Nornicotine application on cockroach dorsal unpaired median neurons induces two distinct ionic currents: Implications of different nicotinic acetylcholine receptors

Delphine Calas-List, Olivier List, Steeve H. Thany

Neuroscience Letters 518 (2012) 64–68

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Nornicotine application on cockroach dorsal unpaired median neurons induces two distinct ionic currents: Implications of different nicotinic acetylcholine receptors

Delphine Calas-List[1], Olivier List[1], Steeve H. Thany*

Laboratoire Récepteurs et Canaux Ioniques Membranaires, UPRES EA 2647/USC INRA 1330, Université d'Angers, 2 Bd. Lavoisier, 49045 Angers, France

ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form 27 March 2012
Accepted 24 April 2012

Keywords:
Insect
Cockroach
Neurons
Nicotinic receptors
Nornicotine

ABSTRACT

The goal of the present study is to examine the agonist action of nornicotine on insect nicotinic acetylcholine receptors. Using patch-clamp techniques on cockroach dorsal unpaired median neurons, we demonstrated that nornicotine induced two distinct ionic currents named types 1 and 2. We found that alpha-bungarotoxin induced a rapid desensitization of type 1 currents whereas type 2 was completely blocked. Interestingly, types 1 and 2 currents were not blocked by the muscarinic antagonist, pirenzepine but by co-application of 1 μM pirenzepine and 0.5 μM alpha-bungarotoxin, suggesting that muscarinic receptors modulated nornicotine-induced current amplitudes. In addition, type 1 current amplitudes were strongly reduced by 20 μM D-tubocurarine and 5 μM mecamylamine which blocked the previously identified alpha-bungarotoxin-insensitive nAChR1 and nAChR2 receptors. Co-application of alpha-bungarotoxin with D-tubocurarine or mecamylamine completely blocked all ionic currents. We propose that types 1 and 2 currents are associated to several nicotinic receptors subtypes, including nAChR1 and nAChR2 receptors. Finally, we conclude that nornicotine could be used as an agonist to identify distinct insect nicotinic receptors.

Nornicotine (Nor) is a tobacco alkaloid and an active nicotine metabolite which is associated to physiological and behavioural effects [3,8,26]. Nor is present in the brain after exposure to nicotine [6,7,11,12,21], it increases dopamine release through nicotinic receptors activation [10,15] and functionally discriminates between different vertebrate nAChR subtypes [22]. Indeed, Nor acts as a potent partial agonist of homomeric α7 receptor while it is a relatively poor agonist for heteromeric α3β4 and α4β2 receptors, leading to the assumption that it could be used as a ligand to characterize nAChR subtypes [22]. Due to the difficulty to express functional nAChR subtype in heterologous system; the subunit composition of native insect nAChRs remains unknown. To date; only one combination using insect nAChR subunit is functional; *i.e. Schistocerca gregaria* αL1 (Sgα1) subunit [17,20]. However; electrophysiological and binding studies performed on insect neurons revealed several nAChRs which could be classified as α-bungarotoxin (αBgt)-sensitive and -insensitive [9,13]. The snake toxin αBgt was commonly used to characterize vertebrate and invertebrate nAChR subtypes [10,13,14]. Thus; it was suggested that such as vertebrates, insect αBgt-sensitive nAChRs could include homomeric receptors [25]. In the cockroach *Periplaneta americana*; αBgt-sensitive and -insensitive nAChR subtypes have been identified [4,5,9,16,23]. Two distinct αBgt-insensitive nAChRs named nAChR1 and nAChR2 were found in the dorsal unpaired median (DUM) neurons. They differ in their pharmacological properties: nAChR1 was blocked by D-tubocurarine (dTC) and nAChR2 by mecamylamine (Mec) [5]. In addition, they are activated by different intracellular pathways involving calcium and second messengers such as PKA and two distinct PKCs [4,5,24]. The αBgt-sensitive nAChRs include the 'mixed' nicotinic/muscarinic receptor which was blocked by αBgt and the muscarinic antagonist, pirenzepine (Pzp) [9,16,18]. In the present study, using patch-clamp methods on DUM neurons, we evaluated for the first time the agonist action of the nicotine metabolite, nornicotine, on insect nAChRs.

Patch clamp recordings were performed on DUM neuron cell bodies isolated from the dorsal midline of the terminal abdominal ganglion of the nerve cord of adult male cockroach *Periplaneta americana*, following enzymatic treatment and mechanical dissociation as previously described [18]. Nor was applied by pneumatic pressure ejection (15 psig. Miniframe, Medical System Corporation, USA). The pressure ejection was made through a glass micropipette, resistance 1.8 MΩ, positioned in solution within 100 μm of the isolated cell body. Nor-induced currents were recorded using the patch-clamp technique in the whole-cell recording configuration

* Corresponding author. Tel.: +33 241 73 52 13; fax: +33 241 73 52 15.
E-mail address: steeve.thany@univ-angers.fr (S.H. Thany).
[1] These authors contributed equally to this work.

0304-3940/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2012.04.058

under voltage-clamp mode with an axopatch 200B (Patch-clamp amplifier, Axon Instruments, Foster City, CA). Signals were digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments) and axoscope 10.2 software (Axon Instruments). The Petri dish containing isolated cell bodies was placed onto the inverted microscope (CK2: Olympus), and continuously bathed with the standard extracellular solution (in mM: NaCl 200, KCl 3.1, $MgCl_2$ 4, $CaCl_2$ 5, sucrose 50, HEPES 10, pH 7.4 adjusted with NaOH) using a gravity perfusion system positioned within 100 μm from the cell body. Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with internal solution containing (in mM): K-D-gluconic acid, 160; NaCl, 10; $MgCl_2$, 1; $CaCl_2$, 0.5; K-fluoride, 10; ATP Mg, 3; EGTA 10; HEPES, 20 and pH adjusted to 7.4 with KOH. Pipettes had resistances ranging from 1.2 to 1.4 MΩ. All compounds tested were purchased from Sigma Chemical Company (France). Statistical analysis was performed using Student's *t*-test. Statistical significance was assessed by a standard Bonferroni's test using GraphPad Software. Results were expressed as means ± SEM. The dose–response curve was derived from the fitted curve following the equation:

$$y = I_{min} + \frac{(I_{max} - I_{min})}{(1 + 10^{(\log EC50 - X)H})}$$

where *Y* is the normalized response, I_{max} and I_{min} are the maximum and minimum responses, '*H*' is the coefficient and EC_{50}, the concentration giving half the maximum response.

As illustrated in Fig. 1, application of Nor induced a transient inward current in cockroach DUM neurons, voltage clamped at −50 mV (membrane potential) [19]. We found that most of the recorded cells responded with high Nor currents (Type 1), while the other cells had lower current amplitudes (Type 2), with respect to the peak current amplitudes (Fig. 1A and B). In each condition, the EC_{50} values were estimated at 19.6 μM and 10.4 μM for types 1 and

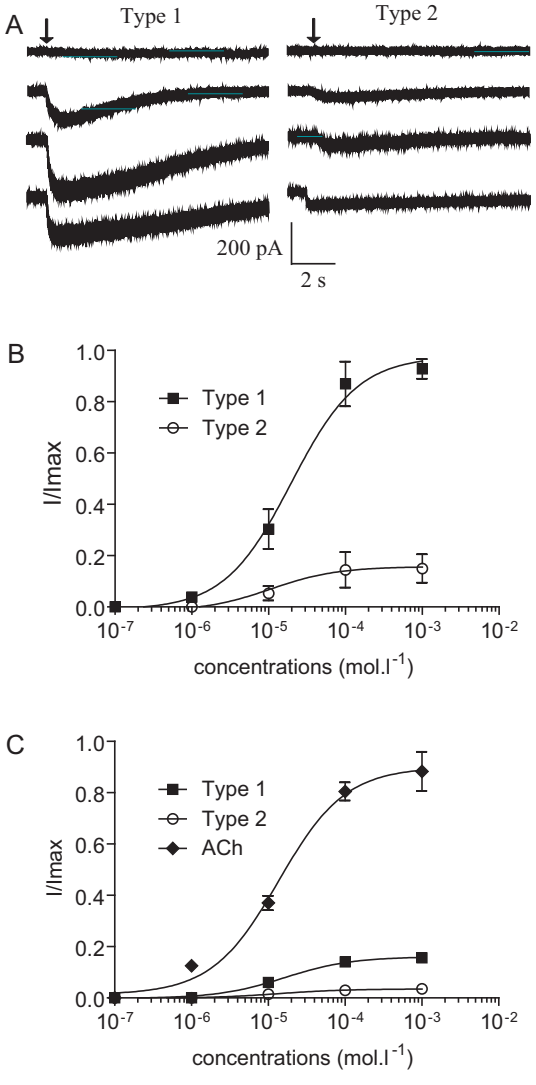

Fig. 1. Effect of Nor on DUM neuron. (A) Typical examples of DUM neurons responses after pressure application of 100 ms Nor at −50 mV holding potential. Currents from the upper trace correspond to 1 μM, 10 μM, 100 μM and 1 mM, respectively. Arrows indicate 150 ms pulse duration. (B) Concentration–response curves for types 1 and 2 currents. Responses were normalized to the maximum peak current elicited by 100 μM Nor. (C) Concentration response–curve comparing the agonist action of ACh and Nor (Types 1 and 2 currents). Current amplitudes were normalized to the maximum peak current amplitude elicited by 1 mM ACh. Data are mean ± SEM ($N = 10$). All currents are measured at −50 mV holding potential.

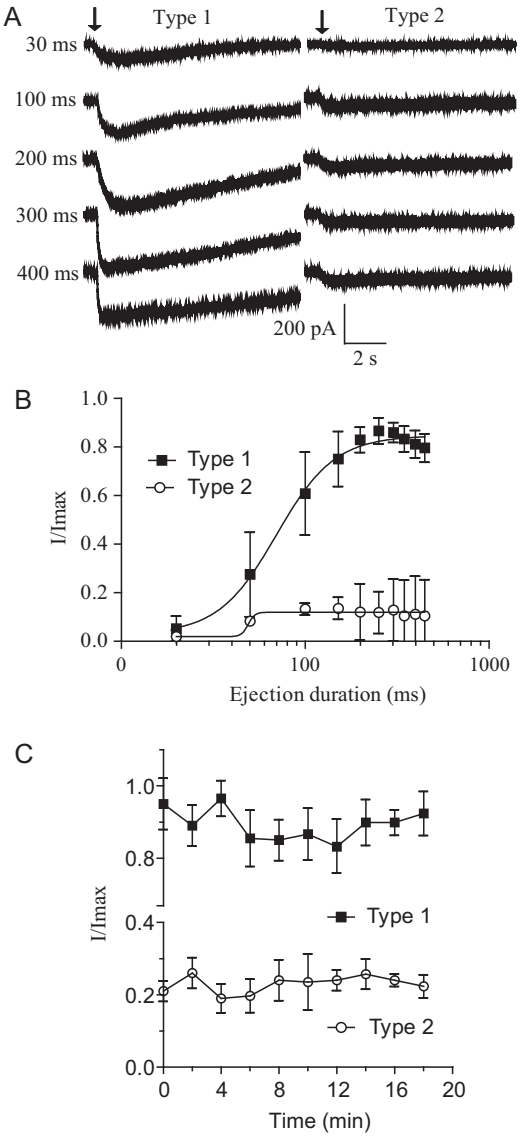

Fig. 2. Successive applications of Nor. (A) Typical examples of type 1 and type 2 current amplitudes at different pulse durations. Arrows indicated 150 ms pulse duration (100 μM Nor). (B) Comparison of type 1 and type 2 current amplitudes. 100 μM Nor was applied at different pulse durations (from 30 ms to 450 ms) and 2 min interval pulse. (C) Nor-induced current amplitudes (types 1 and 2) remained stable after the beginning of the first pulse (100 μM Nor, 150 ms pulse duration, 15 psig). Data are mean ± SEM ($N = 10$) and normalized to the maximum Nor-induced currents at −50 mV holding potential.

2 currents, respectively. Interestingly, the peak current amplitudes of ACh measured in the same condition demonstrated that Nor was a partial agonist of DUM neuron nAChRs (Fig. 1C). These kinds of profiles were previously demonstrated using honeybee antennal lobe neurons. Barbara et al. found that acetylcholine (ACh)-induced currents exhibited two distinct profiles for which some cells elicited low currents and others higher current amplitudes [2]. Unfortunately, the electrophysiological properties of these currents were not investigated, and only ACh-induced higher current amplitudes were examined. Here, the maximal Nor-induced current amplitude was −260 pA at 100 μM and 150 ms pulse duration. We therefore used this concentration to study the agonist action of Nor on DUM neuron nAChRs and all currents were normalized to the Nor maximum response. The mean current amplitudes were −239.3 ± 8 pA and −44 ± 5 pA at 100 μM, for types 1 and 2 currents. Then, to estimate the possible saturation or desensitization of ionic currents, 100 μM Nor was applied with different pulse durations (from 30 ms to 450 ms with 2 min interval pulse), on the same cell [1]. In these conditions, we found a sigmoidal relationship between the ejection duration and the ionic current amplitude for type 1 whereas similar effect was not found with type 2 currents (Fig. 2A and B). These data were in general agreement with previous studies demonstrating that cockroach *Periplaneta americana* expresses two distinct αBgt-sensitive receptors: desensitizing (nAChRD) and non-desensitizing (nAChRN) nicotinic receptors [23]. Indeed, nAChRD and nAChRN could be identified by their peak current amplitudes and we found that types 1 and 2 current amplitudes remained stable 20 min after successive applications of 100 μM Nor (150 ms pulse duration with 2 min interval pulse) (Fig. 2C). Moreover, when Nor-induced currents were investigated using 0.5 μM αBgt, type 1 current amplitudes did not show any significant difference between control (−239.3 ± 8 pA) and αBgt treated cells, whereas type 2 currents were completely blocked. Currents were −248 ± 16 pA and 0 pA for types 1 and 2, respectively (Fig. 3A and B). Interestingly, type 1 currents measured between 150 ms and 450 ms pulse durations showed a strong decrease of peak current amplitudes from −248 ± 16 pA to −90 ± 0.9 pA, indicating a rapid desensitization of nAChRs (Fig. 3C). According to our results, we propose that αBgt affects the desensitization of type 1 currents whereas type 2 is completely blocked. As it was previously suggested that bath applied muscarinic antagonist such as Pzp could block the 'mixed' nicotinic/muscarinic receptors [18], 100 μM Nor was applied under 1 μM Pzp. Neither types 1 and 2 current amplitudes were affected by Pzp, demonstrating that Nor did not bind to DUM neuron muscarinic receptors. The type 1 and 2 Nor-induced currents under Pzp treatment were −266.8 ± 16 pA and −92 ± 13 pA, respectively (Fig. 4A). When Nor was co-applied with 1 μM Pzp and 0.5 μM αBgt, all current amplitudes were strongly reduced to −78 ± 0.1 pA and 0 pA for type 1 and 2 (Fig. 4A). Thus, we propose that Pzp could inhibit the modulatory effect of muscarinic receptors resulting in a strong inhibition of type 1 currents. This hypothesis is supported by the finding that (1) this concentration of Pzp did not block Nor-induced currents, (2) the strong desensitization induced by αBgt

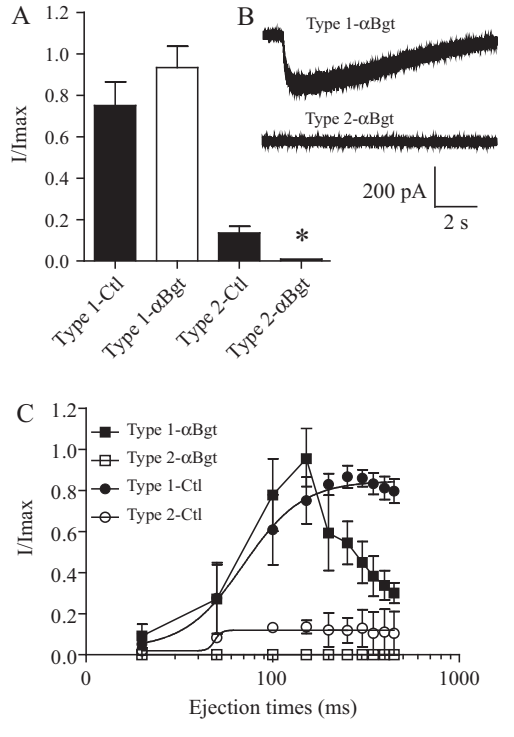

Fig. 3. Effect of 0.5 μM αBgt on Nor-induced currents. (A) Histogram illustrating types 1 and 2 current amplitudes (Ctl = control). Type 1 currents were not affected by αBgt whereas type 2 currents were completely blocked. *$p < 0.05$ using Student's *t*-test ($N = 10$). Data are mean ± SEM, normalized to the maximum Nor-induced currents at −50 mV holding potential and 150 ms pulse duration. (B) Example of Nor-induced currents under 0.5 μM αBgt. (C) Successive applications of 100 μM Nor, using the same cells. Data are normalized using the same condition as indicated above.

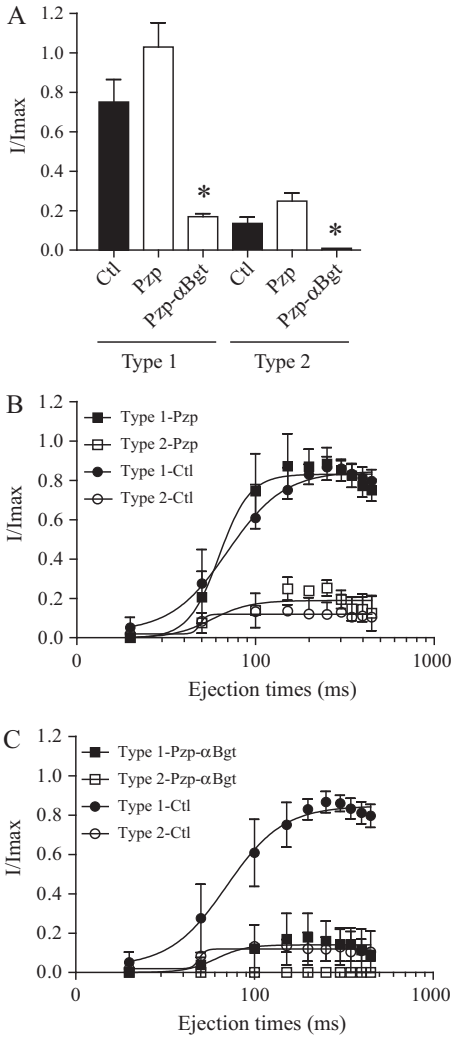

Fig. 4. Effect of 1 μM Pzp on Nor-induced types 1 and 2 currents. (A) Significant decrease of Nor currents (100 μM and 150 ms pulse duration) were found under co-application of Pzp and αBgt ($N = 7$) compared to control (Ctl, $N = 10$). *Student's *t*-test, $p < 0.001$. (B) and (C) Repeated applications of Nor under 1 μM Pzp and co-applications of Pzp and αBgt.

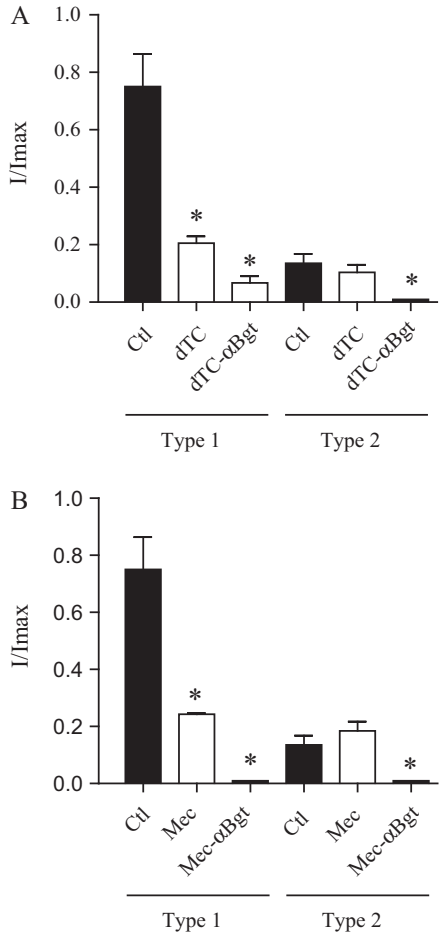

Fig. 5. Effect of dTC and Mec on Nor-induced current amplitudes. (A) Histograms illustrating the decrease of types 1 and 2 currents under dTC and bath application of both dTC and αBgt. In each condition, $p < 0.001$ using Student's *t*-test, $N = 5$. (B) Similar decrease was found with 5 μM Mec or co-applications of Mec and αBgt. *Statistical difference $p < 0.05$, $N = 5$. Ctl = control. Mean current amplitudes are plotted at −50 mV holding potential and 150 ms pulse durations.

(See Fig. 3C) was not observed under Pzp treatment (Fig. 4B) while repeated applications of Nor were completely blocked by both αBgt and Pzp (Fig. 4C) and (3) this hypothesis was in accordance with previous studies demonstrating that the variability of the acetylcholine-induced currents [23] and nicotine-induced current amplitudes through activation of DUM neurons nAChR1 subtype could be modulated by muscarinic receptors [5]. Consequently, we suggested that Nor did not act as an agonist of the 'mixed' nicotinic/muscarinic receptor but that its agonist action was modulated by muscarinic receptors.

In a second set of experiments, we explored the agonist effect of Nor on αBgt-insensitive nAChR1 and nAChR2 subtypes [4,5,24]. Nor was first applied with 20 μM D-tubocurarine (dTC) which blocked nAChR1 [4,5,24]. The relative current amplitude induced by 100 μM Nor was significantly reduced to -61.7 ± 1.7 pA for type 1 currents whereas there was not significant effect on type 2 (current amplitudes were -36.3 ± 0.3 pA, Fig. 5A). As it appeared that type 1 currents were more sensitive to dTC compared to type 2, we proposed that DUM neurons contained two distinct nAChRs which were sensitive (type 1) and insensitive (type 2) to dTC. Thus, bath application of both 20 μM dTC and 0.5 μM αBgt strongly reduced Nor-induced type 1 and 2 currents to -22 ± 3 pA and 0 pA, respectively (Fig. 5A). These results demonstrated that Nor action was associated to αBgt-insensitive nAChRs (*i.e.* type 1 currents) which were blocked by dTC but did not include nAChR1 subtype. The Nor responses were further compared by examining effects on nAChR2 subtype. We first found that 5 μM Mec strongly reduced Nor-induced type 1 to -54.4 ± 4 pA whereas co-application of Mec and αBgt completely blocked all currents (Fig. 5B). In the light of our results and compared to previous electrophysiological studies, we propose that DUM neurons express nAChRs that are blocked by dTC and Mec but are distinct from the previously identified αBgt-insensitive nAChR1 and nAChR2 subtypes [4,5]. Consequently, type 2 currents could be induced through activation of αBgt-sensitive nAChRs while type 1 includes αBgt-sensitive and -insensitive receptors. We have identified several nAChR subunits in the cockroach suggesting different nAChR subtypes (Unpublished data). Our future goal will be to investigate the subunits composition of these receptors and their pharmacological properties. Finally, in the present study we demonstrate that each of these currents can be selectively blocked providing evidence that they are induced by distinct nAChRs and therefore, Nor can be used as an agonist to characterize insect nAChR subtypes.

References

[1] P. Alix, F. Grolleau, B. Hue, Ca^{2+}/calmodulin-dependent protein kinase regulates GABA-activated Cl-current in cockroach dorsal unpaired median neurons, Journal of Neurophysiology 87 (2002) 2972–2982.
[2] G.S. Barbara, B. Grunewald, S. Paute, M. Gauthier, V. Raymond-Delpech, Study of nicotinic acetylcholine receptors on cultured antennal lobe neurones from adult honeybee brains, Invertebrate Neuroscience 8 (2008) 19–29.
[3] J.R. Copeland, A. Adem, P. Jacob 3rd, A. Nordberg, A comparison of the binding of nicotine and nornicotine stereoisomers to nicotinic binding sites in rat brain cortex, Naunyn-Schmiedebergs Archives of Pharmacology 343 (1991) 123–127.
[4] R. Courjaret, F. Grolleau, B. Lapied, Two distinct calcium-sensitive and -insensitive PKC up- and down-regulate an alpha-bungarotoxin-resistant nAChR1 in insect neurosecretory cells (DUM neurons), European Journal of Neuroscience 17 (2003) 2023–2034.
[5] R. Courjaret, B. Lapied, Complex intracellular messenger pathways regulate one type of neuronal alpha-bungarotoxin-resistant nicotinic acetylcholine receptors expressed in insect neurosecretory cells (dorsal unpaired median neurons), Molecular Pharmacology 60 (2001) 80–91.
[6] P.A. Crooks, L.P. dwoskin, Contribution of CNS nicotine metabolites to the neuropharmacological effects of nicotine and tobacco smoking, Biochemical Pharmacology 54 (1997) 743–753.
[7] P.A. Crooks, M. Li, L.P. Dwoskin, Determination of nicotine metabolites in rat brain after peripheral radiolabeled nicotine administration: detection of nornicotine, Drug Metabolism and Disposition: The Biological Fate of Chemicals 23 (1995) 1175–1177.
[8] P.A. Crooks, M. Li, L.P. Dwoskin, Metabolites of nicotine in rat brain after peripheral nicotine administration. Cotinine, nornicotine, and norcotinine, Drug Metabolism and Disposition: The Biological Fate of Chemicals 25 (1997) 47–54.
[9] J.A. David, R.M. Pitman, The pharmacology of alpha-bungarotoxin-resistant acetylcholine rececptors on an identified cockroach motoneurone, Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology 172 (1993) 359–368.
[10] L.P. Dwoskin, P.A. Crooks, Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery, Journal of Pharmacology and Experimental Therapeutics 298 (2001) 395–402.
[11] O. Ghosheh, L.P. Dwoskin, W.K. Li, P.A. Crooks, Residence times and half-liv nicotine metabolites in rat brain after acute peripheral administration of [2'-(14)C]nicotine, Drug Metabolism and Disposition: The Biological Fate of Chemicals 27 (1999) 1448–1455.
[12] O.A. Ghosheh, L.P. Dwoskin, D.K. Miller, P.A. Crooks, Accumulation of nicotine and its metabolites in rat brain after intermittent or continuous peripheral administration of [2′-(14)C]nicotine, Drug Metabolism and Disposition: The Biological Fate of Chemicals 29 (2001) 645–651.
[13] F. Goldberg, B. Grunewald, H. Rosenboom, R. Menzel, Nicotinic acetylcholine currents of cultured Kkenyon cells from the mushroom bodies of the honey bee Aapis mellifera, Journal of Physiology 514 (Pt 3) (1999) 759–768.
[14] C. Gotti, M. Zoli, F. Clementi, Brain nicotinic acetylcholine receptors: native subtypes and their relevance, Trends in Pharmacological Sciences 27 (2006) 482–491.
[15] T.A. Green, P.A. Crooks, M.T. Bardo, L.P. Dwoskin, Contributory role for nornicotine in nicotine neuropharmacology: nornicotine-evoked [3H]dopamine overflow from rat nucleus accumbens slices, Biochemical Pharmacology 62 (2001) 1597–1603.
[16] F. Grolleau, B. Lapied, S.D. Buckingham, W.T. Mason, D.B. Sattelle, Nicotine increases $[Ca^{2+}]i$ and regulates electrical activity in insect neurosecretory cells (DUM neurons) via an acetylcholine receptor with 'mixed' nicotinic–muscarinic pharmacology, Neuroscience Letters 220 (1996) 142–146.

[17] A.K. Jones, J. Marshall, A.D. Blake, S.D. Buckingham, M.G. Darlison, D.B. Sattelle, Sgbeta1, a novel locust (*Schistocerca gregaria*) non-alpha nicotinic acetylcholine receptor-like subunit with homology to the Drosophila melanogaster Dbeta1 subunit, Invertebrate Neuroscience 5 (2005) 147–155.
[18] B. Lapied, H. Le Corronc, B. Hue, Sensitive nicotinic and mixed nicotinic–muscarinic receptors in insect neurosecretory cells, Brain Research 533 (1990) 132–136.
[19] B. Lapied, C.O. Malecot, M. Pelhate, Ionic species involved in the electrical activity of single adult aminergic neurones isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana*, Journal of Experimental Biology 144 (1989) 535–549.
[20] J. Marshall, S.D. Buckingham, R. Shingai, G.G. Lunt, M.W. Goosey, M.G. Darlison, D.B. Sattelle, E.A. Barnard, Sequence and functional expression of a single alpha subunit of an insect nicotinic acetylcholine receptor, EMBO Journal 9 (1990) 4391–4398.
[21] T.P. Moyer, J.R. Charlson, R.J. Enger, L.C. Dale, J.O. Ebbert, D.R. Schroeder, R.D. Hurt, Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry with clinically relevant metabolic profiles, Clinical Chemistry 48 (2002) 1460–1471.
[22] R.L. Papke, L.P. Dwoskin, P.A. Crooks, The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery, Journal of Neurochemistry 101 (2007) 160–167.
[23] V.L. Salgado, R. Saar, Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons, Journal of Insect Physiology 50 (2004) 867–879.
[24] S.H. Thany, R. Courjaret, B. Lapied, Effect of calcium on nicotine-induced current expressed by an atypical alpha-bungarotoxin-insensitive nAChR2, Neuroscience Letters 438 (2008) 317–321.
[25] S.H. Thany, G. Lenaers, V. Raymond-Delpech, D.B. Sattelle, B. Lapied, Exploring the pharmacological properties of insect nicotinic acetylcholine receptors, Trends in Pharmacological Sciences 28 (2007) 14–22.
[26] X. Zhang, A. Nordberg, The competition of (−)-[3H]nicotine binding by the enantiomers of nicotine, nornicotine and anatoxin-a in membranes and solubilized preparations of different brain regions of rat, Naunyn-Schmiedebergs Archives of Pharmacology 348 (1993) 28–34.