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Smoke extracts and nicotine, but not tobacco extracts, potentiate firing and burst activity of  
Ventral Tegmental Area dopaminergic neurons in mice.

Running title: Effect of smoke, tobacco and pure nicotine on VTA DA cells

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## ***Abstract***

Nicotine prominently mediates the behavioral effects of tobacco consumption, either through smoking or when taking tobacco by snuff or chew. However, many studies question the exclusive role of nicotine in these effects. The use of preparations containing all the components of tobacco, such as tobacco and smoke extracts, may be more suitable than nicotine alone to investigate the behavioral effects of smoking and tobacco intake. In the present study, the electrophysiological effects of tobacco and smoke on ventral tegmental area dopamine (DA) neurons were examined *in vivo* in anesthetized wild-type (WT),  $\beta 2$  nicotinic acetylcholine receptor (nAChR) knock-out ( $\beta 2^{-/-}$ ),  $\alpha 4^{-/-}$  and  $\alpha 6^{-/-}$  mice and compared to those of nicotine alone. In WT mice, smoke and nicotine had similar potentiating effects on DA cells activity, but the action of tobacco on neuronal firing was weak, and often inhibitory. In particular, nicotine triggered strong bursting activity, while no bursting activity was observed after tobacco extract administration. In  $\beta 2^{-/-}$  mice, nicotine or extract elicited no modification of the firing patterns of DA cells, indicating that extract act predominantly through nAChRs. The differences between DA cells activation profiles induced by tobacco and nicotine alone observed in WT persisted in  $\alpha 6^{-/-}$  mice but not in  $\alpha 4^{-/-}$  mice. These results would suggest that tobacco has lower addiction generating properties compared to either nicotine alone or smoke. The weak activation and prominent inhibition obtained with tobacco extracts suggest that tobacco contains compounds which counteract some of the activating effects of nicotine and promote inhibition on DA cell acting through  $\alpha 4\beta 2^*$ -nAChRs. The nature of these compounds remains to be elucidated. It nevertheless confirms that nicotine is the main substance involved in the tobacco addiction-related activation of mesolimbic DA neurons.

**Keywords:** nicotine, smoke, tobacco, VTA, dopaminergic cell, tobacco addiction, *in vivo*, nicotinic receptor

**Abbreviations:** DA, dopamine/dopaminergic; HEK293, human embryonic kidney 293 cell line; MAOIs, monoamine oxidase inhibitors; NAc, nucleus accumbens; nAChR, nicotinic acetylcholine receptor; Nic, nicotine; SmE, smoke extract; ToE, tobacco extract; VTA, ventral tegmental area; WT, wild type mice; %SWB, percentage of spikes within bursts;  $\beta 2^{-/-}$  ( $\alpha 6^{-/-}$  or  $\alpha 4^{-/-}$ ),  $\beta 2$  ( $\alpha 6$  or  $\alpha 4$ ) nicotinic acetylcholine receptor knock-out mice

## ***Introduction***

Tobacco addiction is the major cause of preventable death in the world. One hundred million people are expected to die this century from the consequences of this addiction (Peto *et al.*, 1996). Identifying the molecular and cellular mechanisms involved in tobacco addiction is therefore crucial in order to develop novel aids for smoking cessation interventions. Currently, the rewarding effects of smoking are thought to be largely, if not completely, related to the action of nicotine on brain mesolimbic dopaminergic (DA) neurons. Despite thousands of compounds being identified in smoke and tobacco, nicotine is generally acknowledged to be the primary addictive component of tobacco (Wonnacott *et al.*, 2005). Overwhelming evidence suggests that nicotine's actions on mesolimbic DA neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is central to nicotine-reinforced behaviors, and related conditioning processes (Di Chiara, 2000). Nicotine increases DA release in the striatum and NAc (Di Chiara, 1998) by acting through nicotinic acetylcholine receptors (nAChRs) expressed on DA neurons themselves, GABAergic neurons and afferent inputs from various brain structures (Jones *et al.*, 2004; Mansvelder *et al.*, 2002; Wooltorton *et al.*, 2003). Nicotine augments the firing rate and burst activity of DA neurons (Grenhoff *et al.*, 1986; Mamei-Engvall *et al.*, 2006). The nicotine induced increase in DA cell bursting activity causes a phasic release of DA in the NAc that is linked to the addictive properties of nicotine. However, in spite of the evident addictive properties of tobacco, some authors have shown that nicotine alone is not abused (West *et al.*, 2000) and that nicotine replacement products are only moderately effective aids to smoking cessation (Hajek *et al.*, 1999), suggesting that compounds other than nicotine may contribute to tobacco addiction.

Among the thousands of identified compounds in tobacco and cigarette smoke, a number of them can modulate neuronal activities. Studies involving nicotine coupled with specific compounds found in tobacco, such as monoamine oxidase inhibitors (MAOI) or minor tobacco alkaloids, for example anatabine, cotinine, and myosmine, have shown that such associations can increase the motivation for nicotine consumption and nicotine-linked reinforcement learning (Clemens *et al.*, 2009; Guillem *et al.*, 2005). Furthermore, MAOI present in tobacco smoke, e.g. harmaine and norharmaine, have a powerful but highly variable impact on both DA release in the NAc (Baum *et al.*, 1996; Baum *et al.*, 1995) and DA neuron activity (Arib *et al.*, 2010). Therefore, compounds other than nicotine present in tobacco and tobacco smoke can contribute to DA system activation and possibly modulate aspects of the

addictive properties of nicotine.

All these previous studies focused on specific compounds, either alone or in association with nicotine. Another approach to the study of the role of such chemicals is to directly test the effects of tobacco extracts (ToE) or smoke extracts (SmE). These preparations contain all compounds that potentially act upon neuronal activity. Tobacco smoke exposure activates DA neurons (Fa *et al.*, 2000) and induces nicotine dependence (Small *et al.*, 2010) in rats. ToE and SmE have been widely used to investigate the genotoxicity (for review, (DeMarini, 2004)) and cytotoxicity (Bagchi *et al.*, 1998; Bagchi *et al.*, 1999; Tanaka *et al.*, 2007; Yildiz *et al.*, 1999) of tobacco intake. They have also been used to assess whether compounds other than nicotine have effects on neuronal activity, even if such approaches preclude identifying effects of specific compounds. We have previously shown that ToE and SmE effects on serotonergic neurons strongly differ from those of nicotine (Touiki *et al.*, 2007). However, as with nicotine these effects were completely blocked by mecamylamine, a nicotinic receptor antagonist (Touiki *et al.*, 2007). Therefore, tobacco and smoke contain compounds other than nicotine that have physiological effects, and these compounds appear to act through nAChRs, either directly or indirectly.

This paper aims to quantify the effects of ToE and SmE on the firing of VTA DA cells. In order to dissociate the effects of nicotine on VTA DA cells from those of other tobacco compounds, we compared the nicotine-evoked effects on VTA DA neurons to those of ToE and SmE. Given that the activating effects of nicotine on VTA DA neurons are no longer present in mice lacking the  $\beta 2$  receptor subunit of the nAChR (Mameli-Engvall *et al.*, 2006; Maskos *et al.*, 2005), we also tested the effects of ToE and SmE on VTA DA neurons of  $\beta 2^{-/-}$  mice. Finally, we analyzed  $\alpha 4^{-/-}$  and  $\alpha 6^{-/-}$  mice to test the involvement of the two main partners of the  $\beta 2$  receptor subunit of the nAChR.

## ***Methods***

### ***Animals***

Experiments were performed on WT (C57BL/6 strain),  $\beta 2^{-/-}$ ,  $\alpha 4^{-/-}$  and  $\alpha 6^{-/-}$  male mice, weighing between 25 and 30g. In each experiment, mice homozygous for the  $\beta 2$  subunit gene deletion were paired with WT mice. All experiments were performed on male mice between 2 and 4 months of age. The animals were housed in cages containing maximum of six animals, in a 12 hours light/dark cycle and temperature controlled room (23°C) with food and water available ad libitum. All experiments were undertaken in compliance with the directives of the European Community and French law on animal experimentation.

### ***Drugs***

Nicotine tartrate and chloral hydrate were purchased from Sigma-Aldrich, France. For mice, the nicotine solution was prepared as follows: 0.5 mM of nicotine tartrate was dissolved in a 0.9% NaCl solution and adjusted to pH 7.2 using NaOH. ToE and SmE were gifts from Alatis Corporation. ToE was obtained by maceration of tobacco sheets in saline solution 0.9% NaCl. SmE was obtained by bubbling the smoke of 40 cigarettes in saline solution 0.9% NaCl. The pH of both solutions was adjusted to 7.4. Dilutions were made to obtain preparations containing 0.96g/l of nicotine for the ToE solution and 1.08g/l of nicotine for the SmE solution, from which the administered doses were prepared. Doses are always expressed as nicotine base. A preliminary study showed that an intravenous injection of a control solution (0.9% NaCl/0.5 mM of KNa-tartrate) had no effect on the electrophysiological characteristics of DA neurons in animals.

### ***In vivo electrophysiology***

Mice were anesthetized with chloral hydrate (8%), 400 mg/kg i.p. supplemented as required to maintain optimal anesthesia throughout the experiment, and positioned in a stereotaxic frame (David Kopf). Body temperature was kept at 37°C by means of a thermostatically controlled heating blanket. All animals had a catheter inserted into their saphenous vein for intravenous (i.v.) administration of drugs.

An incision was made in the midline to expose the skull. A hole was drilled above the VTA (coordinates: between  $3.5 \pm 0.3$  mm posterior to Bregma and  $0.5 \pm 0.3$  mm lateral to the midline (Paxinos *et al.*, 2004)). Recording electrodes were pulled with a Narishige electrode

puller from borosilicate glass capillaries (with outer and inner diameters of 1.50 and 1.17 mm respectively; Harvard Apparatus Inc.). The tips were broken under microscope control and filled with 2% pontamine sky blue dye in 0.5% sodium-acetate. These electrodes had tip diameters of 1–2  $\mu\text{m}$  and impedances of 4–8  $\text{M}\Omega$ . A reference electrode was placed in the subcutaneous tissue. The recording electrodes were lowered vertically through the hole with a micro drive. Electrical signals were amplified by a high-impedance amplifier (Axon Instruments) and monitored visually with an oscilloscope (Tektronix TDS 2002) and audibly through an audio monitor (A.M. Systems Inc.). When a single unit was well isolated, the oscilloscope sweep was triggered from the rising phase of the action potential and set so as to display the action potential over the entire screen (usually 0.5ms per division). Such continuous observation of the expanded action potential provided assurance that the same single unit was being monitored throughout the experiment. The unit activity digitized at 25 KHz was stored in Spike2 program (Cambridge Electronic Design, United Kingdom).

The electrophysiological characteristics of VTA DA neurons were analyzed in the active cells encountered by systematically passing the microelectrode in a stereotaxically defined block of brain tissue including the VTA. Its margins ranged from 2.92–3.88 mm posterior to bregma, 0.24–0.96 mm mediolateral with respect to the Bregma point, and 3.5–4.5 mm ventral to the cortical surface, according to the coordinates of (Paxinos *et al.*, 2004). Sampling was initiated on the right side and then on the left side. Each electrode descent was spaced 100  $\mu\text{m}$  from the others.

Extracellular identification of DA neurons was based on their location as well as on the set of unique electrophysiological properties that characterize these cells *in vivo*: (1) a typical triphasic action potential with a marked negative deflection; (2) a characteristic long duration ( $>2.0\text{ms}$ ); (3) an action potential width from start to negative through  $R1.1\text{ ms}$  (Fig. Supp. 1A); (4) a slow firing rate (less than 10 Hz and more than 1Hz) with an irregular single spiking pattern and occasional short, slow bursting activity. These electrophysiological properties distinguish DA from non-DA neurons (Floresco *et al.*, 2003; Grace *et al.*, 1983; Sanghera *et al.*, 1984; Steffensen *et al.*, 1998; Ungless, 2004).

After a baseline recording of 10–20 min, 10  $\mu\text{l}$  saline (0.9% NaCl) was injected into the saphenous vein *i.v.*, and after 5-10 minutes, 10  $\mu\text{l}$  of nicotine, ToE or SmE solutions (each containing a concentration of 30 $\mu\text{g}/\text{kg}$  of nicotine) were administered in an identical fashion. The dose of nicotine was selected in accordance with previous studies showing that nicotine is

intravenously self-administered i.v. by mice at this dose (Picciotto *et al.*, 1998). In paired injection protocols, nicotine, ToE or SmE were injected in a random sequence with a delay of 15 to 30 minutes was applied between consecutive injections.

Following the recordings, a small current was applied through the electrode to mark the position of the electrode tip. The animals were then sacrificed and their brain was removed and frozen. Following this, 60µm serial sections were cut and stained, and the position of the electrode verified.

### ***Data analysis***

DA cell firing in-vivo was analyzed with respect to the average firing rate and the percentage of spikes within a burst (SWB, number of spikes within burst divided by total number of spikes). Bursts were identified as discrete events consisting of a sequence of spikes such that: i) their onset is defined by two consecutive spikes within an interval lower than 80msec, whenever ii) they terminated with an interval greater than 160msec (Grace *et al.*, 1984a; Grace *et al.*, 1984b). All data were analyzed using R, a language and environment for statistical computing.

Firing rate  $x$  and %SWB were evaluated on successive windows of 60 sec with a 45 sec overlapping period (Fig. Supp. 1B). In order to quantify nicotine effect, each cell's activity was rescaled by its averaged baseline value  $x_b$  (for the firing frequency) and %SWB<sub>b</sub> (for the bursting) estimated on the 5 minutes period before injection of the substances (Fig Supp 1A). For the firing frequency  $x$ , the rescaling  $X$ , expressed as a % of the baseline, is defined by  $X = x * 100 / x_b$ . For the bursting parameter, we used the difference from the value during baseline ( $Y = \%SWB - \%SWB_b$ ). Saline or substances effects were tested using a comparison of the maximum of variation observed during the baseline and after substance injection. For each neurons, we determined  $X_{bef}$  the maximum of fluctuation before the substance injection (during 5 min used as Baseline), and  $x_{aft}$  the maximum of fluctuation after injection (during 5 min after injection (Fig. Supp. 1C). If  $X_{aft}$  is a positive variation, we select the maximum of positive variation during the baseline for  $X_{bef}$ , and reciprocally if  $X_{aft}$  is a negative variation (Fig. Supp. 2A).

Effects of substances were tested using a Paired non-parametric Wilcoxon test to compare  $X_{bef}$  ( $Y_{bef}$ ) and  $X_{aft}$  ( $Y_{aft}$ ) for firing frequency and %SWB respectively. Differences were considered as significant if  $p < 0.05$ . Data are expressed as mean  $\pm$  standard deviation of the



mean (S.E.M). Total number (n) of observations in each group and statistic used are indicated in text. Mean firing rate and %SWB in different group were compared using Student test of the null hypothesis that the location parameters of the distribution are the same in each of the different groups. Different effects of ToE, SmE and nicotine in paired injections experiment are investigated with a comparison of the xaft induced by the two injections using Paired non-parametric Wilcoxon test.

Substance-evoked responses were classified as excitatory if the % of variation from baseline was greater than 10% for the firing frequency and 5% for %SWB. Responses were classified as inhibitory if the % variation from baseline was greater than -10% for the firing frequency and if it were greater than -5% for %SWB. If the % variation from baseline did not achieve these criteria, the neuron was classified as non-responding. The threshold values were chosen on the basis of the variation observed after saline injections in 30 neurons recorded in WT animals (Fig. Supp. 2B, C).

#### ***Fluorometric imaging plate reader (FLIPR) Membrane Potential analysis of HEK293- $\alpha$ 4 $\beta$ 2 cells line***

We used the FMR (Fluorometric imaging plate reader (FLIPR) Membrane Potential) technique (Molecular Device) to assess the ability of nicotine, smoke and tobacco extracts to activate mouse  $\alpha$ 4 $\beta$ 2 receptor expressed on Stable HEK293- $\alpha$ 4 $\beta$ 2 cell line (Karadsheh *et al.*, 2004). This technique detects by fluorescence the membrane depolarization that follows nAChR activation. A lipophilic and anionic colorant (bis-oxonol) is excited to 530nm and its distribution in the plasma membrane depends on the membrane potential. Thus the depolarization of the membrane induces a stream of colorant inside the cell that causes a rise in the observed fluorescence. Stable HEK293- $\alpha$ 4 $\beta$ 2 cells are distributed in a poly-D-Lysine coated 96 well cell culture plate to a concentration of approximately 20,000 cells per 100 $\mu$ l of medium in each well. 100 $\mu$ l of the colorant 'Red Reagent' (Molecular Devices) was added to each well, which were then incubated at 25°C for 30 minutes. The fluorescence (RFU) was read at 560nm 72h after cell division, using the Tecan infinite 2000 fluorometric imaging plate reader. Fluorescence is induced by a 530nm wavelength excitation light before and 45sec after an addition of 20 $\mu$ l of agonist solution at different concentrations of nicotine (0.1, 1, 5, 10, 50, 100  $\mu$ M in 1xPBS, pH7) with or without  $\alpha$ 4 $\beta$ 2 receptor antagonist (mecamylamine 100  $\mu$ M). Fluorescence measurement are made every second. Two sessions are made per experiment and the presented results are the mean between these two sessions

(n=2 for each agonist concentration). All agonist (six concentration with and without antagonist) were tested during each session. Fluorescence response to agonist is the difference between the mean fluorescence before (evaluated on the 10 second before agonist addition) and the maximum of fluorescence after agonist addition. Specific effect of drug are presented in RFU (see figure 5) as the difference between the fluorescence obtained with drug alone and with drug + mecamylamine. The Bmax and the EC50 are estimated for the three substances fitting the data with a Hill curve.

## ***Results***

### ***SmE, but not ToE, induces nicotine-like responses in WT mice***

We compared, in mice, the changes in VTA DA cell activity evoked by i.v. injections of nicotine alone (30 $\mu$ g/kg), and Smoke (SmE) and Tobacco extract (ToE) containing 30 $\mu$ g/kg of nicotine. Nicotine-evoked modifications of the DA cells firing pattern were described using the firing rate and the percentage of spikes within bursts (%SWB) (see Methods). In WT mice, systemic i.v. nicotine injection caused a large increase of DA cell firing rate (180.76 $\pm$ 26.182%;  $p=0.006$ ;  $n=16$ , Fig. 1A) and of %SWB (18.41 $\pm$ 4.507%;  $p=0.007$ ;  $n=16$ , Fig. 1B) which lasted approximately 600 seconds. Similarly, systemic i.v. injections of SmE elicited a large increase of DA cell firing rate (144.177 $\pm$ 13.022%;  $p=0.0007$ ;  $n=13$ , Fig. 1A) and %SWB (9.56 $\pm$ 4.033%;  $p=0.0225$ ;  $n=13$ , Fig. 1B). Despite an apparent stronger effect of nicotine there was no statistical difference, for the firing rate ( $p=0.16$ ) or for the %SWB ( $p=0.22$ ), between the modification of cell activity induced by nicotine alone or SmE. This was further confirmed by paired injections (see below). We then tested the effects of i.v. injections of ToE on DA cell activity and observed no significant increase in firing rate (111.0565 $\pm$ 15.3675%;  $p=0.468$ ;  $n=18$ , Fig. 1A) or burst response (2.412 $\pm$ 3.73%;  $p=0.589$ ;  $n=18$ , Fig. 1B). ToE elicited changes in firing rate and %SWB that was statistically lower than those produced by nicotine ( $p=0.036$  and  $p=0.014$ ). Overall, nicotine alone and SmE have similar effects on DA cell activity (Fig. 1C). In contrast, ToE fails to evoke a significant response, in spite of the fact that this extract contains the same dose of nicotine.

### ***A greater number of DA cells are inhibited by tobacco than by nicotine***

ToE had a weaker effect on DA neuron activity than nicotine alone, although the amount of nicotine administered with the two preparations was similar. This result does not however indicate that individual cells do not react to ToE injection. A plot of the individual response clearly shows that nicotine, ToE or SmE increase but could also decrease DA cell activity (Fig 2). It also reveals that the absence of statistically significant response to ToE could be explained by an increased number of cells with “inhibitory” response. Thus, we defined a profile of activation based on the increased (+) or the decreased (-) activity following injection. Cells showing a notable modification ( $\pm 10\%$  for the firing rate and  $\pm 5\%$  for %SWB) after injection were classified as either activated or inhibited. The remaining cells were classified as non-responding (N.R) (Supp. Fig1, and see Methods for threshold

definition). The relationship between the firing rate and %SWB alteration was not straightforward and in some cases these two parameters were modified in opposite ways by injection.

Comparison of the ratio of activated/inhibited cells (Fig. 2) demonstrated that nicotine alone modified activity in most of the cells ( $n=15/16$ , N.R=1 for firing rate and  $n=13/16$ , N.R=3 for %SWB) and inhibited a relatively low and constant percentage of cells (12%  $n=2/16$  for the firing rate and 6%,  $n=1/16$  for the SWB). Like nicotine, SmE induced an increase of DA cell firing rate in most of the cells (9/13) without any inhibition (0/13), but only 5 out of 13 showed a %SWB alteration (Fig. 2). A high proportion of no or low responding cell obtained with SmE compared to nicotine can explain the difference between these two substances (see Fig. 1). However, the consequence of these nicotine- and SmE-induced patterns of activation/inhibition is a net increase of both firing rate and %SWB. Similarly and despite the apparent absence of a response evoked by ToE (as shown in Fig. 1), this substance induced a notable modification of activity in most of the cells ( $n=17/18$ , N.R=1 for firing rate and  $n=12/18$ , N.R=6 for %SWB). In contrast to nicotine, ToE inhibited almost half of the responding cells (44%,  $n=8/18$  for the firing rate and 28%,  $n=5/18$  for the %SWB), resulting in an absence of mean response for both firing rate and %SWB (Fig. 2). However, ToE did increase the firing rate (9/19) and %SWB (7/19) of the other half of the cells as well as nicotine or SmE, suggesting that the nicotine contained in the extract still activated DA cells. Altogether, these results suggest that the weak effect of tobacco could be the consequence of an “increased” inhibition of cells by compounds other than nicotine that are contained in tobacco.

### ***Tobacco extract actively inhibits DA cell activity***

At a population level, ToE inhibited a larger number of cells when compared to nicotine. We also show that a pattern of excitation/inhibition coexists in response to ToE, but also to nicotine. DA cells responses are not homogeneous and in order to avoid a sampling effect and firmly conclude on a weaker excitatory effect of ToE, we compared both compounds using paired i.v. injections on the same cell (Figs. 3A, B). Mean and individual responses are plotted in Fig 3C. As observed in a first series of experiments, nicotine, but not ToE, increased both the firing rate and %SWB. Moreover, individual responses also reproduced the previously observed patterns of activation/inhibition induced by ToE and nicotine. ToE injections mainly inhibited cells (8/12 and 6/12, respectively, for the firing rate and %SWB),

whereas nicotine mostly activated cells (10/12 and 5/12). Overall ToE and nicotine have opposite effects on DA cell activity ( $p=0.0004$  and  $0.02$  for firing rate and %SWB;  $n=12$ , Fig 3C): half of the cells are respectively inhibited in frequency by ToE while activated by nicotine (6/12, see neuron1, Fig 3A). For the other cells, ToE seems to have a lower excitatory (3/4, see neuron2, Fig 3B) and a greater inhibitory (2/2, not shown) effect than nicotine on the firing rate. Finally, the effect on %SWB is weak with ToE and increases with nicotine (Fig 3C).

The same experiments (Figs. 4A, B) demonstrated that SmE and nicotine have a similar excitatory effect on DA cell firing rate ( $132.1\pm 20.48\%$  vs  $135.674\pm 18.8\%$  respectively for nicotine and SmE;  $p=0.51$ ,  $n=12$ ; Fig 4C) and %SWB ( $11.28\pm 5.56\%$  vs  $7.85\pm 3.71\%$ ;  $p=0.56$ ,  $n=12$ ) and a similar pattern of excitation/inhibition (excitation: 9/12 and 9/12; inhibition: 3/12 and 3/12 respectively for the firing rate of nicotine and SmE).

These results suggest that some compounds in ToE, but not in SmE, can alter the nicotinic modulation of the VTA DA system, resulting in a larger inhibition and a reduced excitation. In order to determine if these compounds directly interact with VTA DA neurons through the nicotinic receptor, or if they activate a non-nicotinic modulation pathway, we compared in WT and in  $\beta 2^{-/-}$  mice the firing response of DA cells induced by i.v. injections of nicotine alone, ToE and SmE.

### ***ToE and SmE do not induce nicotine-like responses in $\beta 2^{-/-}$ mice***

Previous experiments have shown that nicotine-elicited responses of DA cells are absent in  $\beta 2^{-/-}$  mice (Maskos et al, 2005; Mameli-Engvall et al, 2006). To test whether compounds in ToE and SmE can modulate DA cells despite the absence of  $\beta 2^*$ -nAChRs, we carried out single and paired injections of nicotine, SmE and ToE in  $\beta 2^{-/-}$  mice (Fig 5). Nicotine, SmE and ToE had no effect on the firing rate (nic:  $101.744\pm 8.35$ ;  $p=0.43$ ;  $n=17$ ; SmE:  $102.341\pm 10.74$ ;  $p=0.375$ ;  $n=10$ ; ToE:  $98.62\pm 8.62$ ;  $p=0.19$ ;  $n=14$ ) or on the %SWB (nic:  $1.006\pm 2.28$ ;  $p=0.23$ ;  $n=17$ ; SmE:  $-0.105\pm 2.60$ ;  $p=0.8$ ,  $n=10$ ; ToE:  $-0.234\pm 3.19$ ;  $p=0.6$ ;  $n=14$ ) of DA cells in  $\beta 2^{-/-}$  mice (Fig. 5A). These results are strikingly different from those obtained in WT (Fig. 5B). Paired injections confirmed that there is no difference between the effect induced by nicotine and ToE ( $p=0.84$  and  $p=0.81$  for firing rate and %SWB;  $n=12$ ) or SmE ( $p=0.95$  and  $p=0.1$  for firing rate and %SWB;  $n=8$ ) in  $\beta 2^{-/-}$  mice (Fig. 5C). Altogether, it can be suggested that the increase in firing rate and in %SWB induced by SmE as well as the ToE

evoked inhibition are mediated through a direct action on  $\beta_2^*$ -nAChRs (but see Discussion).

### ***ToE inhibitory effect involves $\alpha_4\beta_2$ nAChRs***

ToE as well as nicotine mediated their effect through  $\beta_2^*$ -nAChRs. We then tried to identify whether  $\alpha_4\beta_2$  or  $\alpha_6\beta_2$  nAChRs are involved in the inhibitory effect of ToE by pairing nicotine and ToE injections on DA cell activity in both  $\alpha_4^{-/-}$  and  $\alpha_6^{-/-}$  mice. Previous experiments have shown that nicotine-elicited responses of DA cells in  $\alpha_6^{-/-}$  mice are similar to those observed in WT, but that burst evoked increased response disappeared in  $\alpha_4^{-/-}$  mice (Exley et al, 2011). In  $\alpha_6^{-/-}$ , nicotine, but not ToE, induced a significant increase of both the firing rate (nic:  $148.866 \pm 16.058$ ;  $p=0.0008$ ;  $n=19$  and ToE:  $98.045 \pm 11.26$ ;  $p=0.83$ ;  $n=19$ ; Fig. 6A) and %SWB (nic:  $6.65 \pm 2.08$ ;  $p=0.01$ ;  $n=19$  and ToE:  $-2.38 \pm 2.8$ ;  $p=0.48$ ;  $n=19$ ) of DA cells. As in the WT, i) ToE injections poorly activated cells in  $\alpha_6^{-/-}$  mice (6/19 and 3/19 respectively for the firing rate and %SWB; Fig 6A) compared to nicotine (14/19 and 9/19); ii) ToE inhibits more cells (7/19 and 5/19 for ToE versus 2/19 and 0/19 for nicotine; Fig. 6A); and iii) ToE and nicotine had opposite effects on DA cell firing ( $p=0.00002$  and  $0.004$  for firing rate and %SWB;  $n=19$ ; Fig. 6A).

In  $\alpha_4^{-/-}$  mice, nicotine and ToE increased DA cells firing rate (nic:  $125.667 \pm 9.7$ ;  $p=0.003$ ;  $n=19$  and ToE:  $125.4 \pm 10.225$ ;  $p=0.0045$ ;  $n=19$ ; Fig. 6B) but none of them induced any significant increase of %SWB (nic:  $0.6 \pm 1.15$ ;  $p=0.615$ ;  $n=19$  and ToE:  $3.525 \pm 1.28$ ;  $p=0.116$ ;  $n=19$ ; Fig. 6B) and only few cells exhibit an increase of %SWB after nicotine (3/19) or ToE (5/19) injection. In contrast to WT and  $\alpha_6^{-/-}$  mice, DA cells in  $\alpha_4^{-/-}$  mice were poorly inhibited by ToE (2/19 and 0/19, respectively for the firing rate and %SWB; Fig. 6B) as well as by nicotine (1/19 and 3/19). Cells are mainly excited by the two substances (14/19 and 12/19, for ToE and nicotine firing rate respectively). As a consequence, ToE and nicotine have similar effect on DA cells firing rate (nic Vs ToE:  $p=0.74$ ,  $n=19$ ) and %SWB ( $p=0.2$ ,  $n=19$ ) in  $\alpha_4^{-/-}$  mice. This result suggests that the  $\beta_2^*$ -nAChRs-dependent inhibitory effect of ToE upon DA cell firing is mediated through  $\alpha_4\beta_2$ -nAChRs rather than  $\alpha_6\beta_2$ -nAChRs.

### ***Agonistic effect of nicotine for $\alpha_4\beta_2$ nAChRs is not modified in ToE and SmE***

$\alpha_4\beta_2$  nAChRs seem to be the key target explaining the characteristic response of ToE. We then tested whether the reduction of the nicotine effect observed for tobacco extract is due to other compounds inhibiting nAChRs. We used HEK cells stably expressing the main high affinity nAChRs of both DAergic and GABAergic cells in the VTA, mouse  $\alpha_4\beta_2$  nAChRs

(Karadsheh et al, 2004), and followed agonist-elicited receptor activation using fluorescence membrane potential indicators. We observed only slight differences on the  $\alpha 4\beta 2$  nAChR activation induced by nicotine, SmE and ToE in the two experiments (nic: Bmax=700, EC50=5; SmE: Bmax=700, EC50=5; ToE Bmax=700, EC50=2 Fig 7A and nic: Bmax=1000, EC50=2; SmE: Bmax=1050, EC50=1; ToE Bmax=1000, EC50=3 Fig 7B)

We conclude that nicotine elicited identical nAChR activation when applied alone, or as smoke or tobacco extracts, and that the altered effect of ToE is not due to a modification of the affinity for the  $\alpha 4\beta 2$  nAChR of nicotine contained in the extract.

## ***Discussion***

Nicotine is generally acknowledged to be the primary addictive component of tobacco (Wonnacott *et al.*, 2005). It is established that nicotine increases DA release in the striatum and NAc (Di Chiara, 1998) by acting through nAChRs at the level of the VTA and by increasing DA cell bursting activity. This increase leads to a phasic release of DA in the NAc that is believed to be linked to the addictive properties of nicotine. However, despite the clear addictive properties of nicotine, the role played by compounds other than nicotine that are present in tobacco and may participate in tobacco addiction is not clear. The present study demonstrates that nicotine and SmE have similar activating effects on VTA DA neurons, which is not the case for ToE. Indeed, while nicotine and SmE strongly activated these cells and promoted burst activity, ToE only produced weak firing and burst-evoked responses. This difference is mainly caused by an inhibitory effect of ToE on DA cell. The present study also showed that the effects of ToE and SmE depend completely on the expression of  $\beta 2^*$ -nAChRs as demonstrated by experiments with KO mutant mice.

### ***Inhibitory effects of ToE***

A high level of inhibited cells and an attenuated excitation are characteristic of ToE's effects on DA cells. Indeed, single injection experiment show that excitation of DA cells induced by ToE is weaker than nicotine, as it does not increase firing rate or the burst activity in WT. Moreover, ToE increases the proportion of inhibited cells compared to nicotine. Paired injections of ToE and nicotine in WT and  $\alpha 6^{-/-}$  mice confirm a smaller excitatory and a greater inhibitory effect of ToE over nicotine alone, suggesting that compounds in tobacco are able to inhibit or reduce the effect of nicotine exposure on DA cell firing. Overall, three hypotheses can be proposed to explain how tobacco components could alter nicotine action: It may first be argued that the diffusion of nicotine in the brain was slower when released from the extracts than when injected alone. It is unlikely that the results of the present study can be explained solely by such a mechanism as nicotine is known to be highly diffusible in organic matter (Wolff *et al.*, 1949) and the responses of neurons, whether activated or inhibited, occurred with a similar latency, almost immediately after nicotine or extract injections. Moreover, the activation of neurons by nicotine, SmE and ToE had a similar shape for equivalent doses. Therefore, the observed differences between the compounds seem more likely to be related to intrinsic differences between the effects of nicotine, ToE and SmE. Two hypotheses therefore remain: First, a compound could directly act on nAChRs to reduce the



excitatory effect of nicotine, acting either directly on nAChRs situated on VTA DA neurons or through the nAChR network. And secondly, the compound could act on non-nicotinic receptors, possibly situated on GABAergic inhibitory neurons, indirectly reducing nicotine-elicited excitatory responses.

### ***Action of ToE on nAChRs***

The results of the present study show that the effects of nicotine, ToE and SmE are absent in  $\beta 2$  KO mice. These results confirm previous findings demonstrating that the  $\beta 2$  subunit of the nAChR is essential for the behavioral effects of nicotine on VTA DA neurons (Mameli-Engvall *et al.*, 2006). These results also extend previous electrophysiological studies showing that the effects of ToE, SmE and nicotine on serotonergic neurons are different in shape, but similarly blocked by mecamylamine, suggesting that functionally active compounds other than nicotine are present in ToE and SmE and act through nicotinic receptors (Touiki *et al.*, 2007). In the present study, paired injection of nicotine and ToE carried out in  $\alpha 4^{-/-}$  and  $\alpha 6^{-/-}$  mice demonstrate that  $\alpha 4\beta 2$  nAChRs are crucial for the action on DA cells of compounds other than nicotine present in ToE. The nature of the functionally active compounds other than nicotine are not known, but some compounds are potential candidates, such as the minor alkaloids nornicotine, anabasine and cotinine, and MAOIs like harmaline and norharmaline, which are tobacco or smoke compounds other than nicotine known to be active on neurons.

The minor alkaloids nornicotine and anabaseine likely act as agonists on nAChRs (Dwoskin *et al.*, 1995). Nornicotine, anabasine and cotinine activate DA neurons (Arib *et al.*, 2010; O'Leary *et al.*, 2008; Reavill *et al.*, 1988) and many minor alkaloids have been shown to enhance nicotine self-administration (Clemens *et al.*, 2009). However, our HEK experiments showed that the  $\alpha 4\beta 2$  nAChR activation by nicotine is not modified by tobacco or smoke compounds, suggesting that these compounds do not directly act as antagonist or agonist at the receptor level, nor interact with nicotine. In addition, being like nicotine agonists at nAChRs, they should activate VTA DA neurons, so that their presence in ToE does not explain the inhibitory effects of ToE on the firing of neurons. Therefore, it is unlikely that the presence in tobacco of minor alkaloids known to act as agonists on nAChRs explains the inhibitory effects of ToE on DA neurons observed in our experiments, unless they specifically target nAChR located on GABA cells (but see below network effect).

The beta-carbolines harmaline and norharmane, which possess MAOI properties, have been shown to dramatically alter VTA DA neuron activity (Arib *et al.*, 2010) and to dose-dependently increase or decrease DA release in the NAc (Baum *et al.*, 1996; Baum *et al.*, 1995). As far as we know, the effects of harmaline and norharmane on nAChRs have never been investigated. However, the effect of harmaline on VTA DA neurons is strongly inhibited by mecamylamine, suggesting that harmaline action on DA cell firing is mediated by a direct or indirect action on nicotinic receptors (Arib *et al.*, 2010). But, similarly to nicotine, harmaline and norharmane appear to predominantly activate VTA DA neurons, although the effect may be dose-dependent, so that their presence in ToE unlikely explains the inhibitory effects of ToE on the firing of DA neurons. In addition, beta-carbolines are mostly products of tobacco combustion, in other words, mostly present in smoke (Poindexter *et al.*, 1962), making it difficult to attribute to harmaline the inhibitory effects of ToE on VTA DA neurons.

### ***Network effect***

Despite a large set of data on the effects of nicotine on VTA DA neurons at the molecular, cellular and circuit levels, the precise mechanisms of how the DA response to nicotine is constructed in the VTA remain controversial. This is mainly due to the fact that DA neuron regulation is subject to many different influences, systems and circuits. It is generally believed that when nicotine arrives in the VTA, it simultaneously stimulates glutamatergic terminals through activation of  $\alpha 7^*$ -nAChRs and activates postsynaptic nAChRs on the DA cell soma (McGehee *et al.*, 1995; Schilstrom *et al.*, 1998). In addition, activation of nAChRs expressed on GABAergic cells will initially increase inhibitory inputs onto DA cells (Mansvelder *et al.*, 2002; Yin *et al.*, 2000). However, the main receptors on GABAergic cells are  $\alpha 4\beta 2^*$  subtypes that are supposed to rapidly desensitize compared to  $\alpha 7^*$ -nAChRs. The DA cells are thereby “released” from GABAergic control (Mansvelder *et al.*, 2002). Mechanisms explaining burst formation are less clear. *In vivo*, somato-dendritic application of NMDA antagonist or NMDA reciprocally inhibits or induces burst firing of DA cell in rats (Charley *et al.*, 1991; Chergui *et al.*, 1993). *In vitro*, the stimulation of NMDA receptors on DA neurons produces a stereotyped form of a calcium-independent rhythmic burst firing (Johnson *et al.*, 1992). Taken together, these results point to the glutamatergic afferents as a key element involved in burst generation. Alternatively, it has been proposed that disinhibition of GABAergic inputs causes the bursting behavior and that cholinergic input can also participate in burst generation (Maskos *et al.*, 2005; O'Brien *et al.*, 1987; Tepper *et al.*, 2007). In a more specific way, bursting behavior of DA cells induced by nicotine does not

seem to involve a presynaptic action of nicotine on glutamatergic afferents via  $\alpha 7^*$ -nAChRs (Mameli-Engvall *et al.*, 2006), but a somatic action on GABA and/or DA cells via  $\beta 2^*$ -nAChRs (Mameli-Engvall *et al.*, 2006; Maskos *et al.*, 2005). Mechanisms likely to influence bursting are therefore numerous but seem to involve a cooperative action of nicotine on nAChRs of both DA and GABA cells. One explanation for the large proportion of DA neurons that are inhibited on administration of ToE and the reduced increase in firing and bursting activity, in comparison to the effects of nicotine administration, could be that certain constituents of ToE act to impair the action of nicotine on both DA and GABAergic neurons. These two kinds of cells have a different stoichiometry of nAChRs. Thus, while DA cells express numerous nAChR subtypes, GABAergic cells mainly express  $\alpha 4\beta 2^*$ -nAChRs. As demonstrated in the  $\alpha 6^{-/-}$  and  $\alpha 4^{-/-}$  mice, the absence of the  $\alpha 6$  subunit does not modify the nicotine and ToE action on DA cell firing whereas the lack of the  $\alpha 4$  subunit suppresses the differences observed between these two substances. Furthermore, HEK experiments indicate that ToE compounds do not alter nicotine's actions on  $\alpha 4\beta 2$ -nAChRs. Assuming that  $\alpha 4\beta 2$ -nAChRs, but not  $\alpha 6\beta 2$ , are expressed on GABA cells, these results could suggest that functional nAChRs on GABA cells are necessary to mediate the ToE inhibitory effect on DA cells. This is compatible with the prominent inhibition of neuronal activity observed with this extract. ToE's effects on DA cells could thus result from an inhibitory influence induced by nicotine's actions on GABAergic cells and a weaker excitatory action of nicotine on DA cell nAChRs. Nicotine and ToE actions on GABA cells need to be investigated further to understand how ToE compounds could modulate the GABAergic afferences to DA cells.

### ***Relation to reinforcing effects***

VTA DA neurons are involved in many functions and phenomena, including tobacco addiction. Tobacco addiction is often called nicotine addiction, and the majority of the literature dealing with tobacco addiction postulates that nicotine is the principal, if not the only, addictive compound in tobacco and smoke. However, administration of tobacco or smoke to rodents is likely to mimic the human smoking situation more accurately than the administration of nicotine alone. The fact that in the present study the effects of ToE on DA neurons differed from those of nicotine alone and SmE may therefore have important implications. In particular, current hypotheses dealing with tobacco addiction state that the addictive properties of tobacco are primarily related to the ability of nicotine to induce burst firing of DA neurons (Balfour, 2002; Di Chiara, 2000). In the present study, nicotine alone and SmE induced consistent burst firing, whereas ToE did not modify the burst firing mode at

the population level. This decrease in the effectiveness of nicotine contained in ToE suggests that some compounds in tobacco reduce the burst-promoting effects of nicotine on DA cells and largely act to inhibit VTA DA cell activity. These results were unexpected and they suggest that tobacco has lower addiction generating properties compared to either nicotine alone or smoke. However, this appears to be in contradiction with the large body of evidence that suggests that smokeless tobacco use in humans can lead to addiction (Hatsukami *et al.*, 1999) and reports that show that minor alkaloids and other compounds with MAOI properties enhance nicotine self-administration in rats (Clemens *et al.*, 2009; Guillem *et al.*, 2005). It nevertheless confirms that nicotine is the main substance involved in the tobacco addiction-related activation of mesolimbic DA neurons.

In summary, nicotine activated almost all the VTA DA cells tested while ToE inhibited a fairly high number of cells and triggered very few bursts. These differences are difficult to explain given that the amount of nicotine injected with ToE was equivalent to the amount of nicotine injected alone and cannot be attributed to alterations in the diffusion of nicotine from ToE. Nevertheless, the prominent abolition of the nicotine-induced increase of VTA DA neuron firing and burst activity upon tobacco administration raises the possibility of the existence of components of tobacco which can attenuate the stimulatory effects of nicotine on these neurons. Given that the effects of smoke did not differ from those of nicotine, these hypothetical compounds would either be destroyed during tobacco combustion, or behave differently after combustion. We have seen that these compounds are probably not the minor alkaloids nornicotine, anabasine and cotinine, nor the beta-carbolines harmane and norharmane, which have all been shown to activate VTA DA neurons (Arib *et al.*, 2010; Dwoskin *et al.*, 1995; O'Leary *et al.*, 2008). The case of harmane is however particular. Harmane seems to activate DA neurons at high doses, and inhibits them at lower doses, following a U-shaped dose-response relationship (Baum *et al.*, 1996; Baum *et al.*, 1995). Harmane is in large part synthesized during tobacco combustion (Poindexter *et al.*, 1962). Therefore, there are lower concentrations of harmane in tobacco than in smoke. A working hypothesis could therefore be that the low concentration of harmane in tobacco would be able to inhibit DA cells, while the high concentration of harmane in smoke would activate them. Further work is necessary to determine whether there are dose-dependent interactions between harmane and nicotine which could be of heuristic value in the context of smoking.

### ***Disclosure/Conflict of Interest***

The authors declare no conflict of interest.

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**Fig. 1 Firing pattern modification of VTA DAergic cells induced by Nicotine, SmE and ToE extract in WT mice.** (A) (Left) Variation of the firing rate of DA cells expressed in percentage from baseline induced by nicotine (black line, n=16), SmE (red line, n=13) and ToE (blue line, n=18), dashed lines indicated the standard error of the mean. All experiments are realized with a 30 $\mu$ g/Kg dose of nicotine. The vertical dashed line indicated the time of injection. (Right) Barplot of means of maximum of firing rate variation induced by nicotine, SmE and ToE Red line indicated the Baseline level. (B) Same representation for the variation from baseline of percentage of spikes within burst. (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001). (C) (Left) Electrophysiological recording of the firing pattern modification induced by nicotine (upper panel). Electrophysiological recording of the same neuron firing pattern during baseline (middle panel) and after nicotine injection (lower panel). (Right) Same for another neuron receiving a injection of SmE.

**Fig. 2 Individual responses analysis.** Individual responses to nicotine (n=16, black), SmE (n=13, red) and ToE (n=18, blue) injections. Each point indicates the maximum variation of firing rate (upper panel) and %SWB (lower panel) from the baseline after injection (xaft, see method). The full dot indicates the mean response. Cells are classified as excited (Excit.), inhibited (Inhib.) and not responding (N.R) for both firing rate and %SWB. The dashed lines indicate the baseline level (100% and 0%) and the range of variation used for the cell classification (110/90 % and -5/5%).

**Fig. 3 Paired injection of ToE and nicotine.** (A) Illustration of paired effect of ToE and nicotine. Evolution of the firing frequency of the neuron 1 in response to ToE and nicotine injection (upper panel, black mark indicate the injection time). Electrophysiological recording of neuron 1 firing pattern during baseline, just after ToE and nicotine injection (lower panel). (B) Same with neuron 2 where ToE induced a weak excitation when compare to nicotine. (C) Maximum of response (xaft) for each cell (n=12) to ToE/nicotine and subsequent nicotine/ToE injection for both the firing rate (left) and the %SWB (right). The full dot indicated the mean response. Depending of their response cells are classified as excited (Excit.), inhibited (Inhibit.) and not responding (N.R) for both the firing rate and the %SWB. The dashed lines indicate the Baseline level (100% and 0%) and the range of variation used for the cell classification (110/90 % and -5/5%). (\*\*\*P<0.001, \*\*P<0.01).

**Fig. 4 Paired injection of nicotine and SmE.** (A) Illustration of paired effect of ToE and nicotine. Evolution of the firing frequency of the neuron 1 in response to ToE and nicotine injection (upper panel, black mark indicate the injection time). Electrophysiological recording of neuron 1 firing pattern during baseline, just after SmE and nicotine injection (lower panel). (B) Same with neuron 2 where ToE induced a weak excitation when compare to nicotine. (C) Maximum of response (xaft) for each cell (n=11) to nicotine/SmE and subsequent SmE/nicotine injection for both the firing rate (left) and the %SWB (right). The full dot indicated the mean response. Depending of their response cells are classified as excited (Excit.), inhibited (Inhibit.) and not responding (N.R) for both the firing rate and the %SWB. The dashed lines indicate the Baseline level (100% and 0%) and the range of variation used for the cell classification (110/90 % and -5/5%). (\*\*P<0.01, \*\*\*P<0.001).

**Fig. 5 Firing pattern modification of VTA DA cells induced by Nicotine, Tobacco and Smoke extract in  $\beta 2$  KO mice.** (A) (Left) Variation of the firing rate of DA cell expressed in percentage from baseline induced by nicotine (Black line, n=17), SmE (Red line, n=10) and ToE (Blue line, n=14) in the  $\beta 2$  KO mice, dashed lines indicates the standart error of the mean. The vertical dashed line indicated the time of the injection. (Right) Same presentation for the Variation of %SWB from baseline (B) Barplot of means of maximum of firing rate variation (left) and %SWB (Right) induced by nicotine, SmE and ToE in WT and  $\beta 2$  KO mice. (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001). The red line indicates the Baseline level. (C) Paired injection of nicotine, SmE and ToE. Maximum of response (xaft) for each cell to nicotine and subsequent SmE (n=8) or/and ToE (n=12) injection for both the firing rate (left) and the %SWB (right). The full dot indicates the mean response. The dashed lines indicate the Baseline level (100% and 0%) and the range of variation used for the cell classification (110/90 % and -5/5%). (\*\*P<0.01, \*\*\*P<0.001).

**Fig. 6 Paired injection of nicotine and ToE in  $\alpha 6$ -/- and  $\alpha 4$ -/- mices.** (A) Maximum of response (xaft) for each cell (n=19) to nicotine/ToE and subsequent ToE/nicotine injection for both the firing rate (left) and the %SWB (right) in  $\alpha 6$ -/- mices. The full blue dot indicated the mean response. Depending of their response cells are classified as excited (Excit.), inhibited

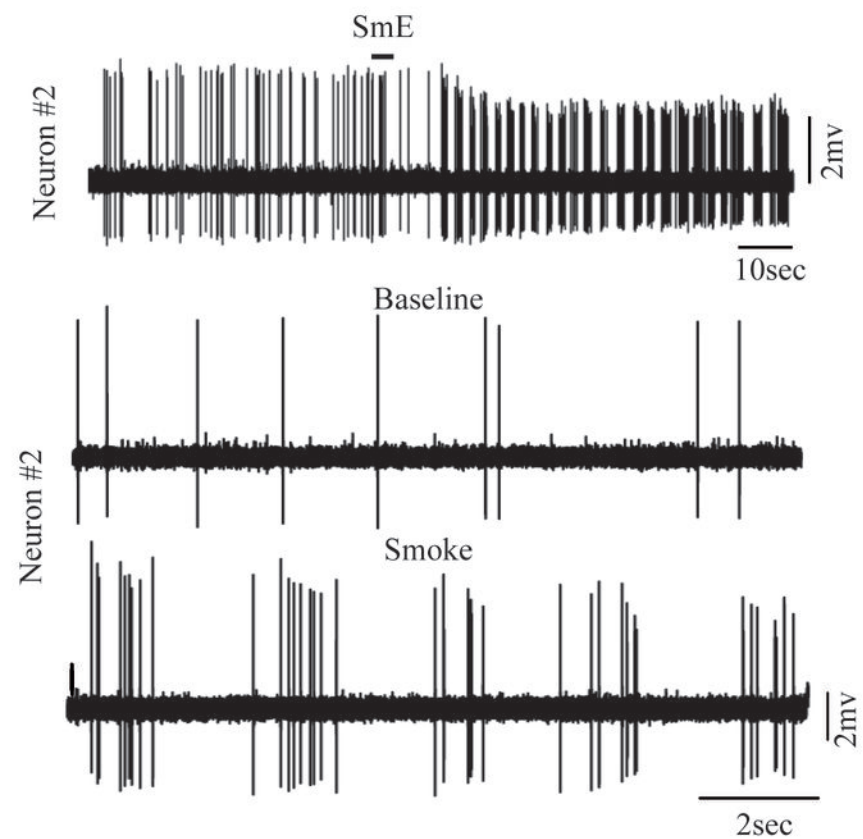
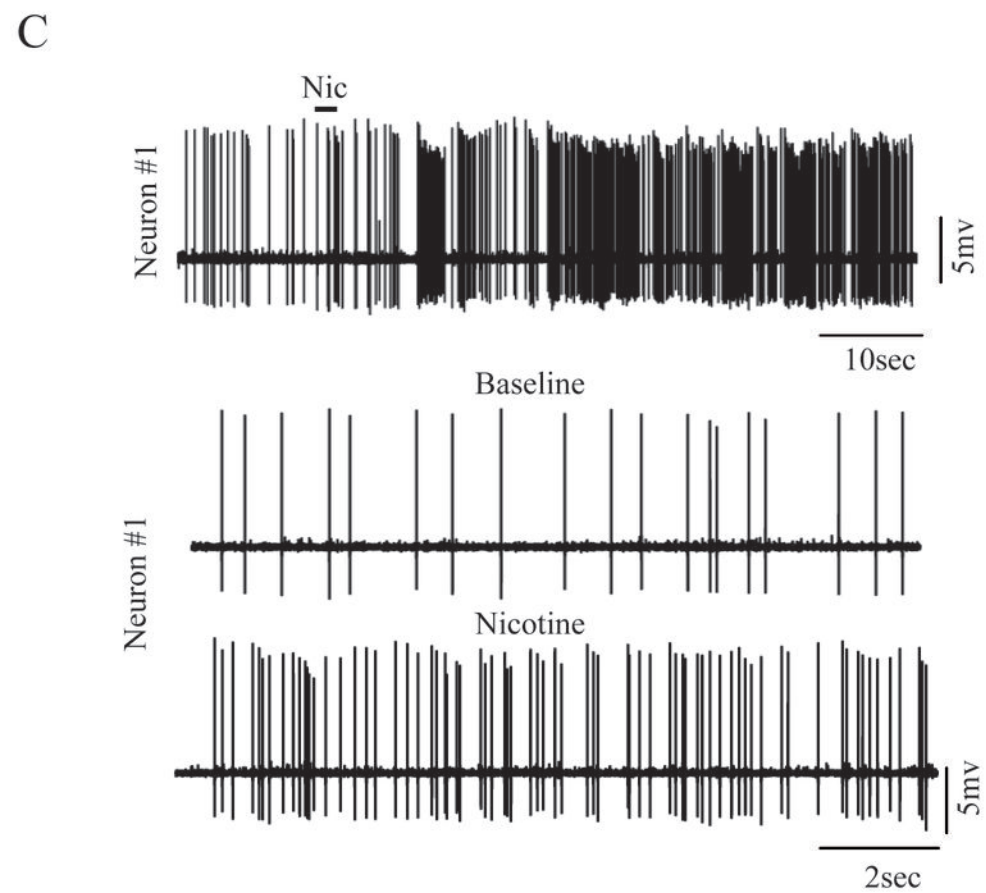
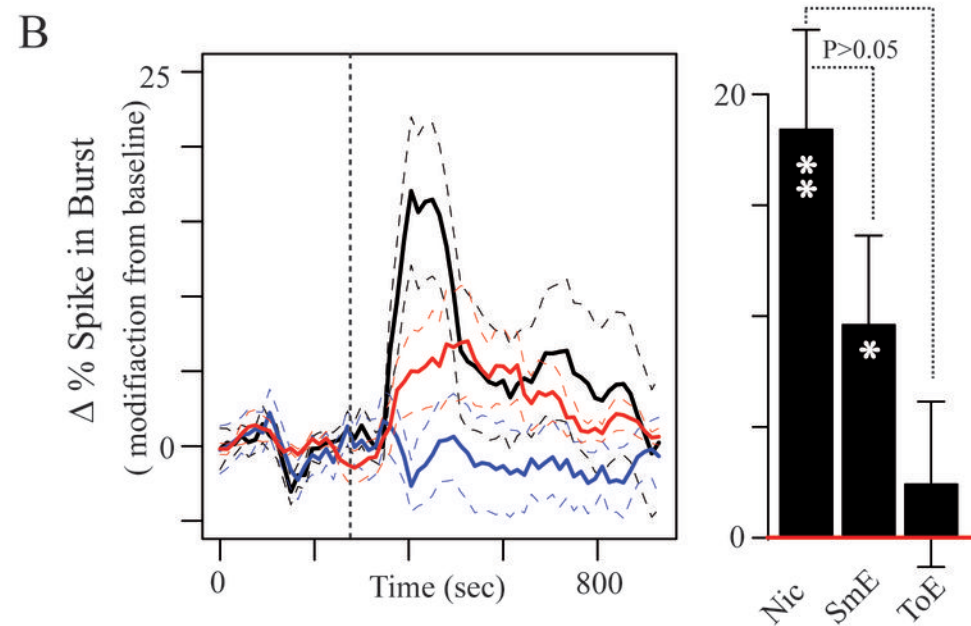
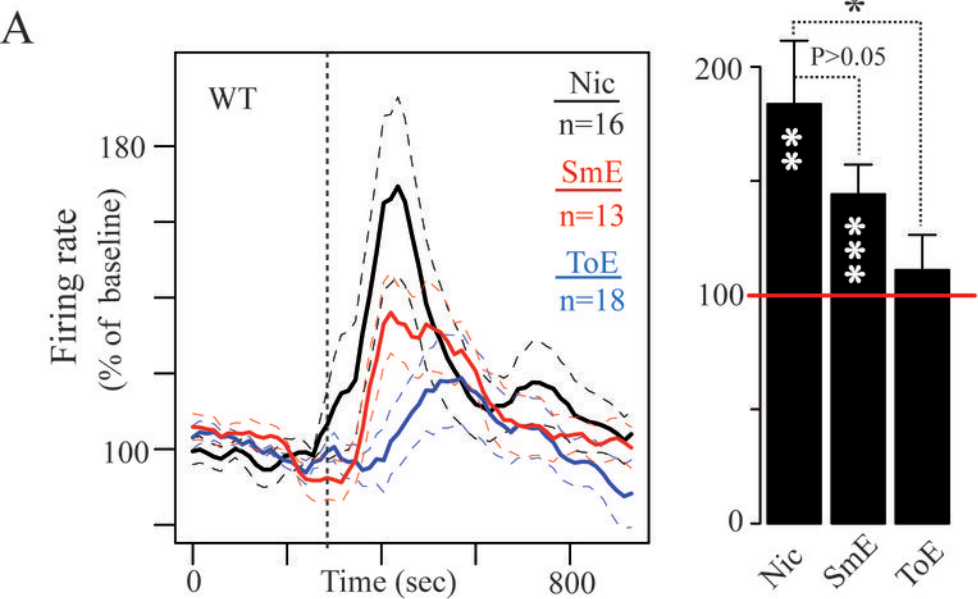
(Inhibit.) and not responding (N.R) for both the firing rate and the %SWB. The dashed lines indicate the Baseline level (100% and 0%) and the range of variation used for the cell classification (110/90 % and -5/5%). (\*\*\*P<0.001,\*\*P<0.01). (B) Same representation for the experiments made in  $\alpha 4$ -/- mice.

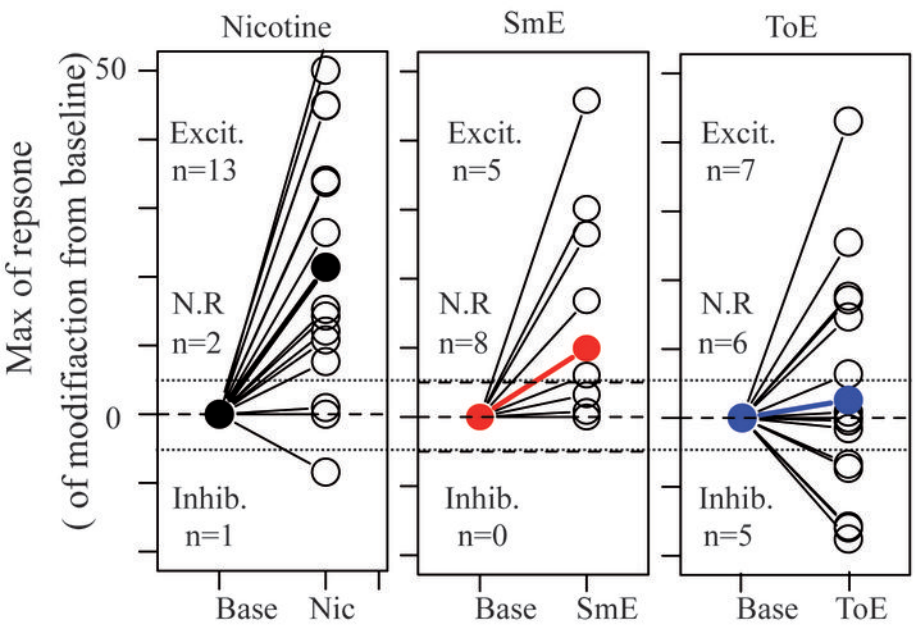
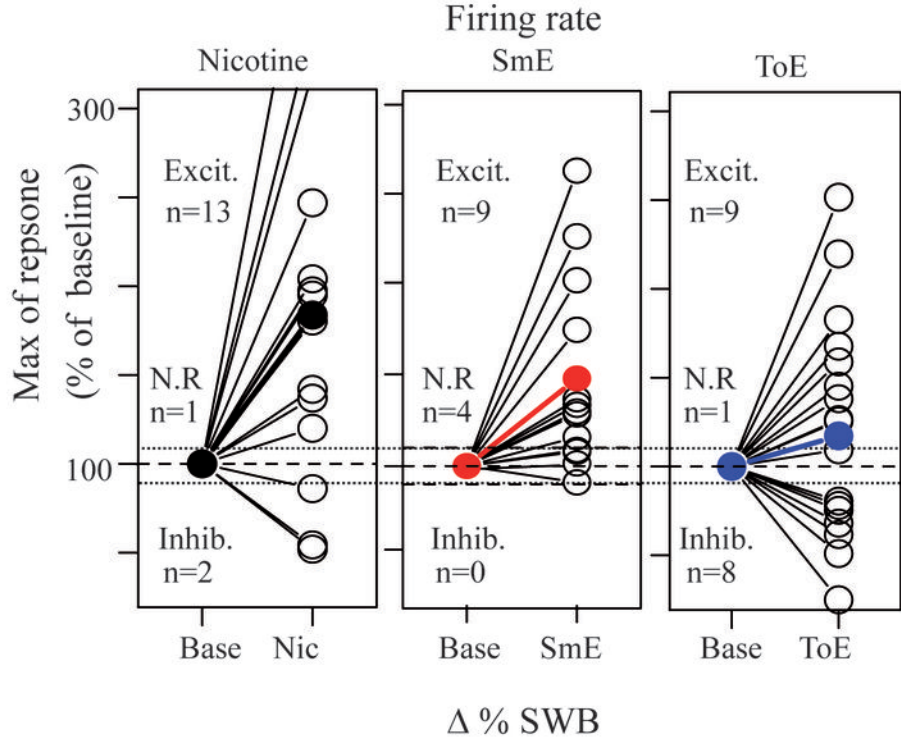
**Fig. 7 Pharmacological study of nicotine contained in Tobacco and smoke extract on HEK293- $\alpha 4\beta 2$  cells.** (A) Nicotine-mediated response of HEK293- $\alpha 4\beta 2$  cells was monitored with ‘Red Reagent’ (Molecular Device) on Fluorometric imaging plate reader (FLIPR). The response in RFU follows the membrane depolarization. The dose response curve of HEK293- $\alpha 4\beta 2$  cells activation is obtained for six doses (0.1, 1, 5, 10, 50, 100  $\mu$ M) of nicotine contained in nicotine (Black points, n=2 per points), ToE (blue points, n=2 per points) and SmE (Red points, n=2 per points) solution. Black, red and blue curve fit the nicotine affinity for the nicotine alone solution, the SmE and the ToE respectively. (B) Same for another experiment.

**Fig. Supp. 1 Analysis procedure.** (A) DA cell identification. Average typical (n=100) extracellular waveform of a DA cell action potential. Red dots indicate the start and negative phase of the action potential. Dashed line indicates the limit of 1.1 msec duration from the start to negative phase use to discriminate DA from Non-DA cells. (B) Estimation of firing frequency. Electrophysiological recording of a DA cell during nicotine injection. Black and blue dashed squares represent three 60 second windows with a 15 second lag used to calculate the firing frequency evolution. Red dashed line indicates the nicotine injection. Same procedure is use for the %SWB estimation. (C) Drug effect quantification: Firing frequency value is indicated before (x; Hz and after rescaling X, % of baseline, see Methods Data analysis). Horizontal dashed line shows the baseline level (xb). Red dashed line indicates the nicotine injection. Blue dot shows the maximum of variation from the baseline before injection (Xbef) and red one the maximum of variation from the baseline after injection (Xaft). Effects of injections are tested by comparing this two values (see method).

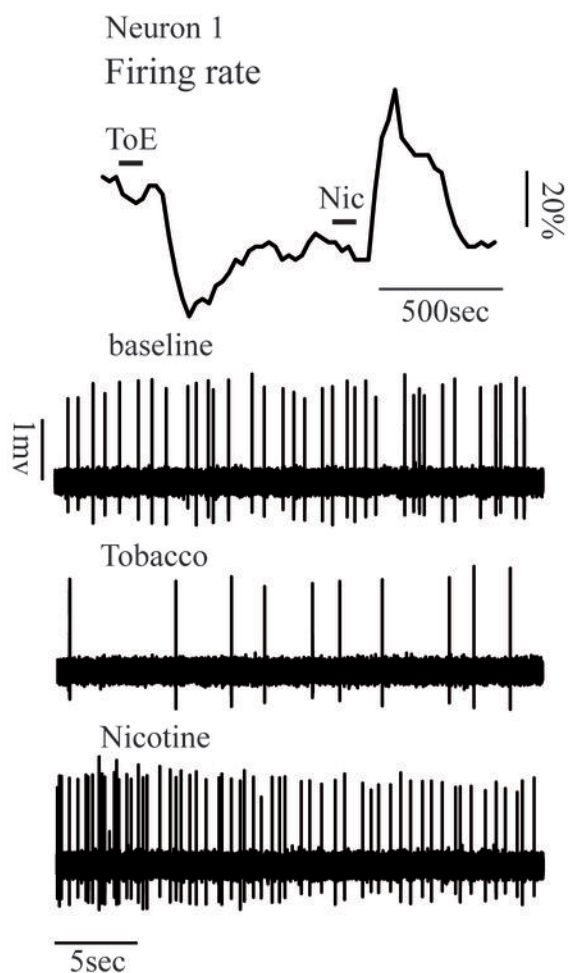
**Fig. Supp. 2 Drug response classification.** (A) Principle of individual response quantification. The two panels illustrate the firing rate (left) and percentage of spike within

burst (right) variation after nicotine injection in two neurons. Neuron#1 shows a negative maximum of variation ( $X_{aft}$ ) in response to nicotine, neuron#2 a positive maximum of variation. Horizontal dashed line shows the baseline level ( $x_b$ ), blue point shows the maximum of variation from the baseline before injection ( $X_{bef}$ ) and red point show the maximum variation from baseline after injection ( $X_{aft}$ ). (B) Saline injection. Variation of the firing rate ( $99.75 \pm 1.05$ ,  $p=0.41$ ;  $n=30$ ; left) and %SWB ( $-0.23 \pm 0.52$ ,  $p=0.47$ ;  $n=30$ ; right) induced by saline injection of 30 neurons used to evaluate the inhibition/excitation threshold in WT mice, dashed lines indicate the standard error of the mean. (C) Inhibition/Excitation threshold. Individual maximum variation ( $X_{aft}$ ) of firing rate (left) and %SWB (right) evoked by saline injection of 30 neurons. Red dashed line indicates mean of positive maximal variation  $+ 2*sd$  ( $102.1+6.61$  and  $0.955+4.28$  respectively for the firing rate and %SWB) and the mean of negative maximal variation  $- 2*sd$  ( $97.42-7.07$  and  $0.115-3.79$ ). Black dashed line shows the Inhibition/Excitation threshold for the Firing rate ( $90/+110\%$ , left) and %SWB ( $-5/+5\%$ , right) used in this study.

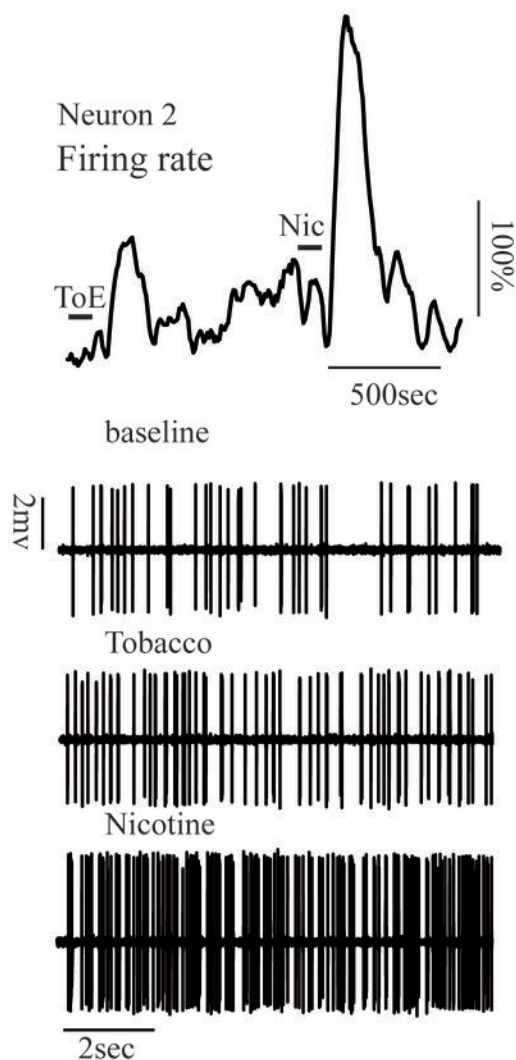




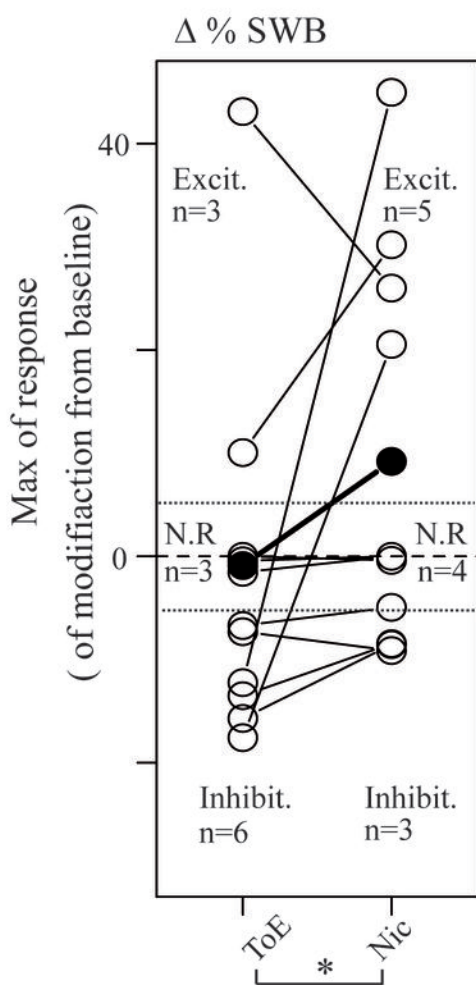
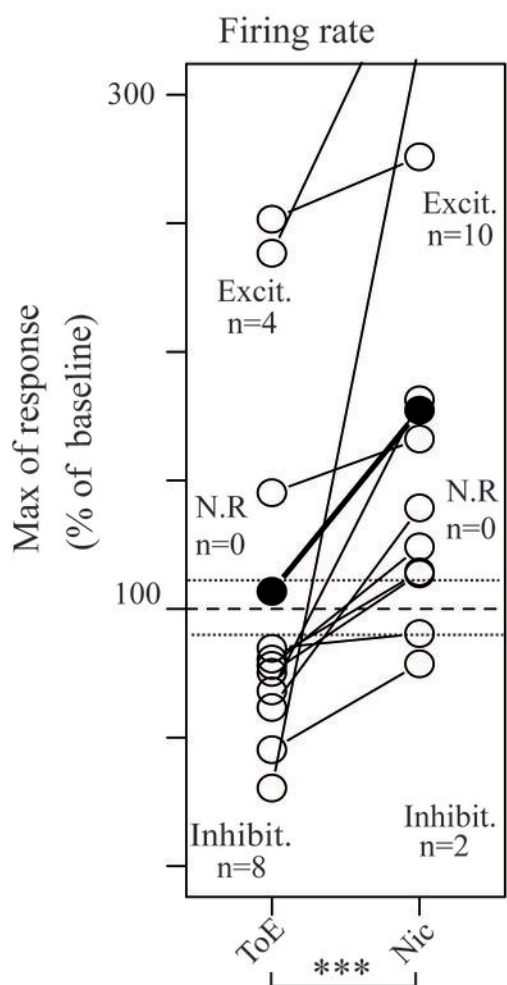
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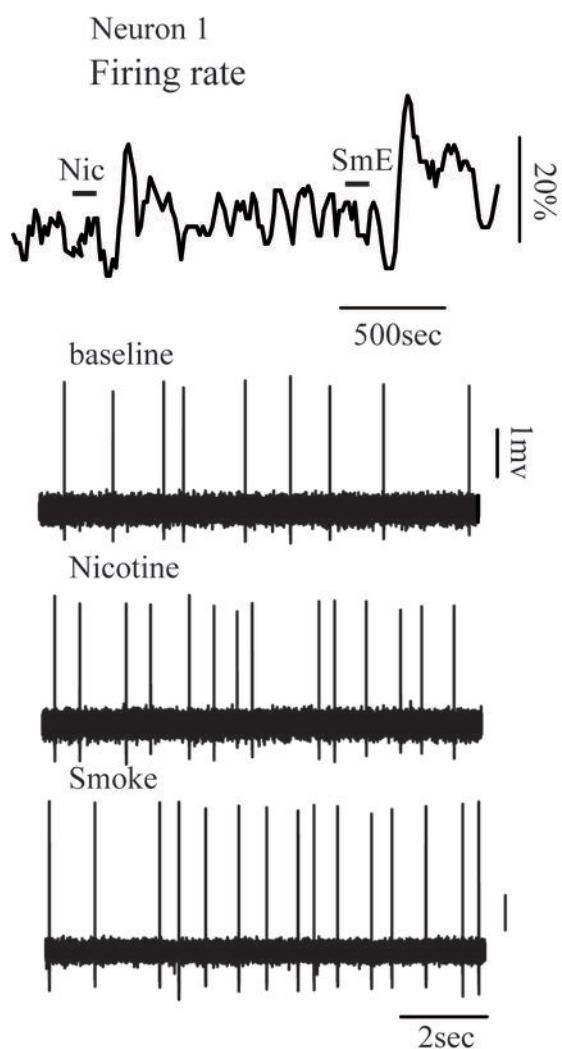


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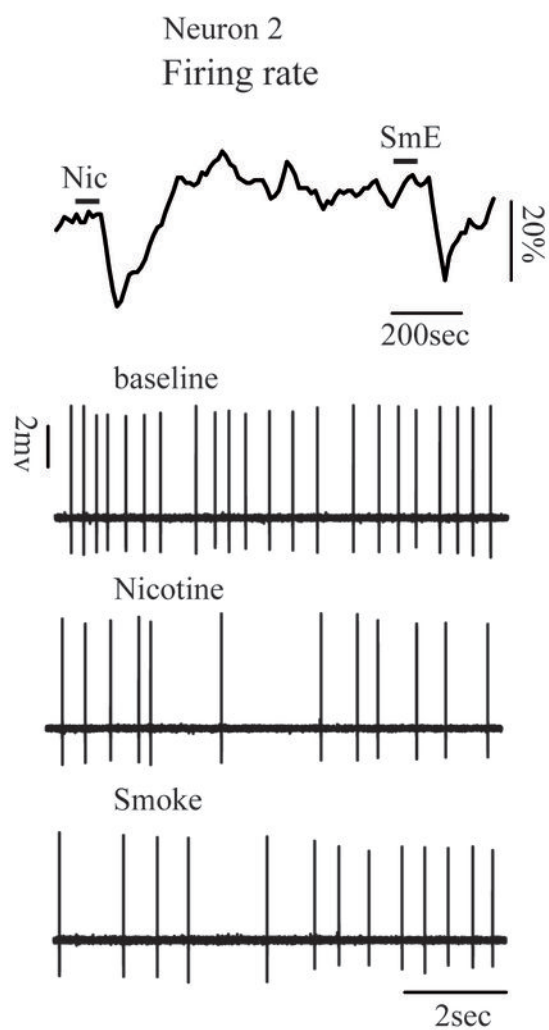




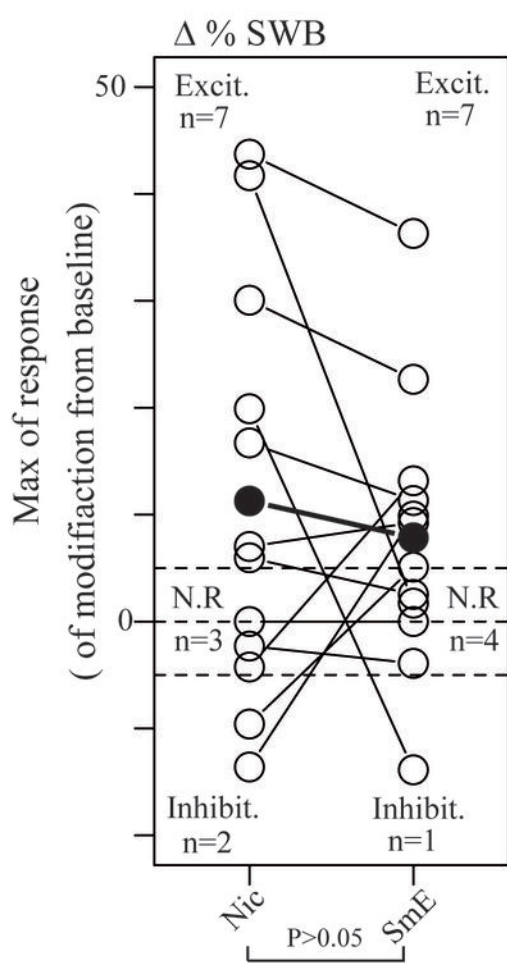
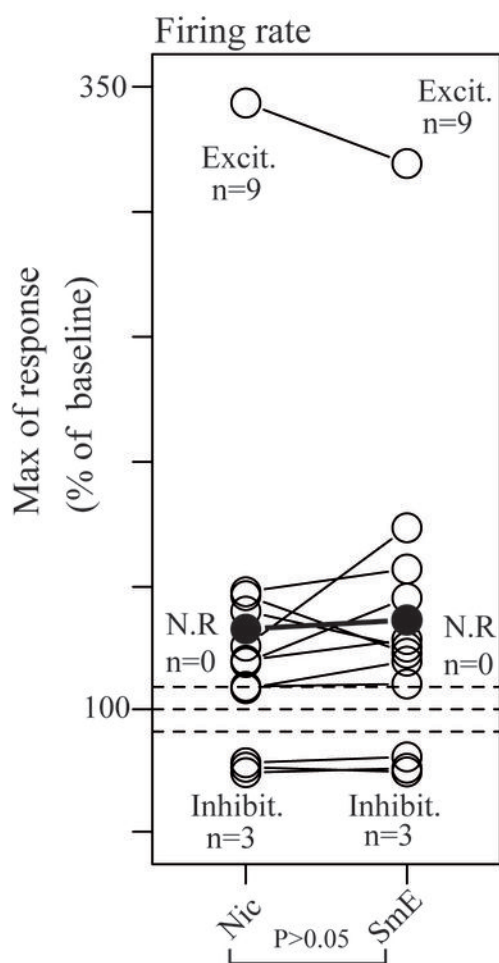
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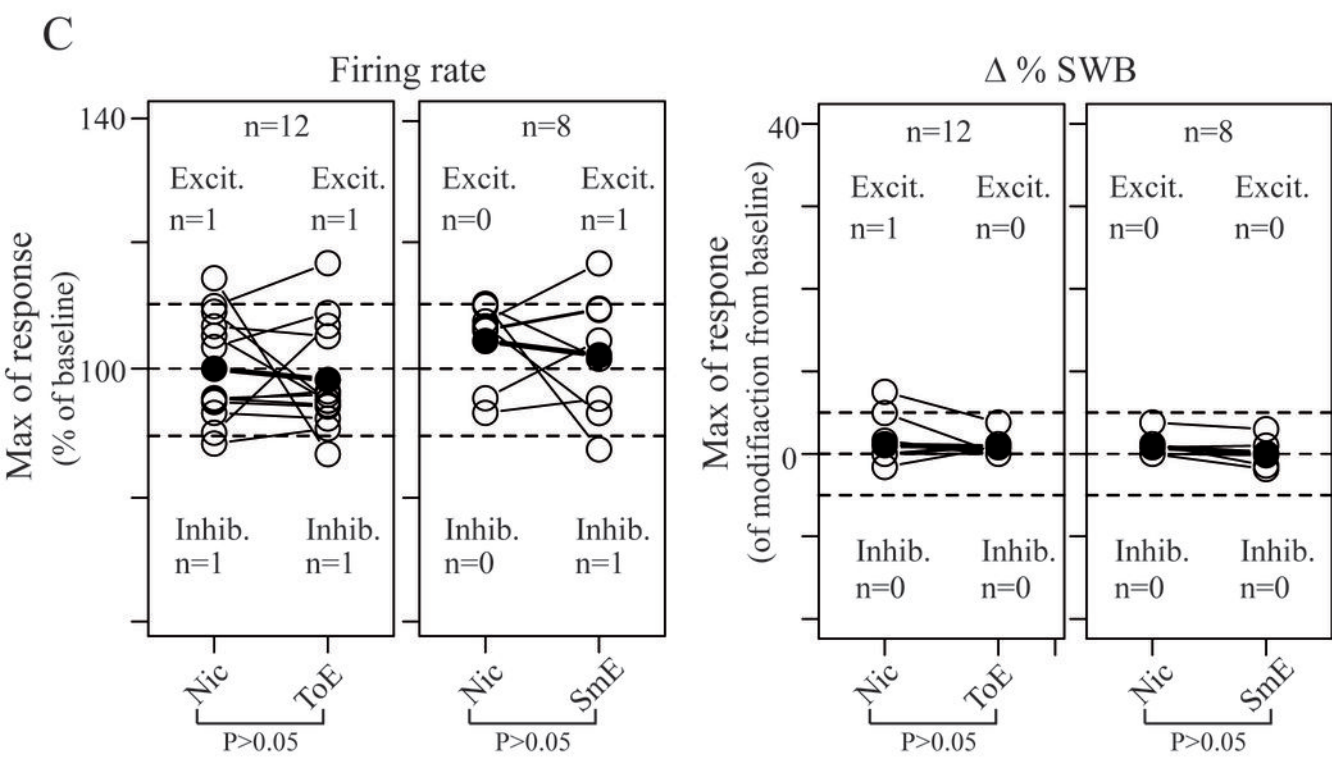
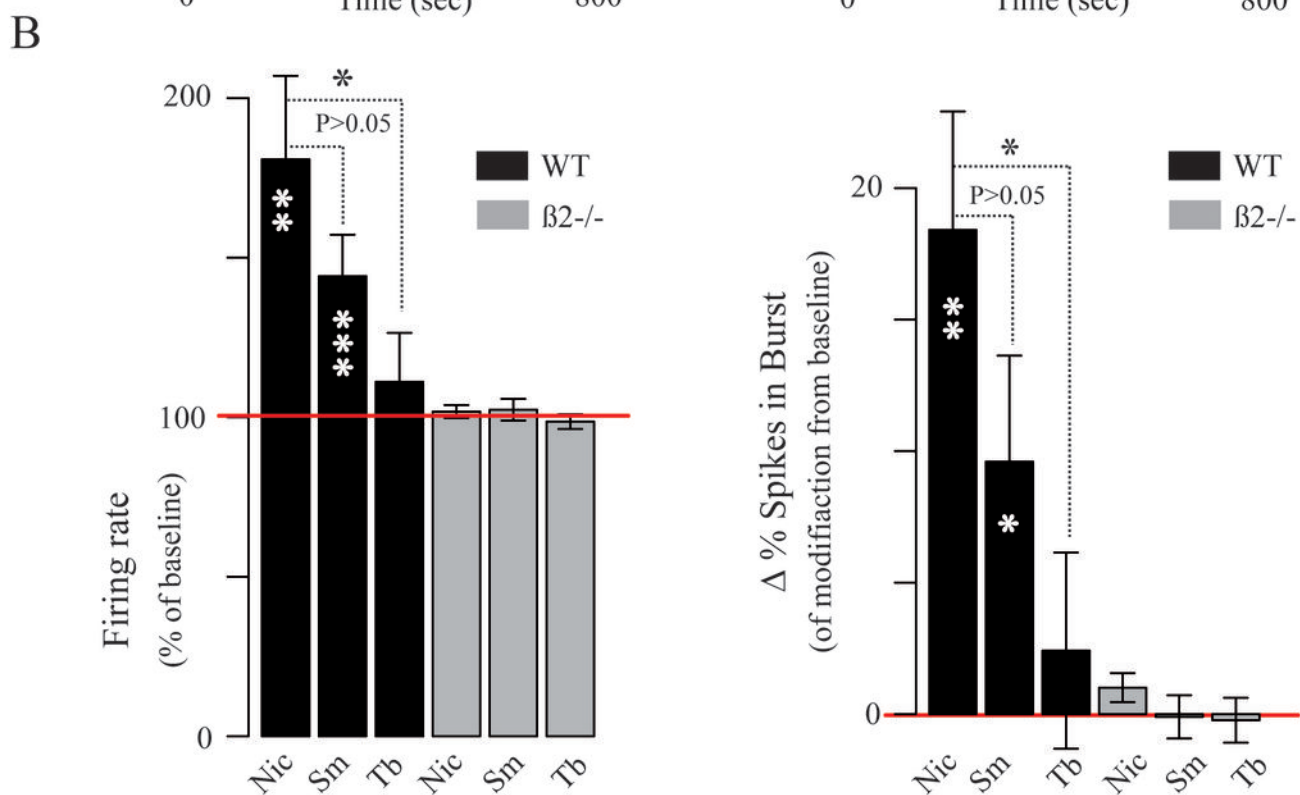
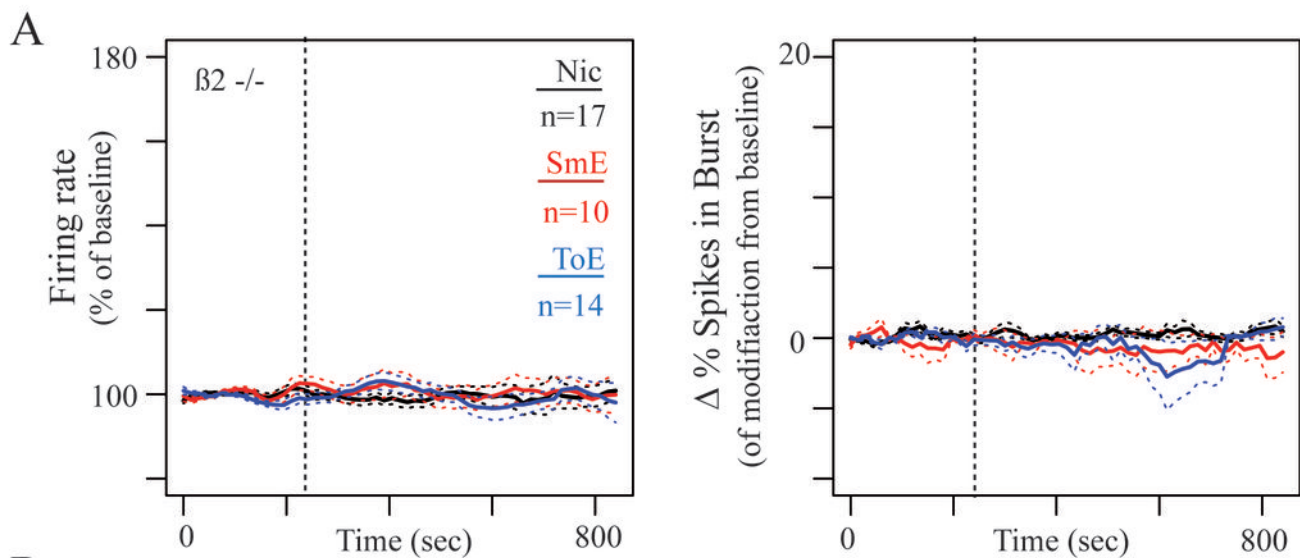


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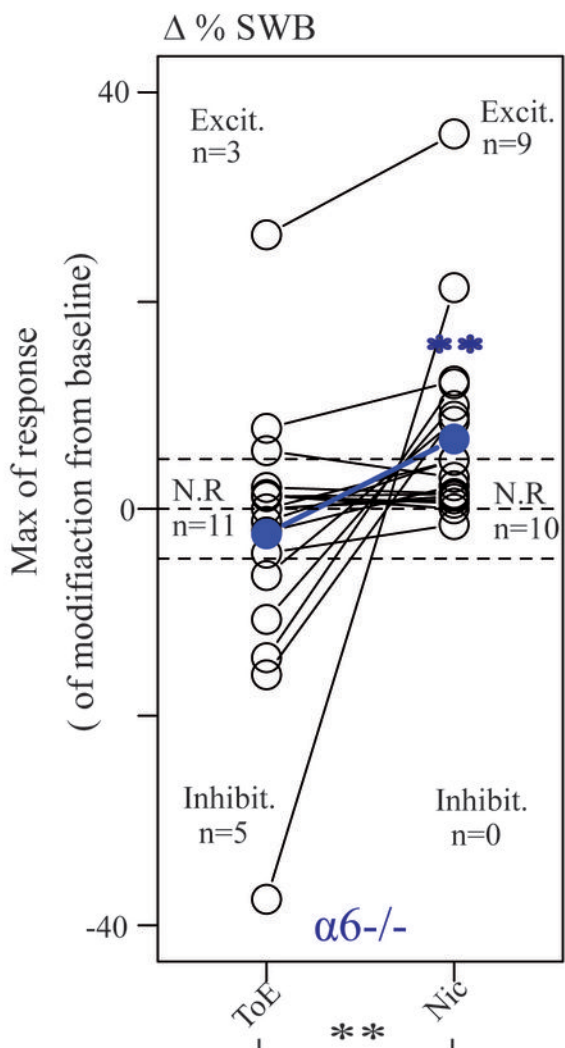
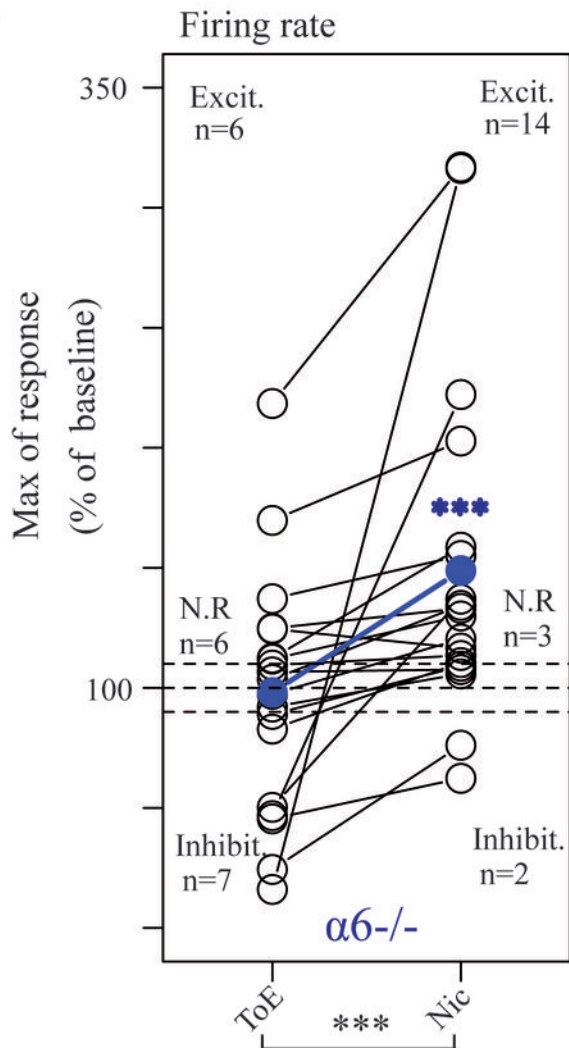


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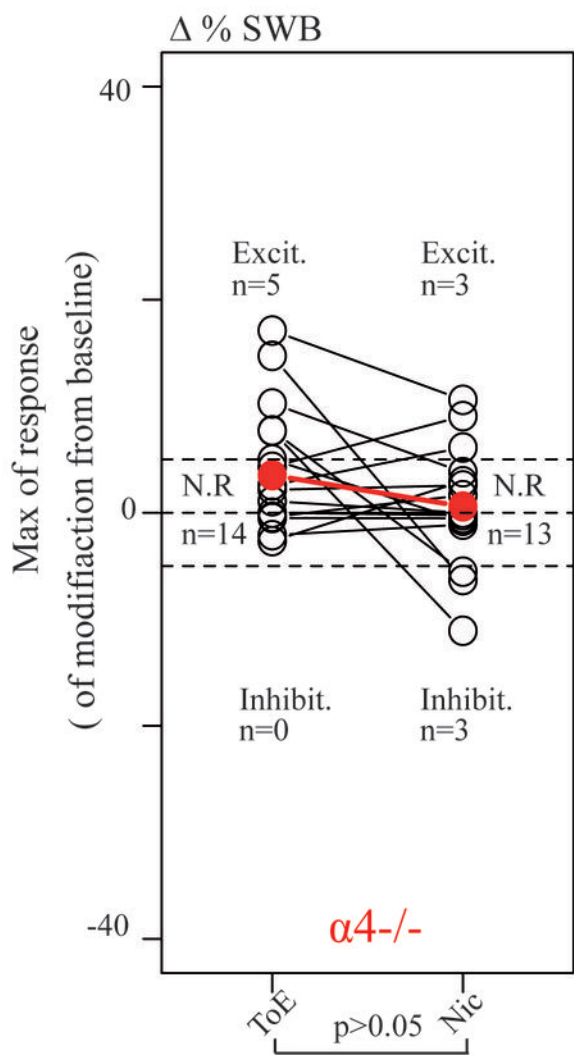
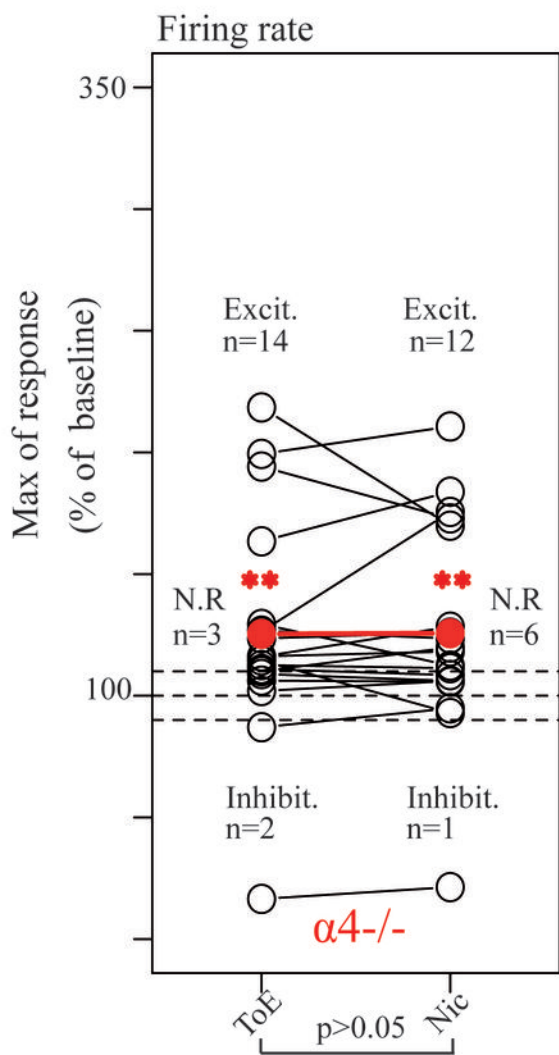




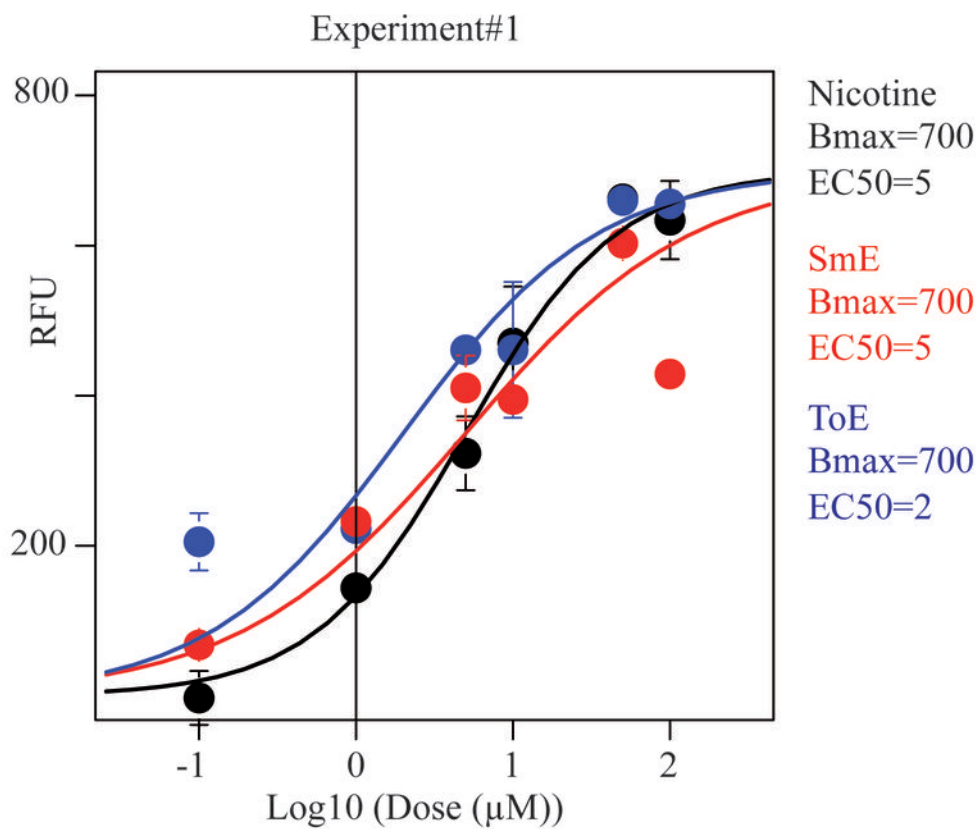
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A



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