VGCCs) and directly through nAChRs. In addition, repetitive exposure to nicotine results in a calcium-dependent plasticity of the nAChR-mediated response. These data suggest that nAChR-mediated plasticity could contribute to adult behaviors involving information processing in the mushroom bodies.

MATERIALS AND METHODS

Fly Strains

Pupae were obtained from the mating of males from the homozygous enhancer trap line OK107-GAL4 (Connolly et al., 1996), and females homozygous for a UAS-GFP transgene. The OK107-GAL4;UAS-GFP pupae exhibit high levels of GFP expression in the cell bodies and processes of the mushroom body Kenyon cells (Su and O'Dowd, 2003).

Primary Pupal Cultures

Heads were removed from animals 55–78 h after pupation and placed in sterile dissecting saline containing 137 m*M* NaCl, 5.4 m*M* KCl, 0.17 m*M* NaH_2PO_4, 0.22 m*M* KH_2PO_4, 33.3 m*M* glucose, 43.8 m*M* sucrose, and 9.9 m*M* HEPES, pH 7.4. The brains were removed and the optic lobes were discarded. Central brain regions were incubated in dissecting saline containing 50 U/mL papain activated by 1.32 m*M* L-cysteine for 10–15 min at room temperature. Brains were washed (4×) in *Drosophila*-defined culture medium, DDM2 (Su and O'Dowd, 2003), followed by mechanical dissociation of each brain in a 5-μL drop of DDM2 on a ConA-laminin-coated glass bottom culture dish (Jiang et al., 2005). The cells were allowed to settle for 30 min. Dishes were flooded with 1.5 mL DDM2 and maintained in a 23°C humidified 5% CO_2 incubator. After 24 h, 0.5 mL of a 3:1 mixture of DDM2 and conditioned Neurobasal medium (cNBM) was added to each dish. The cNBM consisted of Neurobasal medium + B27 supplements (Invitrogen, Gaitherburg, MD) conditioned for 24 h by nonneuronal mouse brain feeder cell cultures (Hilgenberg et al., 1999). Cultures were fed at 4–5 days by removing 1 mL of media from the dish and adding 1 mL of the 3:1 mixture of DDM2 and cNBM.

Calcium Imaging

Cultures were washed in HEPES-buffered salt solution (HBSS) containing 120 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 15 m*M* glucose, 20 m*M* HEPES, and 0.001% phenol red, pH 7.4. Cultures were then incubated in HBSS containing the calcium indicator dye, Fura-2 AM (5 μM, Molecular Probes, Eugene, OR), and the nonionic surfactant pluronic acid (0.1%, Molecular Probes), for 45 min in the dark. Cultures were rinsed with HBSS and left in the dark for an additional 45 min to allow for complete hydrolysis of the AM ester prior to imaging. Cultures were imaged on an inverted Nikon microscope with a 40×, 1.3 numerical aperture, oil-immersion objective. Fluorescent illumination was provided by a 150-W Xenon arc lamp, and specific filters (340 and 380 nm) were used for excitation. All images were acquired at the emission wave-

length 510 nm and recorded with a digital CCD camera (Orca-100, Hamamatsu). The 340/380 ratio images and their pseudocolor representations were generated digitally using Metafluor 4.0 imaging software (Universal Imaging). Data were collected at 4-s intervals.

Estimates of intracellular calcium concentrations were based on the following formula:

$$[Ca^{2+}] = K_d \frac{(R - R_{min})}{(R_{max} - R)} \frac{F_o}{F_s}$$

In this formula, K_d represents the dissociation constant for fura-2:Ca^{2+} (225 n*M*), R is the ratio of emission intensity at 510 nm after excitation at 340 and 380 nm. R_{min} is the ratio in a calcium-free solution, R_{max} is the ratio at saturating free Ca^{2+} (39 μM), F_o is 510-nm emission intensity when exciting at 380 nm in calcium-free solution, and F_s is 510 nm emission intensity when exciting at 380 nm at saturating free Ca^{2+}. Background levels of fluorescence in calcium-free, fura-free solution were determined at each wavelength and subtracted from signals detected. Empirical values for R_{min} (0.46), R_{max} (6.83), and F_o/F_s (10) were obtained from seven independent measurements using the Fura-2 Calcium Imaging Calibration Kit (Molecular Probes). Because the calibration is based on a cell-free system, the values for intracellular calcium levels reported are estimates that are primarily used to compare changes in calcium levels in different experimental conditions.

Data Analysis

Drug-evoked responses were evaluated in all Kenyon cells in a single field of view. Kenyon cells were included in the analysis if they had stable baseline calcium levels of <150 n*M* and a response of >700 n*M* to the calcium ionophore ionomycin (5 μM) at the end of the experiment. As we previously reported, many neurons exhibit low frequency (10–20/h) spontaneous calcium transients mediated by VGCCs (Jiang et al., 2005). To minimize the potential contribution of these transients to evaluation of the response to specific drugs, ensemble averages were generated from records that did not include transients 50 s before or after the drug application. In a typical culture, for example Figure 1, there were 15 GFP+ Kenyon cells in the full field of view, 11 of which fit all three selection criteria; thus the ensemble average response was generated from 73% of the Kenyon cells. The amplitude of drug-induced responses is reported as increases above basal calcium levels (47.1 ± 3.9 n*M*, n = 20 cultures). Only one field of view was examined per culture and the field was chosen based on the presence of healthy neuronal cell bodies and processes in Nomarski images. All experiments were done on cultures between 3 and 6 days *in vitro*, a time period over which the magnitude of the nicotine responses in control cultures was stable.

Recording Solutions

The control recording solution was composed of HBSS and tetrodotoxin (TTX, 1 μM; Alomone Labs) to block voltage-gated sodium channels. Evoked responses were obtained by removal of >95% of the control recording solution and addition of recording solution containing nicotine (0.001–100 μM). Nicotine was washed out after 100 s. The following drugs were added to the base recording solution in specific experiments: *d*-tubocurarine (curare, 20 μM; Sigma), α-bungarotoxin (α-BTX, 0.1–10 μM; Sigma), methyllycaconitine (MLA, 40 n*M*; Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 μM; Sigma), D(−)-2-amino-5-phosphonopentanoic acid (APV, 50 μM; Sigma), picrotoxin

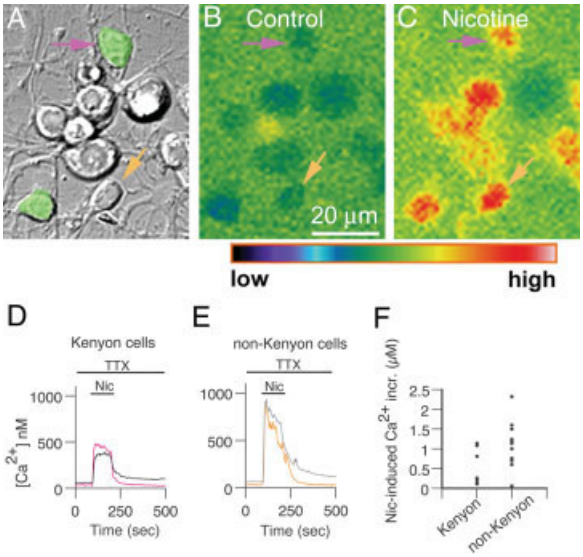

Figure 1 Nicotine induces a rapid and reversible increase in intracellular calcium levels in cultured neurons. A: Kenyon cells identified as GFP+ neurons in a culture prepared from the central brain region of OK107-GAL4;UAS-GFP pupa. Nomarski image with a fluorescent overlay from a culture at 5 DIV loaded with Fura-2. B and C: Calcium (Ca^{2+}) levels are indicated by pseudocolor representation of the 340/380 ratio image—same field of view. In control recording saline (HBSS + TTX), the Ca^{2+} levels were low in the majority of neurons indicated by the blue-green color. Bath application of 10 μM nicotine resulted in a dramatic increase in intracellular Ca^{2+} levels (yellow-red color) in most neurons. D: Responses induced by bath application of 10 μM nicotine. Purple trace is temporal profile from a single Kenyon cell indicated with purple arrow in A–C. Black trace is the ensemble average response from all 11 GFP+ Kenyon cells analyzed in this culture. E: Orange trace is response in the single non-Kenyon cell (GFP−) indicated by the orange arrow in A–C. The ensemble average response from 11 non-Kenyon cells in this culture is shown in gray. Values represent the background corrected average 340/380 ratios, sampled at 4-s intervals, converted to estimates of Ca^{2+} levels based on a standard calcium curve generated in a cell-free system (see Materials and Methods for details). F: The magnitude of the nicotine-induced response was more variable in the general population of non-Kenyon cells when compared with the Kenyon cells (identified as GFP+) in the same culture.

(PTX, 10 μM; Sigma), *Plectreurys* toxin (PLTX, 50 nM; Alomone Labs), cobalt chloride (Co^{2+}, 2 mM; Sigma), thapsigargin (5 μM; Sigma), and ionomycin (5 μM; Sigma). In some experiments, calcium was omitted from the recording solution and these are referred to as "0 Ca^{2+}."

Electrophysiology

Nicotine-evoked currents were recorded in the whole-cell configuration in response to puffer application of the agonist (500 μM, 20 ms), at a holding potential of −75 mV, to block VGCC activation. For cobalt experiments, 2 mM cobalt, in addition to nicotine, was added to the puffer pipette. The puffing electrode was positioned ~20 μm from the cell to be evaluated. Pipette internal solution for analysis of nicotine-evoked currents and voltage-gated calcium currents contained 120 mM D-gluconic acid, 120 mM cesium hydroxide, 20 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1.1 mM EGTA, 4 mM ATP, and 10 mM HEPES, pH 7.2. The external solution was composed of HBSS containing TTX and PTX. All data shown are corrected for the 5-mV liquid junction potential generated in these solutions. Data were acquired with a List EPC7 amplifier, a Digidata 1320A D-A converter (Axon Instruments), a Dell (Dimension 4100) computer, and a pClamp 8 (Axon Instruments) software. Recordings were made at room temperature.

RESULTS

Nicotine Induces a Rapid Increase in Intracellular Calcium

Each primary culture was prepared from the central region of the brain of a single late stage OK107-GAL4;UAS-GFP pupa. Fura-2 AM, a ratiometric calcium imaging dye, was used to monitor calcium levels in the neurons in 3–6–day-old cultures. Kenyon cells are small diameter (3–6 μm), GFP+ neurons [Fig. 1(A)]. GFP+ Kenyon cells represent 10–15% of the total cell population, as shown previously (Su and O'Dowd, 2003). The GFP-negative neurons represent a heterogeneous population of cells from the rest of the central brain region. Most neurons, including Kenyon cells, had low intracellular calcium levels at rest [Fig. 1(B)]. Calcium levels increased dramatically in both Kenyon cells and the general population of GFP-negative neurons upon bath application of 10 μM nicotine [Fig. 1(C)].

To evaluate the magnitude and time course of the nicotine-evoked calcium responses, independent of the contribution of voltage-gated sodium channels, all studies were done in the presence of TTX. Ratio values, sampled at 4-s intervals, were translated to estimates of intracellular calcium concentration, based on a standard calcium curve generated in a cell free system (see Materials and Methods). Plots of the intracellular calcium concentration as a function of time, over the soma region of a Kenyon cell and a GFP-negative neuron, are shown [Fig. 1(D,E)]. Ensemble average nicotine responses were generated by averaging records from all Kenyon cells or an equivalent number of randomly selected GFP-negative non-Kenyon cells in each field of view [Fig. 1(D,E)]. Calcium levels peaked within 20 s and remained elevated above baseline during the nicotine exposure. Intracellular calcium levels declined to basal levels upon removal of nicotine. Although the vast majority of all cultured neurons responded to nicotine (>95%), there was significant variability in the magnitude of the responses, particularly in the non-Kenyon cells [Fig. 1(F)], even when selected for analysis based on soma size that matched the Kenyon cells. This suggests there may be patterns of response that are specific to particular cell types that are not based on size or shape alone. Therefore our study focused on nicotine responses in Kenyon cells that were identified by their GFP expression.

Nicotine-Evoked Response Is Mediated by α-BTX-Sensitive nAChRs

Data representing mean calcium responses obtained from multiple cultures at each concentration show that, as expected for a receptor-mediated response, nicotine evokes a dose-dependent, saturable increase in calcium levels [Fig. 2(A)]. The EC_{50} in Kenyon cells was 280 ± 4 nM. To define the pharmacological properties of the nicotine-induced calcium increase, responses to a saturating concentration of nicotine (10 μM) were measured during bath application of various receptor antagonists. Curare, a specific antagonist of nAChRs in *Drosophila* neurons (Su and O'Dowd, 2003), completely and reversibly blocked the nicotine-evoked increase in calcium levels [Fig. 2(B,D), $^{**}p < 0.01$, Bonferroni post-test; one-way ANOVA]. Bath application of α-BTX [Fig. 2(C,D), $^{***}P < 0.001$, Bonferroni post-test; one-way ANOVA] and MLA [Fig. 2(D), $^{**}p < 0.01$, Bonferroni post-test, one-way ANOVA], antagonists that selectively block calcium-permeable nAChRs in mammals, also completely blocked the nicotine responses. Conversely, neither a cocktail of glutamate receptor antagonists (APV + CNQX) nor the GABAergic antagonist PTX altered the nicotine responses [$p > 0.05$, Bonferroni post-test; one-way ANOVA, Fig. 2(D)]. These data demonstrate that the nicotine-induced increase in intracellular calcium depends specifically on activation of αBTX/MLA-sensitive nAChRs.

Nicotine-Evoked Response Has VGCC-Dependent and VGCC-Independent Components

To determine the source of calcium underlying the nicotine-evoked responses, cultures were exposed twice to saturating concentrations of nicotine either in control recording solution (HBSS + TTX) or in recording solution containing VGCC antagonists. PLTX is an insect-specific calcium channel antagonist that, at a concentration of 50 n*M*, irreversibly blocks most but not all of the voltage-gated calcium currents in cultured Kenyon cells (Jiang et al., 2005). PLTX results in a 70% reduction ($p < 0.001$, Bonferroni post-test, one-way ANOVA) in the peak of the nicotine-evoked response [Fig. 3(A,C)]. To determine whether the residual response was due to calcium flux through VGCCs that were not blocked by PLTX, we examined the effects of 2 m*M* cobalt (HBSS + TTX + Co), a concentration that blocks all voltage-gated calcium currents in *Drosophila* neurons (O'Dowd, 1995). The magnitude of the peak response was reduced by 75% ($p < 0.001$, Bonferroni post-test, one-way ANOVA) but not eliminated in the presence of cobalt, defining both a VGCC-dependent and a VGCC-independent component [Fig. 3(B,C)].

Although these data suggest that the action of cobalt is mediated through its blockade of VGCCs, it is also possible that cobalt could directly alter the activity of nAChRs. To test this possibility, whole cell recordings were used to assess nicotine-evoked currents in the presence and absence of cobalt. Neurons

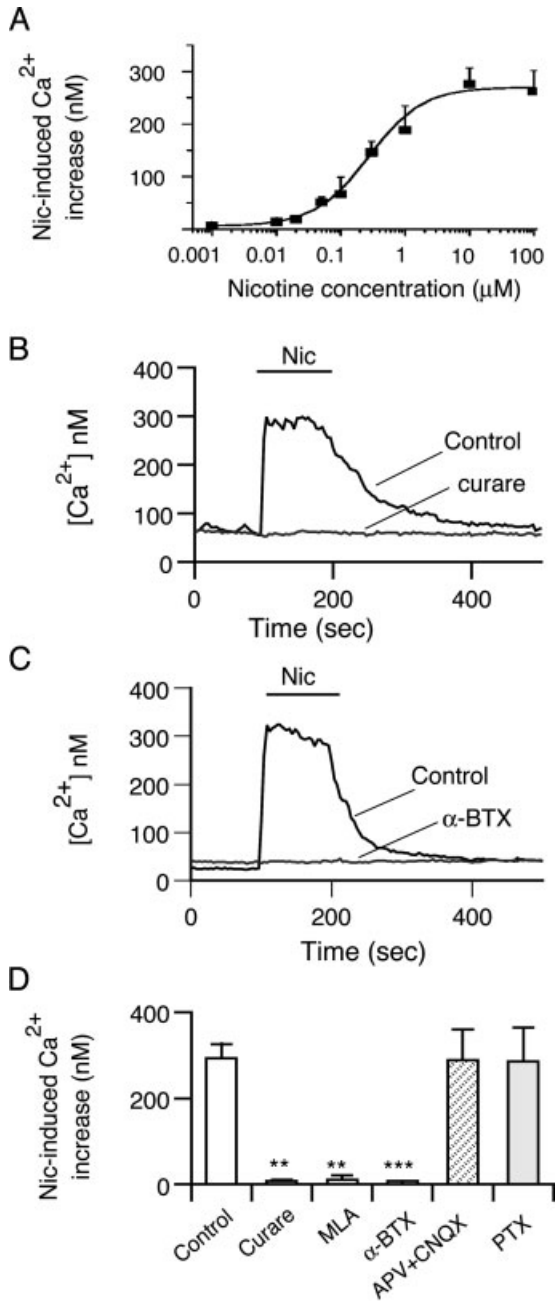

Figure 2 Nicotine induces a dose-dependent saturable increase in intracellular calcium levels in Kenyon cells that is mediated by activation of α-BTX-sensitive nAChRs. A: Increase in intracellular calcium as a function of nicotine concentration. Data fit with a sigmoidal curve indicate EC_{50} of 280 ± 4 n*M*. Each point represents the mean + SEM determined from ensemble average data generated at each nicotine concentration from the number of cultures (*n*) and the total number of neurons indicated in parentheses. Nicotine in *μM*: 0.001 (3, 16); 0.01 (3, 16), 0.02 (5, 31), 0.05 (8, 54), 0.1 (7, 47), 0.3 (9, 65); 1 (10, 58); 10 (22, 136); 100 (10, 57). Nicotine was applied in the bathing solution, HBSS+ TTX. B: Nicotine (10 *μM*)-induced increase in intracellular calcium was reversibly blocked by curare (20 *μM*). Response in the presence of antagonist was obtained prior to control responses, separated by a 10-min wash. Each trace represents the ensemble average nicotine response observed in 10 neurons, in a single culture, in absence or presence of antagonists. C: α-BTX (0.1 *μM*) also blocked the nicotine response. D: The nicotine-induced increase in intracellular calcium was significantly reduced, compared with control, by curare (20 *μM*), α-BTX (0.1–10 *μM*), and MLA (40 n*M*). No significant changes were induced by glutamate receptor antagonists (APV 5 *μM*, CNQX 5 *μM*), nor a GABA receptor antagonist (PTX 10 *μM*). $^{**}p < 0.01$, $^{***}p < 0.001$, Bonferroni post-test, one-way ANOVA. Each bar represents the mean + SEM determined from ensemble average data generated from cultures in indicated conditions: Control ($n = 22$ cultures, 145 cells); curare ($n = 4$ cultures, 26 cells), MLA ($n = 4$ cultures, 22 cells); αBTX ($n = 5$, 38); APV+CNQX ($n = 7$, 49); PTX ($n = 4$, 31). All drugs were applied in HBSS + TTX.

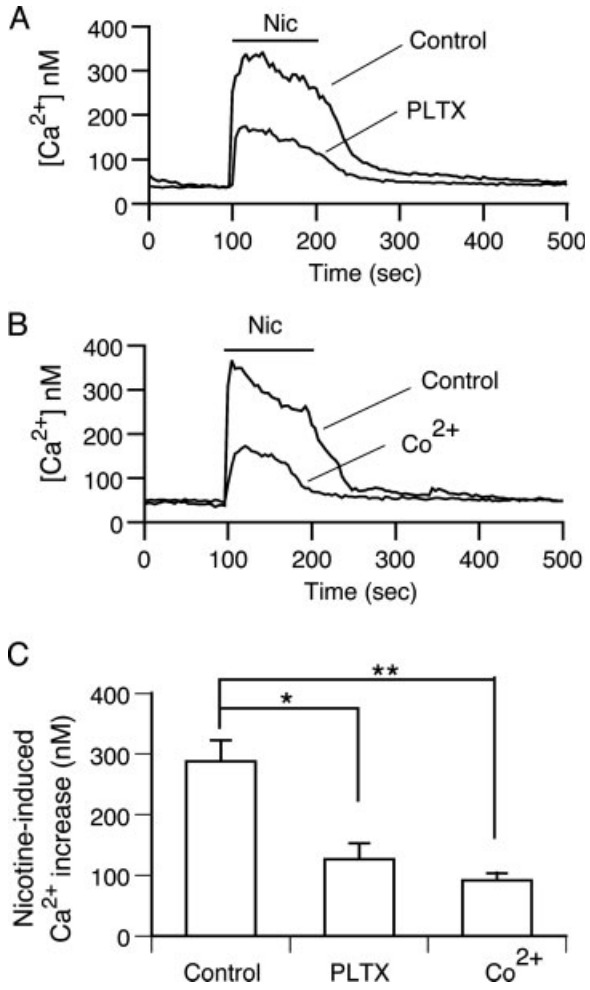

Figure 3 Nicotine response includes a VGCC-dependent and a VGCC-independent component. A: Nicotine-induced increase in intracellular calcium was reduced in the presence of PLTX (50 n*M*), an irreversible calcium channel antagonist. The first nicotine exposure was in control solution followed by PLTX. Traces represent ensemble average nicotine-induced responses from 10 Kenyon cells in a single culture. B: The nicotine-induced increase in intracellular calcium levels in Kenyon cells was reduced but not eliminated by addition of 2 m*M* cobalt, a reversible antagonist that completely blocks VGCCs. Culture was exposed twice to nicotine (10 μM). The first exposure was in 2 m*M* cobalt (Co^{2+}) and the second was in control saline. Traces represent ensemble average nicotine-induced responses from 6 Kenyon cells in a single culture. C: Nicotine responses were significantly reduced but not eliminated by PLTX (50 n*M*) and cobalt (Co^{2+}, 2 m*M*), defining a VGCC-dependent and VGCC-independent component, $*p < 0.05$, $**p < 0.01$, Bonferroni post-test, one-way ANOVA. Bars represent mean + SEM determined from ensemble average data from cultures in indicated solutions: Control ($n = 9$ cultures, 76 cells), PLTX ($n = 6$ cultures, 51 cells), Cobalt ($n = 9$ cultures, 69 cells).

were voltage-clamped at −75 mV to prevent activation of VGCCs and the current response to puffer application of 500 μM nicotine (in a bath volume of 2 mL) was monitored. Data were obtained from Kenyon cells with a capacitance in the range of 1–8 pF (Jiang et al., 2005). The peak nicotine-evoked current density in control external solution (30.8 ± 6.86 pA/pF, mean ± SEM, $n = 15$) was not significantly different ($p > 0.05$, Student's *t*-test) from that observed in the presence of 2 m*M* cobalt (24.5 ± 7.80 pA/pF, mean ± SEM, $n = 11$). For these experiments, cobalt was included both in the external solution and in the puffer pipette. These data indicate that, under these conditions, cobalt does not block current flow through the nAChRs in Kenyon cells.

Thus, the cobalt-sensitive calcium increase defines the VGCC-dependent component and the cobalt-insensitive increase defines the VGCC-independent component of the nicotine response in Kenyon cells.

Nicotine Response Is Reduced in Thapsigargin-Treated Cultures

It has been shown in several systems that activation of ligand-gated receptors can also stimulate calcium release from internal reservoirs (Tsuneki et al., 2000; Chang and Berg, 2001; Sharma and Vijayaraghavan, 2001; Hu et al., 2002; Dajas-Bailador and Wonnacott, 2004). In order to determine whether calcium-induced calcium release from intracellular stores contributes to the nicotine response, cultures were treated with thapsigargin, using a concentration and exposure paradigm similar to those used to deplete internal calcium reservoirs in a number of systems including insects (Thastrup et al., 1990; Chang and Berg, 2001; Oertner et al., 2001; Sharma and Vijayaraghavan, 2001; Hu et al., 2002; Jiang et al., 2005). Cultures were loaded with Fura-2 using the standard procedure, and during the 45-min de-esterification step, 5 μM thapsigargin was added to the HBSS wash. Thapsigargin was also present during assessment of the nicotine response. The mean amplitude of the averaged nicotine response was reduced in cultures treated with thapsigargin, when compared with the averaged nicotine response in control cultures that were processed in parallel [Fig. 4(A,B)]. Examination of multiple dishes revealed a decrease of 50% ($p < 0.05$, Student's *t*-test) in the peak nicotine response of Kenyon cells in thapsigargin versus control cultures [Fig. 4(C)]. These data support the hypothesis that release from internal stores amplifies the nicotine-evoked increase in intracellular calcium.

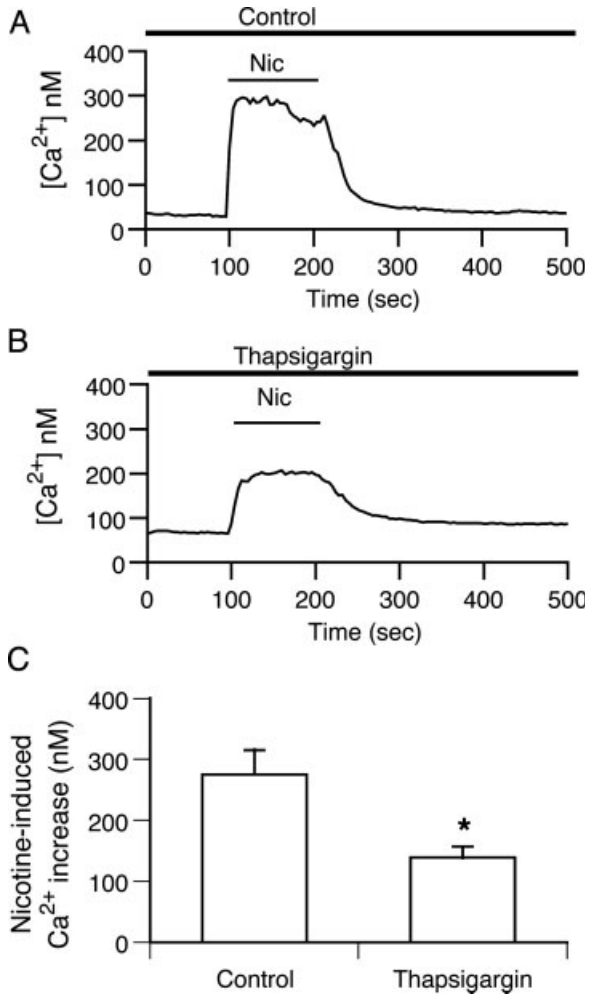

Figure 4 Thapsigargin treatment reduces magnitude of the nicotine response. A: Averaged nicotine-evoked response from 15 Kenyon cells in a control culture. B: The averaged nicotine response from 13 Kenyon cells in a sibling culture following thapsigargin treatment (5 μM, 45 min). C: Nicotine induced-responses were significantly reduced, but not eliminated, by thapsigargin treatment. $*p < 0.05$, Student's t-test. Bars represent mean + SEM determined in control solution ($n = 4$ cultures, 42 cells) and in the presence of thapsigargin ($n = 5$ cultures, 57 cells).

Calcium-Permeable nAChRs

Many (83.2 ± 3.7%, mean ± SEM, $n = 11$ cultures, 112 cells) but not all individual Kenyon cells show an increase in intracellular calcium in response to nicotine in cobalt. The VGCC-independent component of the nicotine response recorded in a Kenyon cell in the presence of cobalt was completely and reversibly blocked by addition of curare, confirming that it is directly dependent on activation of nAChRs [Fig. 5(A)]. To test whether this component involves flux of calcium directly through the receptor, the calcium dependence of the VGCC-independent component was examined. Cultures were exposed to two sequential nicotine stimuli in the presence of cobalt, the first in normal calcium (1.8 mM) and the second in calcium-free solution (0 mM) or in elevated calcium (5 mM). The VGCC-independent component was evaluated only in Kenyon cells that showed an initial nicotine response >100 nM to accurately assess both the potential for a decrease as well as an increase in response magnitude. The VGCC-independent component in these neurons is eliminated in calcium-free solution ($p < 0.001$, Bonferroni post-test, one-way ANOVA) and is twice as large ($p < 0.001$, Bonferroni post-test, one-way ANOVA) in elevated calcium [Fig. 5(B)]. These data demonstrate that there is a VGCC-independent component of the nicotine response in the majority of Kenyon cells, which

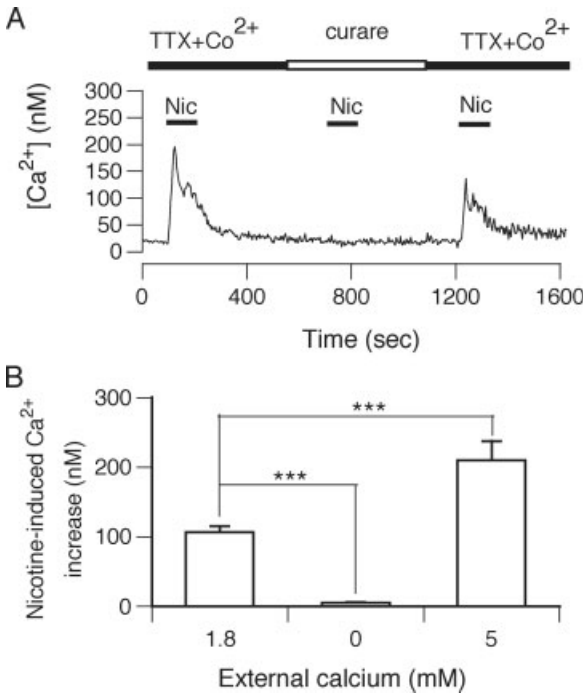

Figure 5 Calcium influx through nAChRs is required for VGCC-independent component of nicotine response. A: Recording from a single Kenyon cell demonstrates that the nicotine response remaining in the presence of Co^{2+} (VGCC-independent) is reversibly blocked by curare. B: Magnitude of the VGCC-independent nicotine response recorded in the presence of TTX+ Co^{2+} with normal external calcium (1.8 mM, $n = 27$ cells) is reduced by removal of calcium (0 mM, $n = 15$ cells) and increased by raising external calcium concentration (5 mM, $n = 12$ cells). Bars indicate mean + SEM, $***p < 0.001$, Bonferroni post-test, one-way ANOVA.

requires the influx of calcium across the membrane directly through nAChRs.

nAChR-Mediated Plasticity

Adult *Drosophila*, exposed to volatilized nicotine for 1 min, show both an acute behavioral response and an increase in behavioral sensitivity to a second nicotine challenge when the interval between exposures is 4 h (Hou et al., 2004). To determine whether activation of nAChRs could induce plastic changes at the cellular level, with a similar time course, we did the following experiment. Sibling cultures were divided into two groups: the experimental group was exposed to a conditioning pulse of nicotine (10 μM) for 5 min (time = 0), whereas the sham group was exposed to a media change without nicotine (sham conditioning pulse) (time = 0). After the conditioning pulse, cultures were washed five times with 2 mL of growth medium and returned to the incubator. The washing protocol diluted the initial concentration of the drug by $>10^5$. Cultures were loaded with Fura-2 just prior to examination of the response to a test pulse of nicotine, at intervals between 1.5 and 12 h after the nicotine or sham conditioning pulse.

The nicotine response in Kenyon cells 4 h after a nicotine conditioning pulse was reduced by 50% ($p < 0.05$, Bonferroni post-test, two-way ANOVA) when compared with that observed in Kenyon cells exposed to a sham conditioning pulse (Fig. 6; 148 ± 42 vs. 347 ± 63 nM in nicotine-conditioned vs. sham-conditioned cultures, mean ± SEM). These results demonstrate that a brief nicotine conditioning pulse causes a transient decrease in the nAChR-mediated calcium response that peaks 4 h after the initial exposure.

In order to determine whether a decrease in density of voltage-gated calcium currents might contribute to the nicotine-induced changes, whole cell recordings were used to evaluate calcium current density in Kenyon cells in cultures 3.5–4.5 h after the conditioning pulse. Whole cell currents were recorded in the presence of TTX in the external solution to block sodium currents and cesium in the internal solution to block potassium currents. The currents recorded in these conditions represent voltage-gated calcium currents based on their complete blockade when 2 mM cobalt is added to the external solution. The peak calcium current density following a sham conditioning pulse (16.2 + 1.9 pA/pF; mean + SEM, $n = 40$ cells from 13 different cultures) was not different from that observed following a nicotine conditioning pulse (16.1 + 1.5 pA/pF; mean + SEM, 26 cells from 6 different cultures). This suggests that it is unlikely that a decrease in calcium current density is the major event underlying nicotine-induced plasticity in the Kenyon cells.

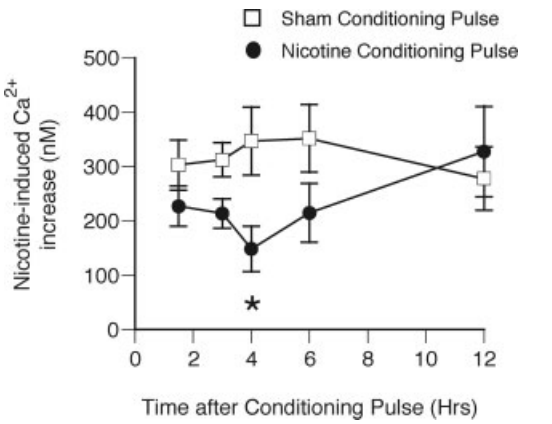

Figure 6 Nicotine conditioning pulse induces a transient decrease in nAChR-mediated calcium response in Kenyon cells. Nicotine responses at 1.5, 3, 4, 6, and 12 h after a 5-min conditioning pulse with 10 μM nicotine (Nicotine Conditioning Pulse) or medium control (Sham Conditioning Pulse). A nicotine conditioning pulse causes a 50% reduction when there is a 4-h interval between the nicotine conditioning and test pulse. *$p < 0.05$, Bonferroni post-test, two-way ANOVA. Data points represent the mean ± SEM determined at each conditioning pulse interval: 1.5 h (Sham: $n = 7$ cultures, 58 cells: Nicotine: $n = 7$ cultures, 56 cells); 3 h (Sham: $n = 6$ cultures, 54 cells; Nicotine: $n = 6$ cultures, 62 cells); 4 h (Sham: $n = 7$ cultures, 70 cell; Nicotine: $n = 7$ cultures, 62 cells); 6 h (Sham: $n = 4$ cultures, 42 cells; Nicotine: $n = 4$ cultures, 43 cells); 12 h (Sham: $n = 4$ cultures, 34 cells; Nicotine: $n = 4$ cultures, 30 cells).

Plasticity Requires Activation of VGCCs and cAMP-Signaling, but Is Protein Synthesis Independent

To determine whether calcium influx through nAChRs and VGCCs is necessary for modulation of the nicotine response, an independent set of cultures was exposed to conditioning pulses of nicotine in the presence and absence of calcium. Similar to data presented in Figure 6, the response to a nicotine test pulse in Kenyon cells 4 h after a 5-min nicotine conditioning pulse in the presence of normal calcium was reduced by greater than 60% ($p < 0.05$, Bonferroni post-test; ANOVA, one-way) when compared with the sham conditioning pulse [Fig. 7(A)]. In contrast, when the nicotine conditioning pulse was given in the absence of external calcium, the response to the test pulse was not reduced. In addition, there was

no depression of the Kenyon cell responses when the nicotine conditioning pulse was done in the presence of 2 m*M* cobalt, which blocks VGCCs but allows calcium influx through the receptor itself [Fig. 7(A)]. These data show that calcium flux across the membrane through VGCCs is necessary for inducing plasticity in the nAChR-mediated response in Kenyon cells and that calcium flow through the nAChR itself is not sufficient.

To determine whether an increase in intracellular calcium caused by a general depolarizing agent in the absence of nicotine is sufficient to downregulate the nicotine response, cultures were exposed to 20 m*M* KCl for 5 min. This high K conditioning pulse depolarizes the neurons and causes an increase in intracellular calcium (308.9 ± 31.3 n*M*, mean ± SEM, n = 18 cultures) similar to the increase induced by exposure to saturating concentrations of nicotine. Four hours after the high K conditioning pulse, the magnitude of the response to the nicotine test pulse was similar to that in the sham group, showing that an increase in intracellular calcium, in the absence of nAChR activation, is not sufficient to trigger the decrease in response of Kenyon cells to nicotine [Fig. 7(A)]. Taken together, these data suggest that activation of the nicotine receptors is required for the modulation of nAChR-mediated responses in Kenyon cells.

The behavioral sensitization induced in the fly by nicotine involves cAMP-dependent signaling and protein synthesis (Hou et al., 2004). To assess whether nicotine-induced plasticity in the Kenyon cells involves cAMP-signaling, the nicotine conditioning pulse was given in the presence of SQ22536 (SQ). This is an inhibitor of the cAMP-synthesizing enzyme adenylyl cyclase and it was used at a concentration (100 μM) that has been shown to be effective in the *Drosophila* neuromuscular junction (Zhang et al., 1999; Kuromi and Kidokoro, 2000) as well as in other systems (Gomes et al., 1999; Araki et al., 2005; Madrid et al., 2005). SQ was added to cultures 10 min prior to the standard 5-min nicotine conditioning pulse. Cultures were washed and maintained in the presence of SQ until the end of the experiment.

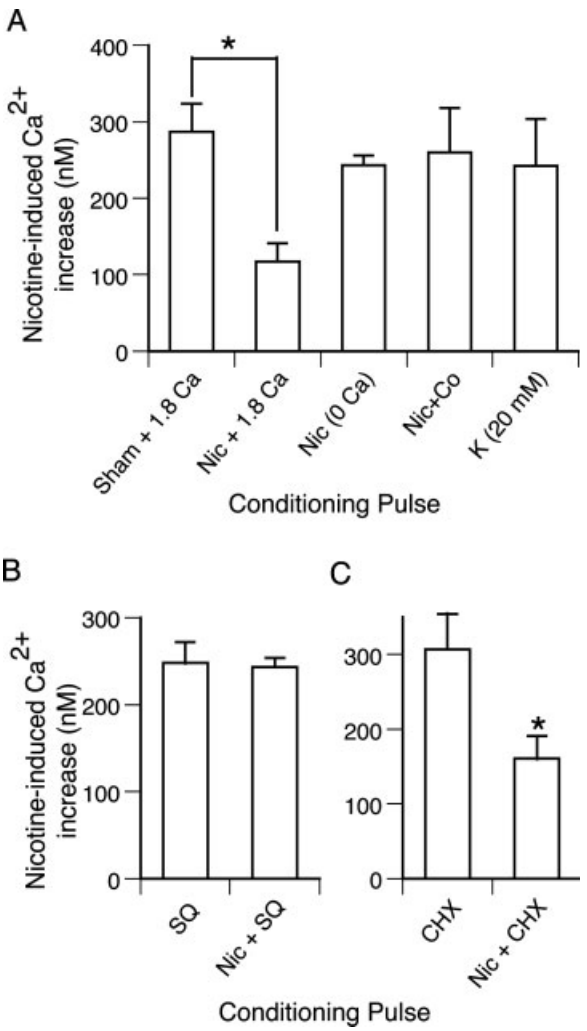

Figure 7 nAChR-dependent plasticity in Kenyon cells requires calcium influx through VGCCs and adenylyl cyclase, but not protein synthesis. Calcium increase induced by 10 μM nicotine challenge, evaluated 4 h after a 5-min conditioning pulse. A: The significant decrease ($^*p < 0.05$, Bonferroni post-test, one-way ANOVA) in response caused by nicotine conditioning pulse in normal calcium (Nic +1.8 Ca^{2+}, n = 15 cultures, 140 cells), compared with a sham conditioning pulse (Sham +1.8 Ca^{2+}, n = 13 cultures, 107 cells), is blocked when pretreatment is done in the absence of calcium (0 Ca^{2+}, n = 3 cultures, 23 cells) or in the presence of cobalt (Nic+Co^{2+}) (n = 5 cultures, 40 cells). An increase in intracellular calcium by a high K^+ conditioning pulse is not sufficient to alter the response to a test pulse of nicotine (K, 20 m*M*, n = 4 cultures, 43 cells). B: Nicotine-dependent decrease is blocked when the nicotine conditioning pulse is done in the presence of the adenylyl cyclase inhibitor (Nic + SQ) (n = 5 cultures, 44 cells) since there is no difference ($p > 0.05$, Student's t-test) when compared with conditioning pulse of SQ alone (SQ) (n = 5 cultures, 41 cells). Both groups were maintained in SQ for the whole experiment. C: In contrast, there is still a 50% decrease ($^*p < 0.05$; Student's t-test) in response to the nicotine test pulse when the nicotine conditioning is done in presence of cycloheximide (Nic + CHX, n = 7 cultures, 54 cells) when compared with a conditioning pulse in cyclohexamide alone (CHX, n = 6 cultures, 48 cells). Both groups were maintained in CHX throughout the experiment. Data represent mean + SEM.

There was no depression induced by a conditioning pulse of nicotine in the presence of SQ (Nic + SQ) [$p > 0.05$, Student's *t*-test, Fig. 7(B)]. This indicates that nicotine triggers cAMP-dependent events required for plasticity of the nAChR-mediated responses in Kenyon cells. The timing and the transient nature of the nicotine-induced plasticity in Kenyon cells are consistent with a cellular mechanism involving cAMP-mediated translational and/or post-translational modifications.

To explore whether nicotine-induced plasticity in the Kenyon cells is protein synthesis dependent, the nicotine conditioning pulse was given in the presence of a protein synthesis inhibitor. Ten minutes prior to the standard 5-min nicotine conditioning pulse, cultures were treated with cyclohexamide, at 100 μM, a concentration previously shown to inhibit protein-synthesis-dependent events in cultured *Drosophila* neurons (Lee and O'Dowd, 2000) and in adult flies (Hou et al., 2004). Following the conditioning pulse, cultures were washed and maintained in cyclohexamide over the next 4 h and during the imaging procedure, in which the responsiveness to a nicotine test pulse was evaluated. There was still a 50% depression in the nicotine response ($p < 0.05$, Student's *t*-test) when the nicotine conditioning pulse was in cyclohexamide (Nic + CHX) when compared with a conditioning pulse of cyclohexamide alone (CHX) [Fig. 7(C)]. This indicates that post-translational modifications, but not protein synthesis, are involved in mediating nicotine-induced cellular plasticity in Kenyon cells.

DISCUSSION

In vertebrate neurons, nicotine generates complex calcium responses resulting from calcium flux directly through nAChRs, particularly the α-BTX-sensitive α7 subtype, through VGCCs opened by nAChR-mediated membrane depolarization, and/or mobilization of intracellular calcium stores (Berg and Conroy, 2002; Dajas-Bailador and Wonnacott, 2004). Here we show that the nicotine-induced increase in intracellular calcium in *Drosophila* neurons is similarly complex. Calcium influx triggered by α-BTX-sensitive nAChR-dependent activation of VGCCs constitutes the major component of the nicotine response in Kenyon cells. A smaller but significant component requires flux of calcium through the receptor itself. Complex calcium responses mediated by calcium flux directly through the nAChRs, and by indirect activation of VGCCs, also occur in neurons from other insects, including locust (Oertner et al., 1999) and blowfly (Oertner et al., 2001; Single and Borst, 2002). Although this is the first evidence that there are calcium-permeable α-BTX-sensitive nAChRs in *Drosophila* Kenyon cells, it is consistent with a voltage-clamp study demonstrating that honey bee Kenyon cells express nAChRs that have a high Ca^{2+}:Na^{+} permeability ratio (Goldberg et al., 1999). Thus it seems likely that synaptic activation of nAChRs in Kenyon cells via cholinergic afferents from the antennal lobe during olfactory associative conditioning contributes to a postsynaptic increase in intracellular calcium that could trigger changes in gene expression required for learning (Waddell and Quinn, 2001). Activation of calcium-permeable nAChRs would result in an increase in intracellular calcium in Kenyon cells even when the stimulus is below threshold for activation of voltage-gated channels, perhaps capable of activating other calcium-dependent signaling cascades.

In a number of vertebrate cell types, calcium flux through α7 nAChRs triggers release of calcium from intracellular stores (Tsuneki et al., 2000; Chang and Berg, 2001; Sharma and Vijayaraghavan, 2001; Hu et al., 2002; Dajas-Bailador and Wonnacott, 2004). Here we report that the VGCC-independent component in Kenyon cells is reduced but not eliminated by thapsigargin. This suggests that the VGCC-independent component in *Drosophila* neurons is amplified by calcium release from intracellular stores.

Nicotine-induced increases in intracellular calcium mediated by activation of nAChRs in vertebrates have been linked to a variety of events associated with cellular plasticity, including regulation of receptor number, neurotransmitter release, neuronal excitability, and gene expression (Berg and Conroy, 2002; Dajas-Bailador and Wonnacott, 2004). Our data demonstrate that nicotine can also induce calcium-dependent plasticity of nAChR-mediated responses in *Drosophila* Kenyon cells: a brief conditioning pulse of nicotine resulted in a decrease in responsiveness to a second nicotine challenge 4 h later. Exposure to nicotine when calcium channels were blocked was insufficient to modify the nicotine response in Kenyon cells. This argues that nicotine-induced plasticity requires an increase in calcium that surpasses the influx mediated by the receptor itself, involving the VGCC-dependent component that includes flux of calcium through these channels and release from intracellular stores. However, an increase in intracellular calcium, independent of activation of nAChRs, did not generate plasticity in Kenyon cells. This suggests that while calcium flux through VGCCs is necessary for plasticity to occur, it is not sufficient. These findings suggest that activation of nAChRs is a

key element for induction of plasticity in Kenyon cells. While our data indicate that conditioning pulses of nicotine do not regulate calcium current density, future experiments will be necessary to determine whether the plasticity involves changes in the number or properties of the nAChRs themselves, as has been reported in the rodent CNS (Dani and De Biasi, 2001; Buisson and Bertrand, 2002; Mansvelder and McGehee, 2002).

Two previous studies showed that adult *Drosophila* exhibit a series of stereotyped behaviors in response to acute exposure to volatilized nicotine, with a rapid onset of hyperactivity and spasmodic movements that can lead to hypo- or akinesia at higher concentrations (Bainton et al., 2000; Hou et al., 2004). In addition to its ability to alter spontaneous behaviors, nicotine also impairs startle-induced negative geotaxis, a robust evoked behavior. Our data showing that nicotine elicits a rapid increase in intracellular calcium levels in virtually all cultured neurons suggests that nicotine-induced changes in spontaneous and evoked behaviors are likely to involve neurons throughout the CNS.

The ability of nicotine to inhibit startle-induced negative geotaxis in *Drosophila* is enhanced by a second exposure to nicotine (Hou et al., 2004). This behavior, termed hyper-responsiveness, was seen when the interval between the two nicotine exposures was 4 but not 2 or 8 h, and is analogous to sensitization in vertebrates, an enhancement of drug responsiveness with repeated exposure to a constant dose of the drug (Nestler, 2001). Challenging the cultured neurons with a similar stimulus paradigm induced a change in the response of Kenyon cells to nicotine that peaked when the interval between exposures was 4 h. The depression in the response was eliminated by removal of calcium, addition of VGCC antagonists, or blockade of cAMP-signaling, during the conditioning pulse. The time course, dependence on influx of calcium, cAMP-signaling, and expression of this effect in a neuronal population implicated in generation of behaviors associated with repetitive exposures to other drugs of abuse are consistent with nicotine-induced plasticity in Kenyon cells contributing to behavioral plasticity induced by repeated exposure to nicotine in the animal (Scholz et al., 2000; Cho et al., 2004).

However, there are two observations suggesting that cellular or circuit level plasticity at other sites is also required to mediate the behavioral plasticity induced by nicotine. First, in contrast to behavioral plasticity in the fly (Hou et al., 2004), cellular plasticity in Kenyon cells is not dependent on new protein synthesis. Second, nicotine causes a decrease in response to a second nicotine exposure in Kenyon cells as opposed to the behavioral effect characterized by an increase in responsiveness. Therefore, if the nicotine-dependent cellular plasticity in Kenyon cells contributes to the behavioral plasticity, the link is not a translation of a change in activity of this single cell type via a simple linear circuit. Although unknown, the neural circuits through which Kenyon cells influence motor output are likely to be complex, including interneurons containing biogenic amines. Consistent with this hypothesis is the finding that acute behavioral responses to nicotine depend on intact dopaminergic signaling (Bainton et al., 2000).

Calcium imaging and electrophysiological studies in identified vertebrate neuronal populations, in dissociated cell culture and acute slices, have been important in defining the cellular changes that translate drug-induced alterations in molecular signaling to behaviors (Berke and Hyman, 2000; Laviolette and van der Kooy, 2004). Here we demonstrate the ability to begin exploring the cellular mechanisms underlying responses to drugs of abuse in Kenyon cells, neurons that are involved in mediating behavioral plasticity in *Drosophila*. Future studies in *Drosophila*, employing genetic and pharmacological manipulations, should provide additional insights into the cellular mechanisms by which repetitive exposure to nicotine alone, or in combination with other drugs of abuse, regulates neuronal function (Nichols, 2006).

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