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The role of intracellular calcium regulatory pathways in the modulation of insects' nicotinic acetylcholine receptors

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List of abbreviations

(A)

AC:	Adenylate cyclase
ACE:	Acetamiprid
ACh:	Acetylcholine
AChBP:	Acetylcholine-binding protein
ALS:	Alpha-like subunit
AMP:	Adenosine monophosphate
cAMP:	Cyclic adenosine monophosphate
AMPK:	AMP-activated protein kinase
2-APB:	2-Aminoethoxydiphenyl borate
aPKC:	Atypical PKC
α-Bgt:	α -bungarotoxin

(C)

CaM:	Calmodulin
CaMK:	Ca ²⁺ /CaM-dependent protein kinases or CaM-kinases
CaMKK:	CaMK-kinase
cAMP:	Cyclic adenosine monophosphate
CdCl₂:	Cadmium chloride
Cdz:	Calmidazolium
cGMP:	Cyclic guanosine monophosphate
CICR:	Calcium-induced calcium release
CLT:	Clothianidin
cPKC:	Classical PKC
CREB:	cAMP response element-binding protein

(D)

DIN:	Dinotefuran
DM:	Dorsomorphin
DMPP:	1,1-dimethyl-4-phenylpiperzinium iodide
d-TC:	d-tubocurarine
DUM:	Dorsal unpaired median

(G)

GC: Guanylate cyclase

(H)

HVA: High voltage-activated

HEK-293: Human embryonic kidney cell line

5-HT₃: 5-hydroxytryptamine

(I)

IMI: Imidacloprid

IP₃: Inositol-triphosphate

IP₃R: Inositol-triphosphate receptors

(K)

KN-62: Isoquinolinesulfonamide derivative and a potent inhibitor of CaMKII

(L)

LGICs: Ligand-gated ion channels

(M)

Mec: Mecamylamine

MLA: Methyllycaconitine

(N)

nAChR: Nicotinic acetylcholine receptors

nAChD: Desensitizing nAChR

nAChN: Non desensitizing nAChR

NAM: Negative allosteric modulator

NHD: Neurohormone D

NIC: Nicotine

NIT: Nitenpyram

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

NPPB: 5-nitro-2-(3-phenylpropylamino) benzoic acid

nPKC: Novel PKC

(P)

PAM: Positive allosteric modulator

PaCaMKII-E: *Periplaneta americana* CaMKII isoform E

PDE: Phosphodiesterase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PKG: GMP dependent protein kinase

PLC: Phospholipase

(R)

RE: Endoplasmic reticulum

RyR: Ryanodine receptors

(S)

SAM: Silent allosteric modulator

SFX: Sulfoxaflor

STO-609: 7-Oxo-7H-benzimidazo[2,1-a]benz[de]isoquinoline-3-carboxylic acid

(T)

TAG: Terminal abdominal ganglia

THI: Thiacloprid

TMX: Thiamethoxam

TRP γ : Transient receptor potential

(V)

VGCC: Voltage-gated calcium channel

Préambule

Ces dernières années, l'utilisation des pesticides a évolué rapidement. Ils sont aujourd'hui considérés comme l'une des clés de voûte de l'agriculture moderne. Leur importance vient de leur rôle dans la protection des cultures contre une variété de ravageurs et de maladies d'origine fongique, végétale ou animale.

Cependant, de nombreuses controverses concernant l'utilisation de certains pesticides, notamment les néonicotinoïdes, sont apparues ces dernières années en raison de leurs effets nocifs potentiels sur les pollinisateurs, en particulier les abeilles mellifères. Par exemple, l'utilisation des néonicotinoïdes a été liée au déclin des populations d'abeilles mellifères, ce qui a suscité de nombreuses inquiétudes quant à leur impact sur l'environnement.

En réponse à ces préoccupations, l'Union européenne a interdit en 2018 l'utilisation de trois néonicotinoïdes: la clothianidine, l'imidaclopride et le thiaméthoxame. Cependant, cette mesure a affecté de nombreux agriculteurs qui comptent sur ces produits pour protéger leurs cultures contre les ravageurs et les maladies. En l'absence d'alternative efficace, l'interdiction des néonicotinoïdes pourrait entraîner une baisse des rendements agricoles et des pertes économiques. Par conséquent, les agriculteurs et la société sont désormais confrontés à un défi de taille : trouver des alternatives aux pesticides ou synthétiser de nouveaux composés qui n'ont aucun effet sur les organismes non cibles.

Dans tous les cas, une meilleure compréhension des récepteurs nicotiniques neuronaux des insectes qui sont la principale cible de ces composés est nécessaire. Pour mieux comprendre le mode d'action de ces composés et les mécanismes de résistance développés par les insectes. Au sein du laboratoire, nous avons entrepris d'étudier les mécanismes de régulation intracellulaire des récepteurs nicotiniques neuronaux des insectes dans l'optique de mieux comprendre comment ces récepteurs sont activés par les insecticides néonicotinoïdes et de leurs dérivés (sulfoximines, butenolides).

Dans cette thèse, notre objectif a été d'étudier comment les voies intracellulaires dépendante du calcium modulaient les nAChRs exprimés sur les neurones DUM de la blatte *Periplaneta americana*. En particulier, nous nous sommes intéressés aux deux sous-types de récepteurs, nAChR1 et nAChR2, insensibles à l' α -bungarotoxine (α -Bgt)

Abstract

In recent years, the use of insecticides has evolved rapidly. They are now considered one of the main keystones in modern agriculture. Their importance comes from their role in protecting crops against pest insects.

However, many controversies have arisen in recent years regarding the use of certain insecticides such as neonicotinoids, due to their potential harmful effects on pollinators, especially honeybees. For example, the use of neonicotinoids has been linked to the decline of honeybee populations, raising significant concerns about their environmental impact. Indeed, they act as activators of insect neuronal nicotinic acetylcholine receptors.

In response to these concerns, in 2018, the European Union banned the use of three neonicotinoids: clothianidin, imidacloprid, and thiamethoxam. However, this measure has affected many farmers who rely on these products to protect their crops from pests and diseases. Consequently, farmers and industries now face a significant challenge in finding natural alternatives to pesticides or synthesizing new compounds that do not affect non-target organisms.

Recently, two new families of insecticides have been marketed, butenolides and sulfoximines which are also the subject of several controversies.

In this thesis, our objective was to investigate how calcium-dependent intracellular pathways modulate nAChRs expressed on DUM neurons of the cockroach *Periplaneta americana*. In particular, we focused on the two receptors, nAChR1 and nAChR2, which are insensitive to α -bungarotoxin (α -Bgt) and regulated by different intracellular calcium pathways. We found that a new pathway involving CaMKK modulates nAChR1 and nAChR2. In addition, flupyradifurone-induced currents were associated to an increase in intracellular calcium levels through endoplasmic reticulum

Substantial summary in French (Résumé substantiel en Français)

Ces dernières années, l'utilisation des insecticides a évolué rapidement. Ils sont aujourd'hui considérés comme l'une des clés de voûte de l'agriculture moderne. Leur importance vient de leur rôle majeur dans la protection des cultures, en éliminant les insectes ravageurs.

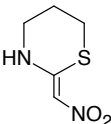
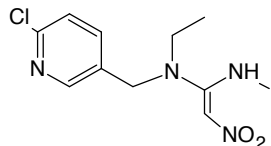
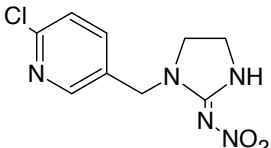
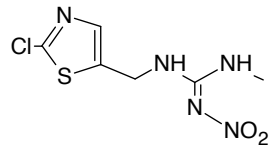
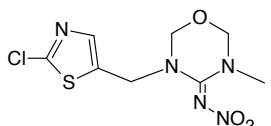
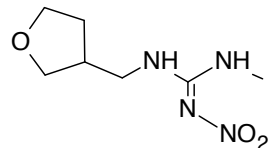
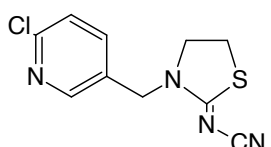
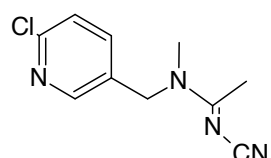
Cependant, de nombreuses controverses concernant l'utilisation de certains insecticides, notamment les néonicotinoïdes, sont apparues ces dernières années en raison de leurs effets nocifs potentiels sur les pollinisateurs, en particulier les abeilles mellifères. Par exemple, l'utilisation des néonicotinoïdes a été liée au déclin des populations d'abeilles mellifères, ce qui a suscité de nombreuses polémiques quant à leur impact sur l'environnement.

En réponse à ces préoccupations, l'Union Européenne a interdit en 2018 l'utilisation de trois néonicotinoïdes : la clothianidine, l'imidaclopride et le thiaméthoxame. Cependant, cette mesure a affecté de nombreux agriculteurs qui comptent sur ces produits pour protéger leurs cultures contre les ravageurs et les maladies. En l'absence d'alternative efficace, l'interdiction des néonicotinoïdes pourrait entraîner une baisse des rendements agricoles et des pertes économiques. Par conséquent, les agriculteurs et les industriels sont désormais confrontés à un défi de taille : trouver des alternatives aux pesticides ou synthétiser de nouveaux composés qui n'ont aucun effet sur les organismes non cibles.

Les insecticides néonicotinoïdes sont une classe particulière d'insecticides développés ces 40 dernières années et qui trouvent leur origine dans la structure de la nicotine avec laquelle il partage au départ le noyau benzénique (**Table 1**). Le premier néonicotinoïde synthétisé a été la nithiazine en 1978 par Soloway et ses collègues. Cette molécule présentait un fort potentiel insecticide mais n'a pu être commercialisée du fait de sa grande photo-instabilité. Ensuite, fut développé l'imidaclopride par Kagabu et ses collègues du groupe Bayer, au Japon, en 1979. Cette molécule fut commercialisée à partir des années 90, notamment comme la substance active du Gaucho®. Des néonicotinoïdes de seconde génération tel que le nitenpyrame en 1988 ou encore l'acétamipride en 1989 ont ensuite été développés. L'imidaclopride est le chef de file des néonicotinoïdes. Il est la substance active d'insecticides à usage agricole comme le Gaucho®, ou à usage vétérinaire comme Advantage®. Il s'agit de l'insecticide le plus vendu dans le monde à raison d'environ 1 milliard d'euros de chiffre d'affaires. En France, le Gaucho® était très utilisé en enrobage de semences de tournesol, de maïs et de colza. Il y a quelques

années, il fut accusé d'être responsable du dépeuplement des colonies d'abeilles. Son autorisation de mise sur le marché a alors été retirée en juillet 2004. En réaction à la polémique engendrée par le Gaucho[®], les industries phytosanitaires ont mis sur le marché des néonicotinoïdes de deuxième génération tel que l'acétamipride, commercialisé en France sous le nom de Supreme[®] (autorisation de mise sur le marché en décembre 2004, source : Ministère de l'Agriculture).

Table 1: Classification des insecticides néonicotinoïdes en fonction du pharmacophore (Modifié d'après Jeschke *et al.* 2008).

pharmacophores [-N-C(E)=X-Y]	composés à cycle de 5 et 6 éléments	composés à chaîne ouverte
nitroenamines/nitrométhylènes [-N-C(E)=CH-NO ₂]	nithiazine 	nitenpyrame 
	imidaclopride 	clothianidine 
N-nitroguanidines/N-nitro-imines [-N-C(E)=N-NO ₂]	thiaméthoxame 	dinotefuran 
N-cyano-amidines/N-cyano-imines [-N-C(E)=N-CN]	thiaclopride 	acétamipride 

Suite aux polémiques soulevées par l'utilisation des insecticides néonicotinoïdes, une nouvelle génération d'insecticides, présentant des structures chimiques différentes, mais activant les récepteurs nicotiniques neuronaux des mammifères a été mise sur le marché. Il s'agit des buténolides et des sulfoximines, dont les plus connus sont respectivement le flupyradifurone et le sulfoxaflor (**Figure 1**).

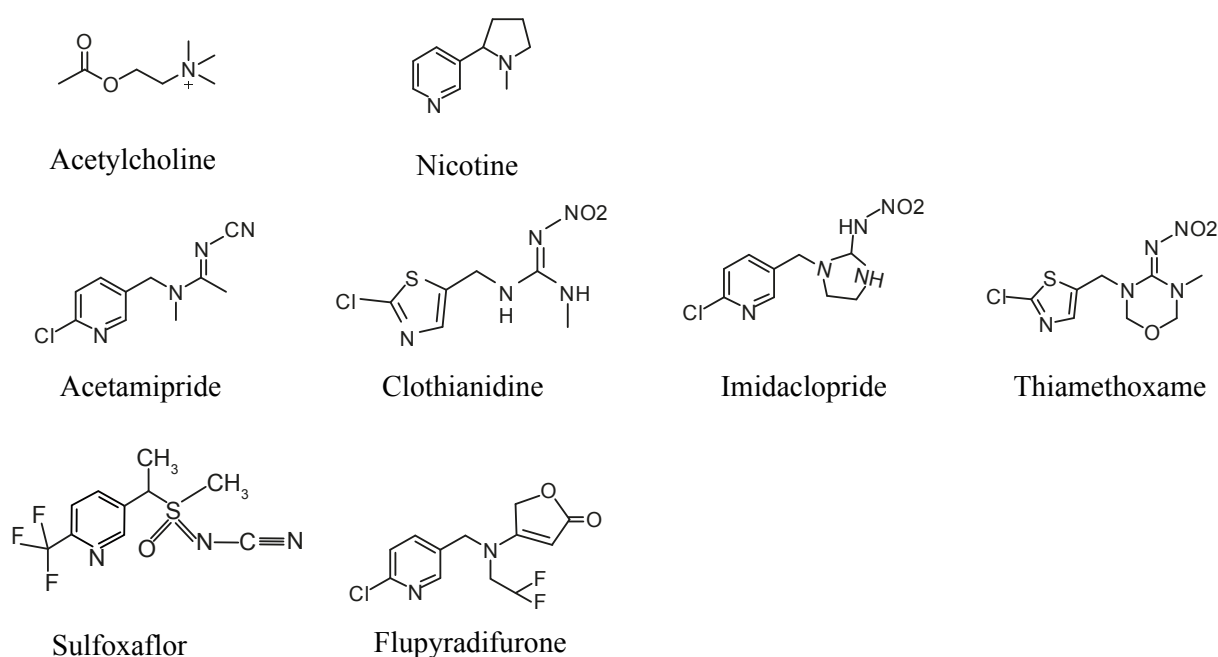


Figure 1: Structures chimiques de l'acétylcholine, nicotine, clothianidine, acetamipride, imidaclopride, thiamethoxame, sulfoxaflor et flupyradifurone.

Le Flupyradifurone est un insecticide systémique, organochloré qui a été interdit dans le traitement des semences à cause de son effet potentiel sur les pollinisateurs comme l'abeille *Apis mellifera*. Des travaux récents ont mis en évidence le fait que le flupyradifurone activait les récepteurs nicotiniques neuronaux des insectes comme les insecticides néonicotinoïdes. Les récepteurs à l'acétylcholine de type nicotinique sont ainsi une cible majeure pour les insecticides néonicotinoïdes et leurs dérivés, les sulfoximines et les buténolides.

En effet, les insecticides néonicotinoïdes agissent comme des agonistes des récepteurs neuronaux à l'acétylcholine de type nicotinique des insectes (**Figure 2**). Ce sont des récepteurs canaux impliqués dans la neurotransmission rapide au niveau synaptique. Le récepteur nicotinique est un pentamère dont le site de liaison de l'acétylcholine est situé à l'interface entre deux sous-unités. Il existe deux types de sous-unité, dite α (car elle possède deux cystéine adjacentes) et non- α (encore appelée β). Par conséquent, le site de liaison de l'acétylcholine est

formé d'une partie principale constituée par la sous-unité α , alors que la partie secondaire du site de liaison est constituée soit d'une sous-unité α ou d'une sous-unité β .

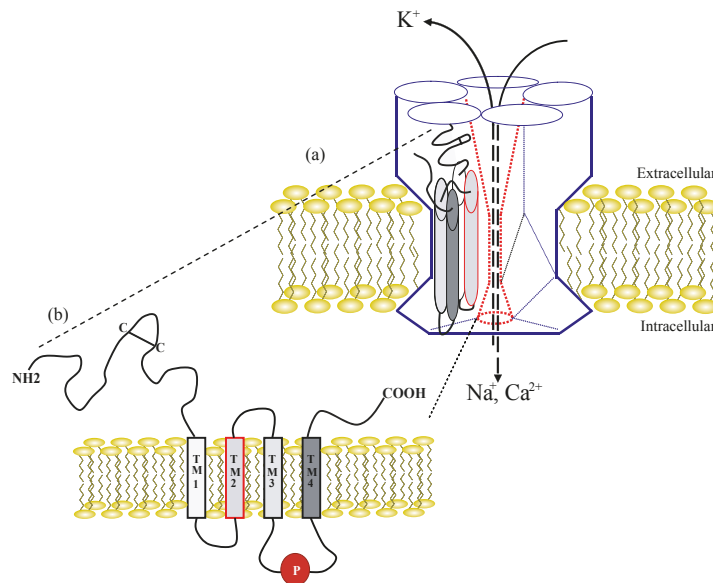


Figure 2: Structure pentamérique du récepteur nicotinique au sein de la membrane plasmique avec les 4 segments transmembranaires. Le site potentiel de phosphorylation situé entre les segments 3 et 4 est représenté en rouge (Modifié d'après Thany *et al.* 2007).

La composition en sous-unité détermine le sous-type de récepteur présent dans le système nerveux des insectes et également la pharmacologie du récepteur. Il existe deux sous-types de récepteurs : homomérique, formé uniquement de sous-unité α , c'est le cas du récepteur $\alpha 7$ qui est généralement bloqué par une toxine, l' α -bungarotoxine. Ce récepteur est décrit chez les mammifères comme étant très perméable aux ions calcium. Le deuxième sous-type de récepteur est dit hétéromérique. Il est constitué de sous-unité α et β dont la stoechiométrie et le nombre peut varier, offrant une complexité importante sur le nombre de sous-type de récepteur présent dans le système nerveux des insectes et la pharmacologie associée. Par exemple, les sous-types présents peuvent être du genre $\alpha 2\beta 2$, $\alpha 4\beta 2$, $\alpha 1\alpha 8\beta 1$, etc. Leur ouverture entraîne le passage d'ions sodium, potassium et calcium de part et d'autre de la membrane plasmique du neurone. Ils sont également impliqués dans les processus de mémorisation et d'apprentissage chez les insectes.

Ces récepteurs ont connu ces 30 dernières années une importance majeure car ils sont la cible des insecticides néonicotinoïdes et de leurs dérivés.

Des travaux de recherche antérieurs ont démontré qu'il existait des récepteurs nicotiniques d'insecte qui étaient insensibles aux insecticides néonicotinoïdes alors que l'insecte n'avait

jamais été mis en contact avec l'insecticide. Ainsi, chez la blatte *Periplaneta americana*, il a été mis en évidence deux populations différentes de récepteurs nicotinique nommés nAChR1 et nAChR2, sensible et insensible à l'imidaclopride. En plus d'avoir des compositions en sous-unités différentes, ces deux types de récepteurs sont modulés par des voies de régulation intracellulaire dépendantes du calcium différentes. Le sous-type nAChR1 serait modulé par une PKA et deux PKC différentes alors qu'une modification de la concentration en AMPc dans le milieu intracellulaire s'accompagne d'une modification de la sensibilité des récepteurs nAChR1 et nAChR2 vis-à-vis de la clothianidine. De manière générale toute modification des voies de régulation intracellulaire dépendantes du calcium semble impacter la sensibilité des récepteurs nicotiniques neuronaux de la blatte face aux insecticides néonicotinoïdes.

Nous avons émis l'hypothèse que des voies de régulation intracellulaire dépendante du calcium pouvaient être un mécanisme supplémentaire permettant aux insectes d'augmenter leur insensibilité (résistance) vis-à-vis des insecticides. Dans ce contexte, plusieurs voies de régulation intracellulaire ont été mises en évidence lors d'études précédentes (**Figure 3**). L'un des points de départ du postulat était une augmentation du calcium intracellulaire et une activation du complexe calcium/calmoduline. Les récepteurs nicotiniques étant perméable aux ions calcium, nous avons émis l'hypothèse que l'entrée du calcium via ces neurones participait en partie au modification de calcium intracellulaire et donc à une activation des voies de régulation intracellulaire dépendantes du calcium.

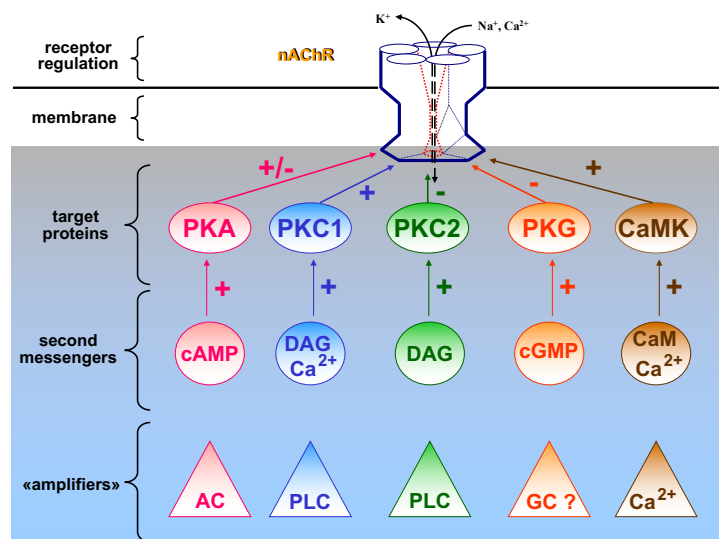
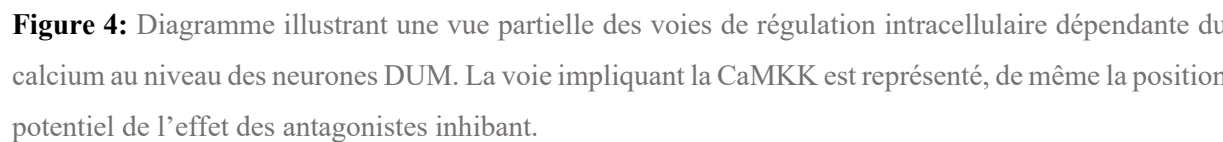


Figure 3: Voies de régulation intracellulaire dépendantes du calcium et impliquées dans la modulation des propriétés pharmacologiques des récepteurs nicotiniques neuronaux expérimentés au niveau des neurones DUM de la blatte *Periplaneta americana* (Modifié d'après Thany *et al.* 2007).

Pour étudier l'impact de ces voies de régulation dans la sensibilité/insensibilité des récepteurs nicotiniques neuronaux des insectes vis-à-vis des insecticides néonicotinoïdes et de leurs dérivés, nous avons pris comme modèle les neurones DUM, situé dans le 6^e ganglion abdominal de la blatte *Periplaneta americana*. Deux techniques ont été utilisées au cours de cette thèse, la technique de patch clamp en condition cellule entière, pour étudier les courants induits par la nicotine et le flupyradifurone sur les récepteurs nicotiniques présents au niveau des neurones DUM. La technique d'imagerie calcique en utilisant une sonde Fura-2 afin d'étudier les variations de calcium intracellulaire suite à l'activation des récepteurs nicotiniques par la nicotine et le flupyradifurone.

Nous avons dans un premier temps, mis en évidence l'existence d'une voie de régulation intracellulaire impliquant une CaMKK (**Figure 4**). Cette voie n'avait jamais été étudiée à ce jour chez les insectes, surtout sa capacité à moduler la pharmacologie des récepteurs nicotiniques exprimés dans les neurones DUM. En effet, en utilisant un inhibiteur de la CaMKK, le STO, nous avons démontré que l'inhibition de la CaMKK entraînait une réduction des courants induits par la nicotine sur les neurones DUM de la blatte, particulièrement lorsque le STO est co-appliqué avec l' α -bungarotoxine (α -Bgt), une toxine qui est connue chez les mammifères pour bloquer préférentiellement les récepteurs nicotiniques homomériques. Cette réduction est contrebalancée par l'application intracellulaire d'un activateur de l'AMPK, le A-76. Le A-76 est également capable d'empêcher la réduction des courants induits par la nicotine, observée avec un autre inhibiteur de l'AMPK, la dorsomorphine. Pour confirmer ces résultats, et différencier l'implication des deux sous-types nAChR1 et nAChR2, nous avons utilisé un activateur sélectif du sous-type nAChR2, le LMA10203. Les courants induits par le LMA10203 ont également été réduits en présence de STO-609. Cette diminution était également inhibé en présence de A-76. Ces résultats suggèrent une implication du récepteur nAChR2 dans la régulation des récepteurs nicotiniques par la voie CaMKK. En utilisant l'imagerie calcique, nous avons constaté que le prétraitement des neurones DUM avec l' α -Bgt bloquait complètement l'augmentation de calcium intracellulaire induite suite à une activation des récepteurs nicotiniques par l'application de nicotine. Cet effet était confirmé en présence de STO-609 et était complètement différent de ce que nous observions avec le courants nicotiniques en électrophysiologie patch clamp. Par contre, l' α -Bgt n'avait aucun effet sur l'augmentation de calcium intracellulaire induite suite à une application de LMA 10203. Mais, la mecamlamine, un inhibiteur des récepteurs nicotiniques, inhibait complètement l'augmentation du calcium intracellulaire induite par le LMA10203.



nicotiques neuronaux exprimés sur les neurones DUM et surtout une implication potentielle des stocks de calcium contenus dans le réticulum endoplasmique.

Chapter I: General introduction

I. Nicotinic acetylcholine receptors (nAChRs) in vertebrates and invertebrates

I.1 History

During the late 19th and early 20th centuries, researchers started to explore the physiological effects of nicotine. Initially, nicotine stimulant properties were noted, leading to its use as a recreational substance. However, it was not until the mid-20th century that scientists started unraveling the molecular mechanisms behind nicotine's actions.

Starting from curare, a plant-derived toxin known for its muscle relaxant properties, was first discovered by the indigenous people in South America. Claude Bernard identified its site of action that cause neuromuscular block in the mid-19th century (Bowman *et al.*, 2006).

Later, between 1906 and 1907 in Cambridge, the English physiologist John Newport Langley studied the actions and interactions of nicotine (NIC) and curare on chicken and frog muscles, including chronically denervated frog muscle. In 1908, he published the "receptive substances" concept for the first time in the Journal of Physiology, explaining how NIC interactions with these substances can induce muscle contraction (Langley *et al.*, 1909; Maehle *et al.*, 2004).

In the early 20th century, researchers like Sir Henry Hallett Dale and Otto Loewi made significant contributions to the understanding of neurotransmission. Dale, along with Loewi, were awarded the Nobel Prize in Physiology or Medicine in 1936 for their work on the chemical transmission of nerve impulses. Loewi's experiments with the parasympathetic vagus nerve stimulation and acetylcholine release in the frog's heart provided crucial insights into the chemical nature of synaptic transmission and the discovery of acetylcholine (ACh). Dale subsequently demonstrated that ACh was released at the neuromuscular junction and played a role in voluntary skeletal muscle contraction (Dale *et al.*, 1936).

In the 1970s, researchers investigated novel models such as the electric eel *Electrophorus electricus* and the torpedo fish *Torpedo marmorata*. These fish possess an electric organ equipped with billions of nerve endings that produce ACh, a neurotransmitter involved in various physiological processes. The investigation of these unique electric organs provided valuable insights into the functioning and characteristics of nicotinic acetylcholine receptors (nAChRs).

Building on earlier research on snake venoms by Lee in 1970, Changeux and colleagues conducted experiments using α -Bungarotoxin (α -Bgt) on the electric eel. α -Bgt was found to block the action of a cholinergic agonist, carbamylcholine, both *in vivo* (on the isolated electroplaque) and *in vitro* (on membrane fragments). This inhibition allowed the identification and characterization of the acetylcholine receptor (Kasai *et al.*, 1970). Subsequent research involved the purification of the protein (Kasai *et al.*, 1971; Meunier *et al.*, 1974; Olsen *et al.*, 1972) and the determination of its molecular weight (Hucho *et al.*, 1973), which contributed to a deeper understanding of the structure and function of nAChRs.

The efforts to determine the amino acid sequence of the ACh-binding subunit of nAChRs began in the late 1970s. A significant contribution came from the work of Devillers-Thiery and colleagues (Thiery *et al.*, 1979). As molecular biology techniques advanced, researchers aimed to clone the genes encoding the various subunits of nAChRs, including the ACh-binding subunit (Thiery *et al.*, 1983; Giraudat *et al.*, 1982). In 1984, the first functional nAChR from *Torpedo californica* were successfully cloned and found to be consisted of four subunits, α , β , γ , and δ based on their molecular weights (Mishina *et al.*, 1984).

I.2 Structure of neuronal nicotinic acetylcholine receptors (nAChRs)

I.2.1 nAChRs molecular composition

Our knowledge about nAChRs structure comes from studies conducted on the electric organ of *Torpedo californica* (Karlin, 2002). High-resolution (4Å) observations and electron microscopy revealed a pentameric structure with total length of ~ 160 Å normal to the membrane plane (**Figure 5**) (Unwin, 1995). Neuronal nAChRs are composed of five subunits and are classified into two subgroups based on their subunit composition: heteromeric nAChRs containing α and β subunits like $\alpha 4\beta 2$ nAChRs, and homomeric nAChRs containing only α subunits, like $\alpha 7$ nAChRs. All α subunits are characterized by the presence of two adjacent cysteines (Cys192 and Cys193) essential for acetylcholine binding (Kao *et al.*, 1984; Karlin *et al.*, 1995; Rubboli *et al.*, 1994; Sargent, 1993; Zouridakis *et al.*, 2009).

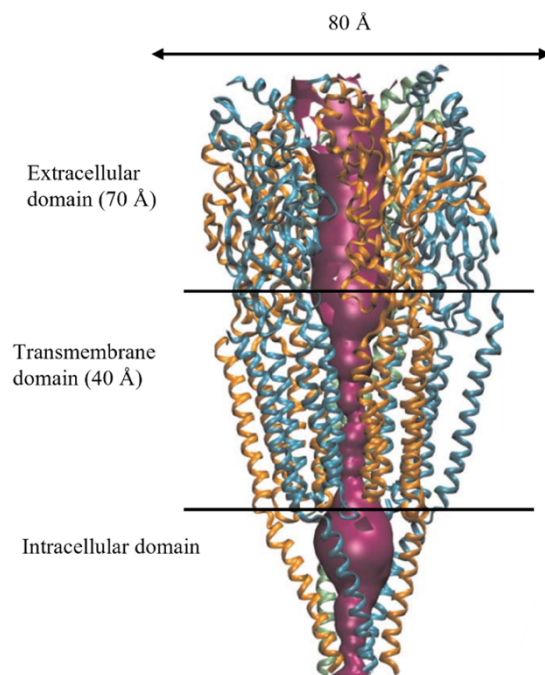


Figure 5: Structural representation of the ACh receptor of the torpedo electric organ. The α subunits are illustrated in orange, and the non- α subunits are illustrated in light blue. The channel pores are represented in purple (modified from Corry *et al.* 2006)

All nAChRs subunits share a basic scaffold composed of: (1) an 70 Å extracellular domain (ECD), with N-terminal domain involved in the binding site of ACh, (2) a hydrophobic transmembrane domain (TMD), that forms a water-filled narrow pore, containing the channel gate, (3) a large intracellular domain (ICD) between the third and fourth transmembrane helix that varies significantly from one subunit to the other, and finally a short extracellular C terminus (**Figure 6**) (Albuquerque *et al.*, 2009; Hucho *et al.*, 1996; Unwin, 2005; Wu *et al.*, 2015).

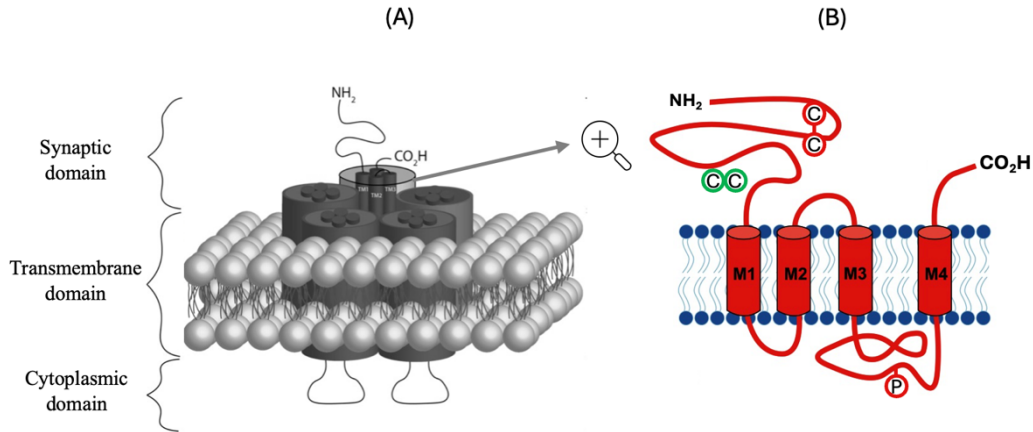


Figure 6: Structure of nAChR subunits. (A) Diagram of ACh receptor organization across the membrane. (B) Representation of the four transmembrane domains composing a receptor subunit. In the N-terminal part lies the disulfide bridge characteristic of ligand-gated ion channels (LGICs) (C) and the two adjacent cysteines (C) characteristic of the α subunits (modified from Posadas *et al.* 2013)

The extracellular domain of each nAChR subunit consists of an N-terminal α -helix and a core of ten β -strands that form an $\alpha\beta$ -sandwich structure organized around a hydrophobic core of 10 β -strands, joined through the Cys-loop, and has around 200 amino acids long relatively hydrophilic extracellular amino terminal. The 15-amino acid cystine loop is necessary for a complete nAChR assembly and for the conformational change of α subunit and the addition of unassembled subunits (Green *et al.*, 1997; Unwin, 2005).

The TMD of each nAChR subunit is made up of four membrane spanning α -helical domains (M1–M4). The M2 α -helices of all five subunits are ~ 40 Å long and shape the central conduction path, whereas the M1, M3, and M4 α -helices coil around each other and form an outer ring, shielding the inner ring of the M2 domains from the membrane lipids (Unwin, 2005).

The intracellular domain consists of two cytoplasmic loops: (1) a short intracellular segment between M1 and M2 domains that contain a single cysteine residue, and (2) a large cytoplasmic loop between M3 and M4 domain that contain 11 cysteine residues. The M3-M4 loop varies in length between the different subunits (110–270 amino acid residues) and contains a curved α -helix (MA) prior to the M4 helix. The MA α -helices of all five subunits shape the wall of the intracellular vestibule, which similarly to the extracellular vestibule is 20 Å wide (Amici *et al.*, 2012; Unwin, 2005). Each subunit of the nAChR has unique sequences in M3-M4 cytoplasmic loop, which act like fingerprints. Residues in the intracellular end of M4 were found to affect

nAChRs electrophysiological and pharmacological properties (Kracun *et al.*, 2008; Zouridakis *et al.*, 2009). For example, nAChRs expression was abolished when amino sequences were removed from two regions of the M3-M4 loop: a 29-amino acid segment near the N-terminus adjacent to M3 and an 11-amino acid segment near M4. These regions were found to be crucial for nAChR expression (Hales *et al.*, 2006; Valor *et al.*, 2002).

In addition, several phosphorylation sites in the M3-M4 cytoplasmic loop of the $\alpha 4$ subunit have a crucial role in regulating agonist selectivity of $\alpha 4\beta 2$ nAChRs. S516D and T536A mutations in the $\alpha 4$ subunit of $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes, significantly reduced the receptors' sensitivity to nicotine without affecting their sensitivity to ACh (Labiosa *et al.*, 2015).

[1.2.2 nAChRs subunit's diversity in both mammals and insects](#)

While our knowledge about nAChRs subunits is largely based on research conducted in mammals, the structural characteristics identified in these studies are thought to be conserved across species, including insects.

In mammals, two groups of nAChRs have been identified: muscular and neuronal. Muscular nAChRs are heteromeric receptors consisting of five subunits: one $\alpha 1$ and four non- α subunits ($\beta 1$, δ , γ , ϵ) (Albuquerque *et al.*, 2009), while neuronal nAChRs are composed of 12 subunits ($\alpha 2$ - $\alpha 10$, $\beta 2$ - $\beta 4$) that can form a diverse set of receptors with distinct composition (Albuquerque *et al.*, 2009; Millar *et al.*, 2009). The majority of these subunits can only co-assemble with other types of subunits to generate functional receptors (Zhao *et al.*, 2020). For example, in rats nAChRs, $\alpha 2$, $\alpha 3$, and $\alpha 4$ subunits can assemble with the $\beta 2$ subunit to form functional $\alpha 2\beta 2$, $\alpha 3\beta 2$, and $\alpha 4\beta 2$ receptors (Boulter *et al.*, 1987; Kouvatsos *et al.*, 2016; Wada *et al.*, 1988). However, $\alpha 7$, $\alpha 8$, and $\alpha 9$ have shown to form homomeric receptors rather than heteromeric ones, along with their ability to assemble with other subunits and form heteromeric receptors (**Figure 7**) (Azam *et al.*, 2003; Elgoyhen *et al.*, 1994; Millar *et al.*, 2009; Zhao *et al.*, 2020).

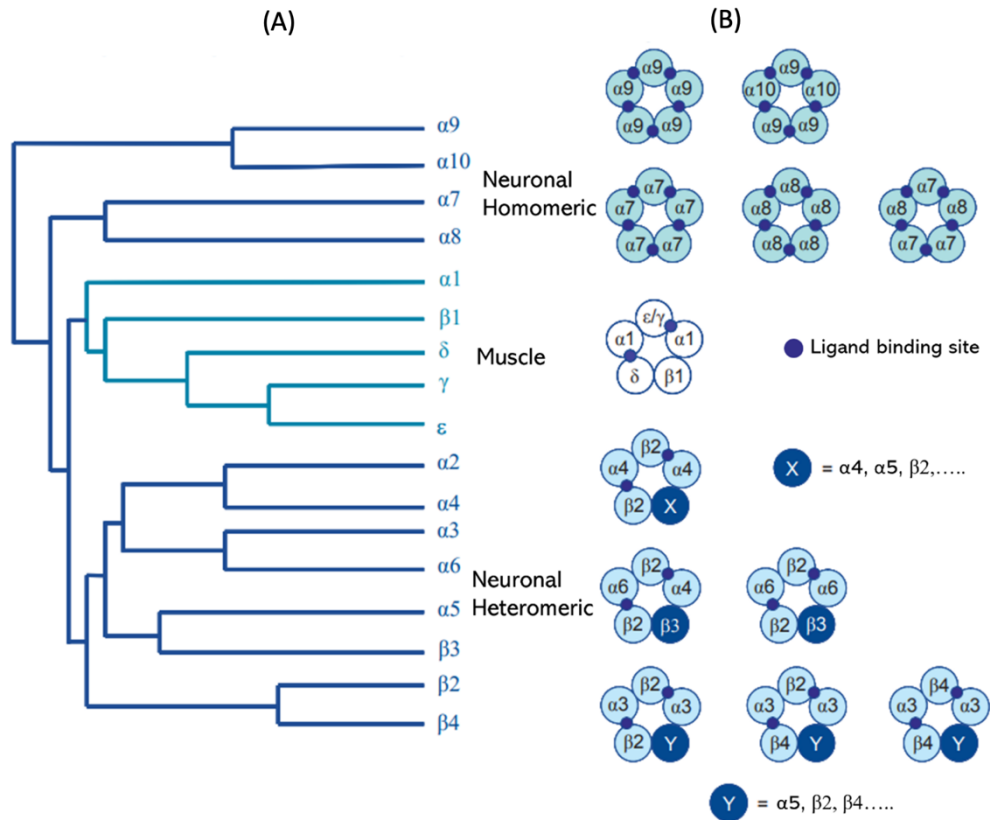


Figure 7: Heterogeneity of vertebrates nAChRs. (A) Phylogenetic relationship of all cloned vertebrate nAChRs subunits to date. (B) Viable subunit combinations, with agonist binding sites shown in dark blue (modified from Wonnacott *et al.* 2007).

Contrary to mammals, insects have only neuronal nAChRs. In fact, their synaptic transmission at the neuromuscular junction is glutamatergic (Usherwood, 1994; Vo *et al.*, 2010). Using sequence data from various vertebrate nAChRs, different orthologous genes have been identified in insects. The first studies conducted on *Drosophila melanogaster* identified the first insect α subunit, named alpha like subunit (ALS), which was later renamed as the alpha subunit (α), and subsequently various other subunits were identified. Currently, 10 subunits have been identified in *D. melanogaster*: 7 of the α type (Dm α 1-Dm α 7) and 3 of the non- α type (Dm β 1-Dm β 3) (Bossy *et al.*, 1988). Genes encoding nAChR subunits have also been identified in other insects, for example, 6 genes in the aphid *Myzus persicae* (Bass *et al.*, 2011; Sgard *et al.*, 1998), 10 in the mosquito *Anopheles gambiae* (Jones *et al.*, 2005), 11 in the aphid *Acyrtosiphon pisum* (Dale *et al.*, 2010; Liu *et al.*, 2013), 11 in the honeybee *Apis mellifera* (Jones *et al.*, 2006), 12 in the butterflies *Bombyx mori* (Shao *et al.*, 2007), 14 in the mosquito *Aedes aegypti* (Matthews *et al.*, 2018), and 16 in the parasitoid wasp *Nasonia vitripennis* (Jones *et al.*, 2010). To date, the largest families of nAChR-subunit encoding genes were identified in the genomes of *Blattella germanica* and *Periplaneta americana*, with 17 and 19 genes,

respectively (Jones *et al.*, 2021). In addition, studies showed that insects nAChRs diversity can be significantly enhanced through alternative splicing and mRNA editing, which produce species-specific subunit isoforms (Ho *et al.*, 2020; Jones *et al.*, 2007; Jones *et al.*, 2021; Millar *et al.*, 2007; Vo *et al.*, 2010).

Insect nAChRs subunits can be classified based on their sequence homology which refers to the degree of similarity in the amino acid sequences of drosophila nAChRs subunits (Jones *et al.*, 2007). Dmel $\beta 1$, Amel $\beta 1$, and Agam $\beta 1$ subunits are the most conserved, with 80% homology. However, divergent subunits with less than 20% homology have also been identified in many insect species, such as *D. melanogaster* (Dmel $\beta 3$) (Lansdell *et al.*, 2000), *A. gambiae* (Agam $\alpha 9$) (Jones *et al.*, 2005), *A. mellifera* (Amel $\alpha 9$ and Amel $\beta 2$), *A. pisum* (Apis $\alpha 9$, Apis 10, and Apis $\beta 2$) (Jones *et al.*, 2006), and *T. castaneum* (Tcas $\alpha 9$ and Tcas $\alpha 10$). While the other insect species have between 1 and 7 divergent subunit genes, *B. germanica* and *P. americana* possess eight and ten divergent nAChR subunits, respectively (Jones *et al.*, 2021). Compared to the other subunits, divergent ones have a shorter intracellular loop between the M3 and M4 segments (Jones *et al.*, 2010). Studies also showed that $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 8$, and $\beta 1$ subunits diverge from a common ancestor but distinct from that of vertebrates, whereas $\alpha 5$, $\alpha 6$, and $\alpha 7$ subunits are very close phylogenetically and share a common ancestor with vertebrate $\alpha 7$ and $\alpha 8$ (**Figure 8**) (Thany *et al.*, 2007).

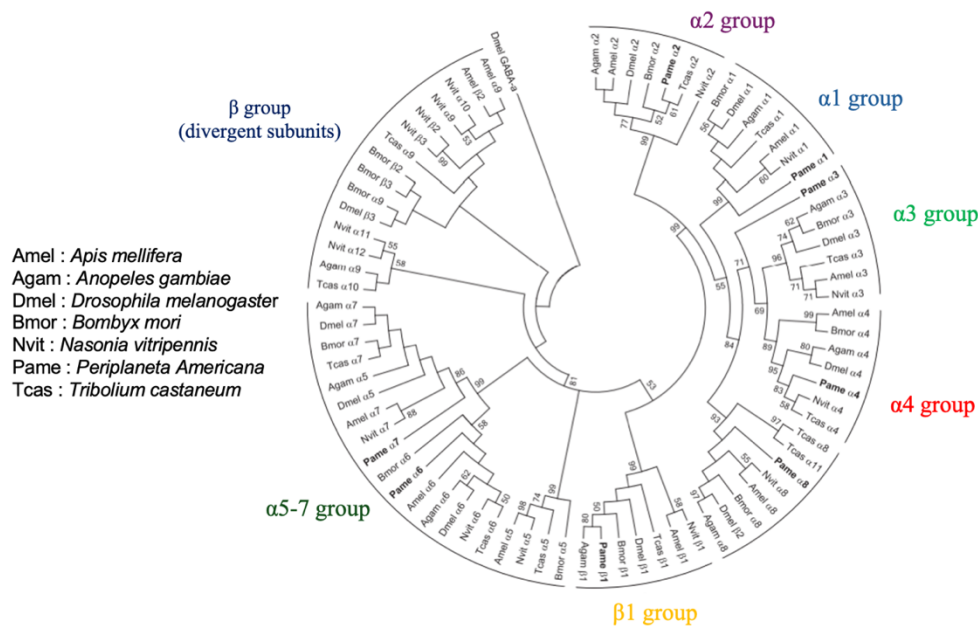


Figure 8: Phylogenetic tree of nAChRs subunits in different insect species (modified from Taillebois & Thany 2016).

I.2.3 The ion channel function of nAChR

The five subunits of nAChRs are arranged around a central ion-conducting pore that becomes permeable to cations including Na^+ , K^+ and Ca^{2+} when the receptors are activated (Wang *et al.*, 2008). These subunits possess different amino acids forming two types of rings, charged and uncharged:

- The negatively charged rings, consisting of negatively charged amino acid residues lined in the inner wall of the 20 Å extracellular vestibule in the extracellular domain present at the M2 level which allow the ionic selectivity of the channel (Arias, 1997; Karlin, 2002; Zouridakis *et al.*, 2009).
- The uncharged rings, composed of valine (V), leucine (L), serine (T), and threonine (S) and located in the extracellular and intracellular domains (Arias, 1997; Imoto, 1988; Karlin, 2002).

The main lining of the ionic pore is provided by α helices of the M2 segments in each subunit with additional contribution from the α helices of the M1 segments where the pore widens. The central pore has a diameter of approximately 25 Å at the extracellular entrance (synaptic entry) of the channel and narrows to about 6 Å at the transmembrane level. It extends 60 Å from the membrane surface into the synaptic cleft (**Figure 9**) (Corringer *et al.*, 2000; Dani, 2015; Unwin, 2012). Site-directed mutagenesis experiments have shown that the M2 domain lines the ion channel and contains specific residues important for the ion selectivity, permeability and channel gating of the receptors (Zoli *et al.*, 2015). For example, in mouse neuronal nAChRs expressed in *Xenopus* oocytes, when leucine to alanine substitution (L9'A) was introduced in the M2 of $\alpha 4$ subunit, nAChRs sensitivity to both ACh and nicotine was increased, and nicotine concentration required to gate the receptor was decreased (Albuquerque *et al.*, 2009; Fonck *et al.*, 2005).

In addition, E237, L254, and L255 residues were found to be crucial for the receptor's Ca^{2+} permeability in chicken $\alpha 7$ neuronal nAChRs expressed in *Xenopus* oocytes (Bertrand *et al.*, 1993; Galzi *et al.*, 1992). L254R/T and L255R/T/G mutations reduced Ca^{2+} permeability and affected the affinity for ACh, the rates of both currents onset and desensitization. On the other hand, E237A mutation abolishes Ca^{2+} permeability without significantly affecting other pharmacological properties of the ACh responses (Bertrand *et al.*, 1993; Galzi *et al.*, 1992). Furthermore, mutations of the highly conserved L247 residue decreased desensitization rate,

increased the affinity for ACh, and abolished current rectification in chicken $\alpha 7$ neuronal nAChRs expressed in *Xenopus* oocytes (Revah *et al.*, 1991).

Similar experiments were performed in both *Drosophila melanogaster* and *Nasonia vitripennis*, demonstrated that changes residues in the M2 of Dm $\alpha 6$ and Nv $\alpha 6$ subunit can alter the ion channel properties of the receptor. Indeed, alternative splicing of exon 8, which introduces an asparagine (N) residue at a position homologous to L254 in the chicken $\alpha 7$ subunit within the M2 region of the Dm $\alpha 6$ subunit, can influence the receptor's ion selectivity, permeability, and gating mechanisms (Grauso *et al.*, 2002; Jones *et al.*, 2010).

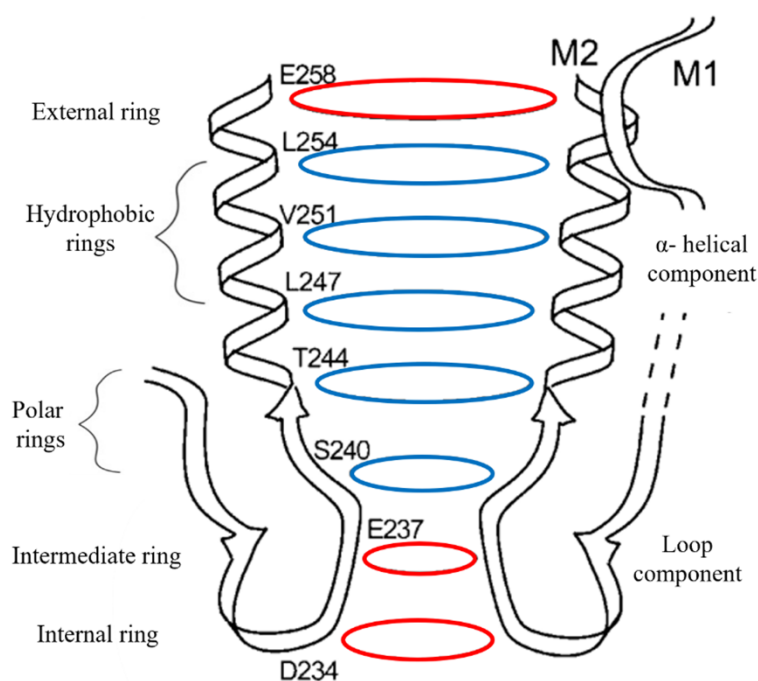


Figure 9: A model for the structural and functional organization of the $\alpha 7$ - receptor ion channel. The rings (circles) are formed by several homologous amino acids residues facing the lumen of the ion channel. Negatively charged rings are in red, while positively charged rings are symbolized in blue (modified from Corringer *et al.* 2000).

I.2.4 The conformational states of nAChRs

When an agonist binds to the orthosteric site of nAChRs, it significantly impacts the transition rates between various nAChRs conformational states. In general, nAChRs can exist in three primary conformational states: closed, open, and desensitized (**Figure 10**). The rate constant between these states is highly dependent on the subunits combination and the agonist's molecular structure (Dani, 2015; Hurst *et al.*, 2013).

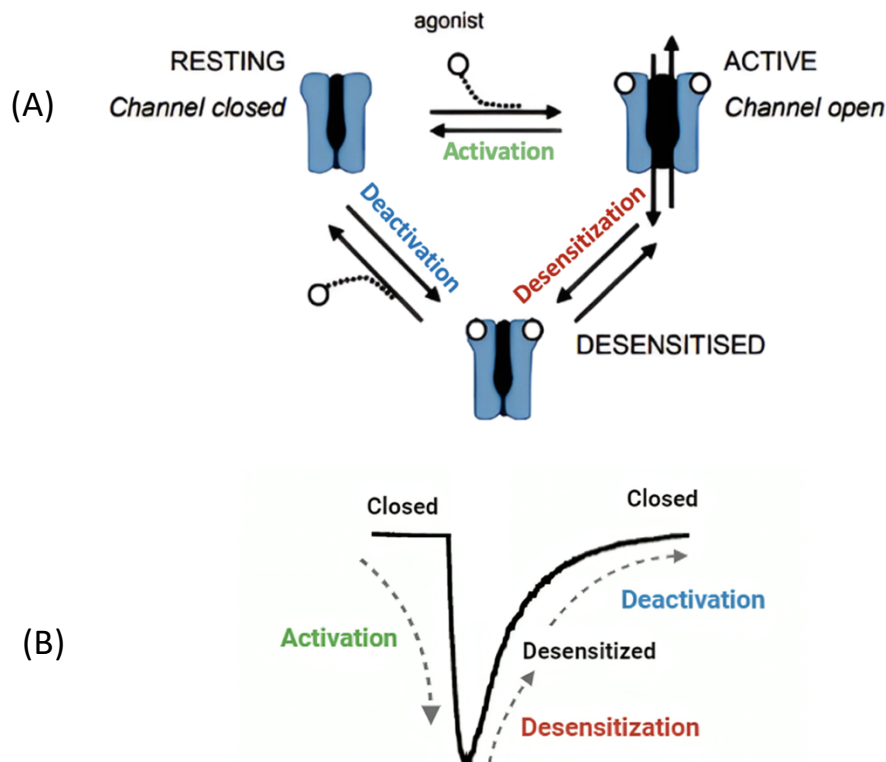


Figure 10: (A) Relationship between the major conformational states of nAChRs. (B) a representative trace of an inward current after an agonist pulse application displaying the three key states: activation, desensitization, and deactivation (modified from Wonnacott *et al.* 2007).

In the open (activation) state, agonist binds and stabilizes the open conformation of the nAChR channel allowing the permitting of small cations for several milliseconds before closing back to a resting state (deactivation) or to a non-conducting state (desensitization) (Giniatullin *et al.*, 2005).

Desensitization is a reversible reduction in nAChRs response where nAChRs become desensitized due to sustained agonist exposure or/and increasing agonist concentration. Both

desensitization rate and the recovery from desensitization rate are highly dependent on the subunit composition of nAChRs as well as on the agonists chemical properties. For example, in mammals, $\alpha 7$ nAChRs have a high desensitization rate (in milliseconds), while non- $\alpha 7$, like $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors, have a slower desensitization rate (in seconds) (Dani, 2015; Giniatullin *et al.*, 2005).

At the resting state, when nAChR is ligand-free (closed state), the channel has a funnel shape with a narrower part formed by the Gly240, Glu241, Lys242 residues of the α loops, and the Met243 and Thr244 residues of the M2 segment (Wilson *et al.*, 2001).

When a ligand binds to nAChRs, the receptors undergo a conformational change, resulting in the opening of the channel which induces the rotation of the β sheets in the extracellular domain, followed by a 15-16° clockwise rotation of the helices in the M2 segments. The residues of the ionic barrier then move apart, and the channel diameter at this level increases from 3 to 8 Å, allowing ions to pass through (Sansom, 1995; Unwin *et al.*, 2002).

In this transition from closed to activated pre-open intermediate state, studies showed that loop C from the principal face plays a crucial role. Indeed, in the resting nAChR when no agonist is binding, the loop C found to be in an extended (open) conformation, whereas in the presence of full agonists, loop C is contracted (closed) capping the entrance to the binding cavity, and trapping the agonist (Arias, 2012; Sine *et al.*, 2006).

When the ligand is applied for a prolonged period at the binding site, the receptor enters the non-conducting (desensitized) state. During this phase, the ionic barrier extends from Gly240 to Leu250 rather than being confined to the Gly240-Leu242 region. The receptor then returns to a closed state and regains its sensitivity to the ligand (Wilson *et al.*, 2001) (**Figure 11**).

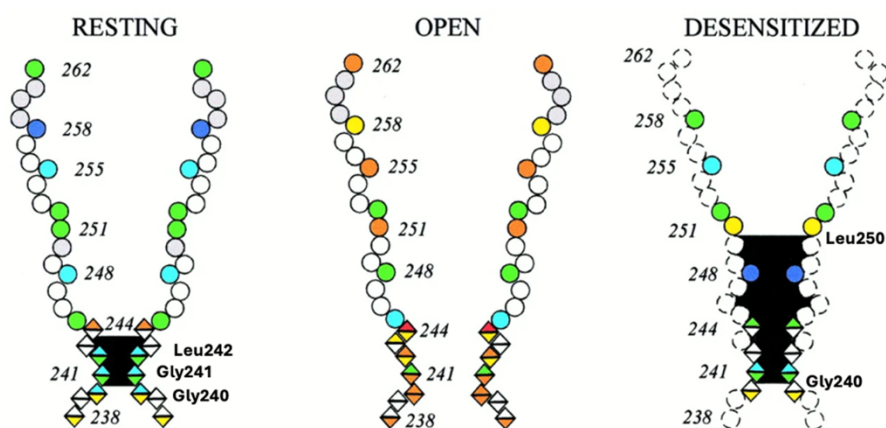


Figure 11: Conformational changes of nAChR and the arrangement of pore residues in the closed, open, and desensitized states. The black shading indicates the extent of the ion barrier, with the residues defining this barrier shown in bold (modified from Wilson *et al.* 2001).

Cations flowing across the cell membrane results in an inward current, which can be measured using electrophysiological techniques like patch clamp (Dani, 2015; Fieber *et al.*, 1991). I-V curve, or current-voltage curve, is a fundamental graph illustrating the relationship between the current (I) that flows through a nAChRs and the different applied voltages (V) across these channels. This relationship is crucial for understanding the behavior of nAChRs ion channels under different conditions. The I-V curve can have different phases, a linear phase where the current changes linearly with the applied voltage, and the I-V curve appears as a straight line; and a non-linear phase, where the I-V curve can have S, bell, or another nonlinear shape on a graph (Christophe *et al.*, 2002; Palma *et al.*, 1999; Stanchev *et al.*, 2011; Weltzin *et al.*, 2010).

1.2.5 Ligand binding sites

➤ The orthosteric binding sites

Orthosteric ligands are compounds that can bind to the same site as that for the endogenous neurotransmitter ACh, so called orthosteric sites. Orthosteric binding sites are located in the extracellular domain at the interface between two adjacent subunits, the principal and the complementary sites (**Figure 12**) (Changeux, 2018). In all nAChRs, the principal subunit is α , while the complementary subunit is α or non- α subunit (Ho *et al.*, 2020; Wu *et al.*, 2015). As an example, in the mammalian brain, the heteromeric $\alpha 4\beta 2$ receptors can carry two or three

orthosteric binding sites at the α/β subunit and α/α interfaces, whereas homomeric $\alpha 7$ nAChRs have five potential orthosteric binding sites at the α/α subunit interfaces (Barrantes, 2023).

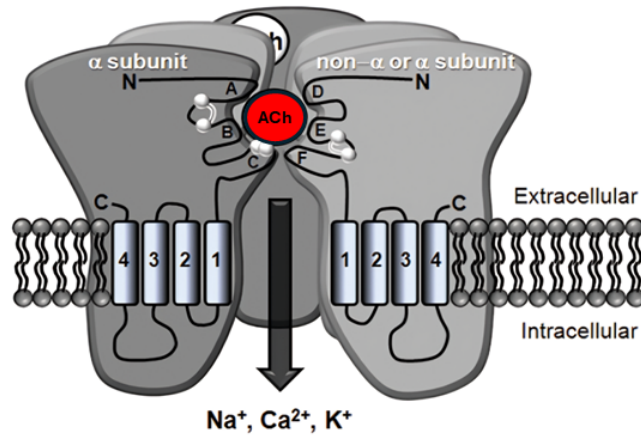


Figure 12: Representation of the ACh (in red) binding site at the interface between two subunits. The site is formed by loops A, B, C of the α subunit (characterized by the two adjacent cysteines) and loops D, E and F of the α or non- α subunit (modified from Jones *et al.* 2010).

Residues from loops A, B, and C of the principal subunit, as well as β -strands from loop D, E, and F of the complementary subunit contribute to ligand recognition (**Figure 13**) (Ho *et al.*, 2020; Wu *et al.*, 2015). Each ligand-binding site has two sides:

- The positive/front side: it can determine the ligand affinity and it is produced by an α subunit, like $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, or $\alpha 9$ in mammals. These α subunits contain the essential Cys-Cys loop. In addition to this loop, key tyrosine residues in the α subunit, $\alpha\text{Tyr } 93$, $\alpha\text{Tyr } 190$, and $\alpha\text{Tyr } 198$, are essential for ligand binding, along with tryptophan at the 149 position ($\alpha\text{Trp}149$). However, there is an exception with $\alpha 5$ subunit, where the essential Tyr 190 is replaced by Asp 190. This substitution explains the $\alpha 5$ subunit's inability to bind ligands (Albuquerque *et al.*, 2009; Karlin, 2002).
- The negative/back face: it can determine ligand selectivity and it can be composed by α or β subunits. For example, in mammals, it is composed by at least three amino acids of each the $\alpha 10$, $\beta 2$, $\beta 4$ subunits. As in the positive face, there are required residues for binding which are L112, M114, and Trp53 (Albuquerque *et al.*, 2009).

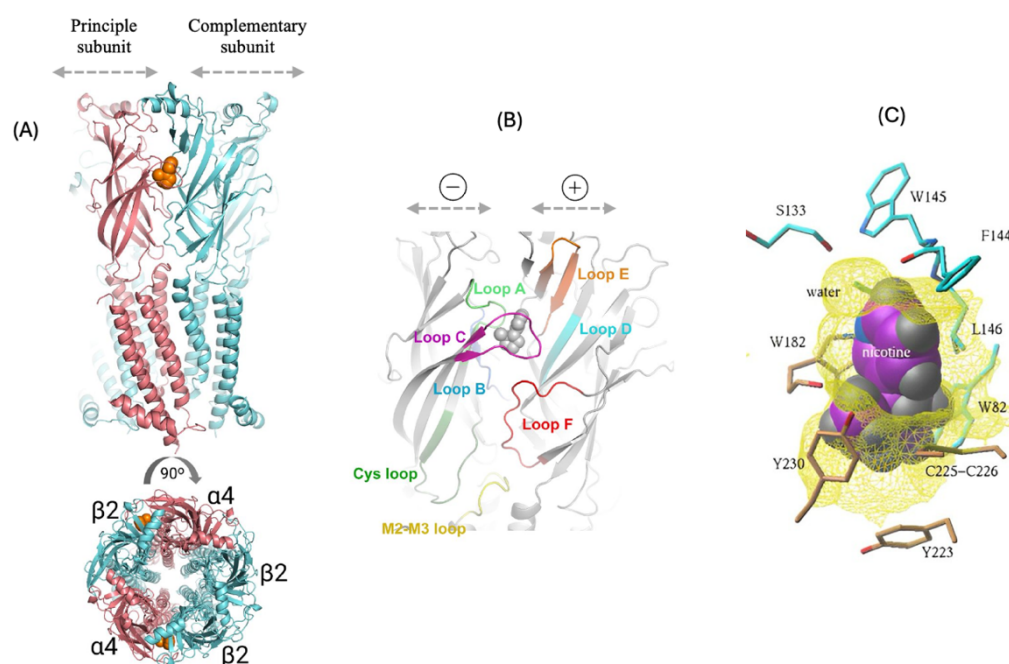


Figure 13: X-ray structure of the human $(\alpha 4)_2(\beta 2)_3$ receptor. (A) Lateral and top view of $(\alpha 4)_2(\beta 2)_3$ stoichiometry as the receptor is formed by two $\alpha 4$ (red) and three $\beta 2$ subunits (blue), with nicotine molecules occupying the two orthosteric binding sites (orange) (B) Structural motifs involved in agonist binding, loop A, loop B, and loop C from the principal subunit, and loop D, loop E, and loop from the complementary subunit. (C) Nicotine docked to the agonist binding site of human nAChR $\alpha 4\beta 2$ (modified from Oliveira *et al.* 2022 and Schapira *et al.* 2002).

Orthosteric ligands may also have different efficacy towards different nAChRs stoichiometries. For instance, ACh and nicotine bind with high affinity to $(\alpha 4)_2(\beta 2)_3$ stoichiometry, while they bind with low affinity to $(\alpha 4)_3(\beta 2)_2$ stoichiometry (**Figure 14**) (Eaton *et al.*, 2014; Pandya *et al.*, 2011).

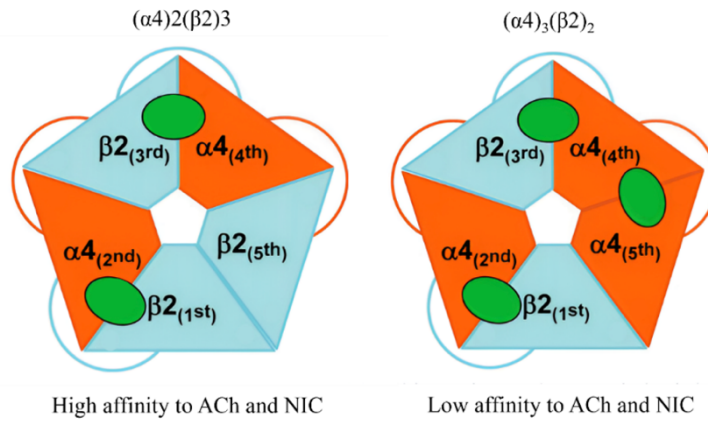


Figure 14: Schematic for both $\alpha 4\beta 2$ -nAChRs stoichiometries, with the orthosteric binding sites illustrated in green (modified from Eaton *et al.* 2014).

The discovery and crystallization of Acetylcholine binding protein (AChBP) allowed the study of the ACh binding site of nAChRs and ligand-protein interactions in more details (Brejc *et al.*, 2001; Smit *et al.*, 2001). AChBP is a soluble protein first isolated from the freshwater snail *Lymnaea stagnalis* (Ls-AChBP) which can bind to ACh, and modulate synaptic transmission at the cholinergic synapses (Brejc *et al.*, 2001). To date, different AChBPs have been identified, for example, Ac-AChBP from the marine mollusk *Aplysia californica* (Hansen *et al.*, 2005), Bt-AChBP from the freshwater snail *Bulinus truncates* (Celie *et al.*, 2005), and the marine worm *Capitella teleta*. (Billen *et al.*, 2012). Compared to nAChRs, AChBP is most closely related to the α subunit. It forms a homopentamer of 210 residues in length, homologous to the N-terminal extracellular domain of nAChRs, but it lacks a transmembrane domain or intracellular domain. Therefore, AChBP is used as an example of the N-terminal domain of an α -subunit nAChRs (Brejc *et al.*, 2001). Nearly all residues that are conserved within the nAChRs family are present in AChBP, including those that are relevant for ligand binding (Brejc *et al.*, 2001). Moreover, AChBP binds many nAChRs agonists and antagonists such as acetylcholine, nicotine, d-tubocurarine and α -bungarotoxin. For that reason, Ls-AChBP and Ac-AChBP are used as models to study interactions of nicotinic receptors with their ligands (Brejc *et al.*, 2001).

➤ [The allosteric modulatory sites](#)

Allosteric modulators are ligands able to modulate nAChRs function by binding to sites that are topographically distinct from orthosteric sites and known as allosteric sites (**Figure 15**) (Chatzidaki *et al.*, 2015). These allosteric sites are distributed throughout each domain of the receptor molecule. For example there are: 1) Ca^{2+} binding site, located in the extracellular domain (ECD) where Ca^{2+} can potentiates most neuronal nAChRs; 2) Transmembrane binding (TM) sites, located at the transmembrane domain (TMD), where the positive or negative allosteric modulators (PAMs or NAMs) can bind; and 3) intracellular sites, located in the intracellular domain (ICD) and play important roles in nAChRs function like, clustering, stabilization, and modulation (Changeux, 2018).

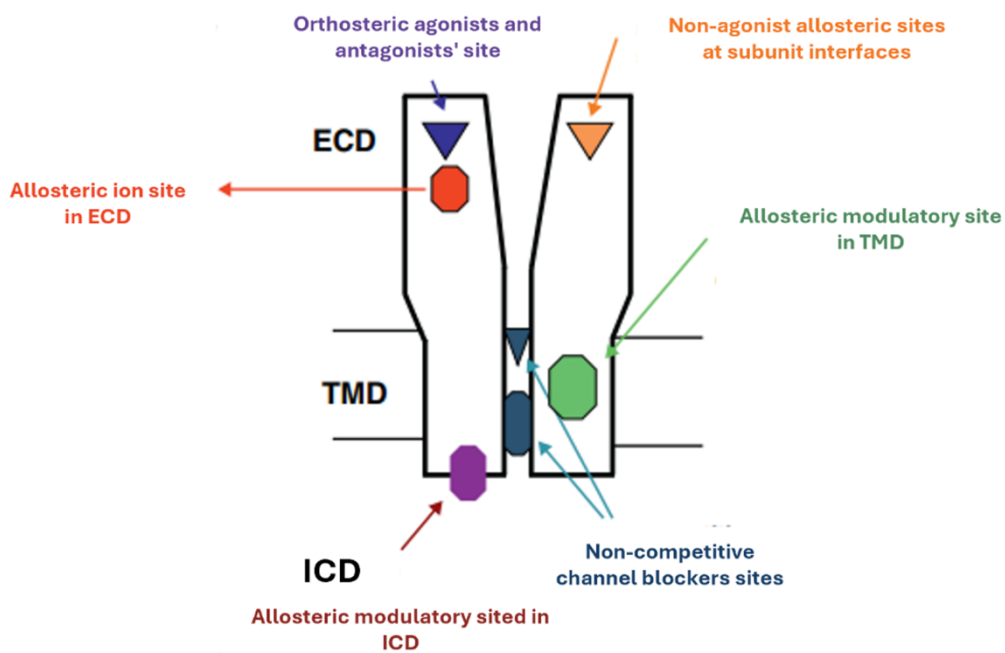


Figure 15: Diagrammatic representation of the different classes of orthosteric and allosteric sites in pentameric ligand-gated ion channels. ECD: extracellular domain; TMD: transmembrane domain; ICD: intracellular domain (modified from Changeux *et al.* 2013).

The MWC model has been proposed for ion channel's activation and deactivation by Jacques Monod, Jeffries Wyman, and Jean-Pierre Changeux in 1965 (Monod *et al.*, 1965). This model was the first to suggest that the allosteric ligand-gated ion channels exist in reversible equilibrium between a few, at least two, global conformational states, namely a relaxing (R) open-channel state and a tensed (T) closed-channel state, even in the absence of agonist. In this model the effect of the ligand is to stabilize the conformation of the receptor for which it has the highest affinity (Cecchini *et al.*, 2015).

While ACh binding for example shifts the equilibrium towards the R state, allosteric modulators can stabilize either the T or R state, thus influencing the receptor's activity (**Figure 16**).

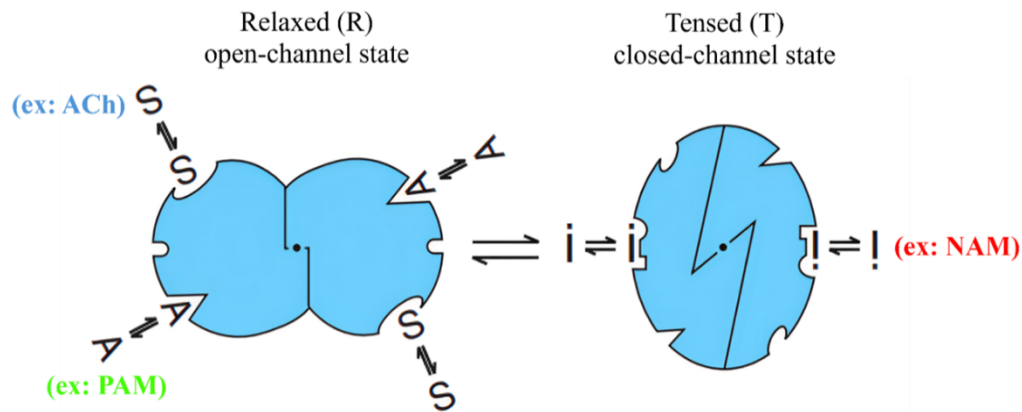


Figure 16: The MWC model illustration. Initial models of nAChRs transitions between two states, R (relaxed) and T (tensed). Three classes of molecules are shown that bind to nAChRs: A (activator), I (inhibitor), and S (substrate); ACh, acetylcholine; PAM, positive allosteric modulator; NAM, negative allosteric modulator (modified from Changeux *et al.* 2005).

Allosteric modulators can have positive or negative effects on the nAChRs, by increasing or decreasing the response induced by agonist, respectively (Pandya *et al.*, 2011). Depending on their modulatory effects and binding sites, they were classified into two groups:

- **Positive allosteric modulators (PAMs):** they are identified as ligands that stabilize the open ion conducting conformation (R state) of the receptor channel induced by the agonist. They increase agonist efficacy by reducing the energy barrier between closed and open states or by increasing the energy barrier between open and desensitized states (Chatzidaki *et al.*, 2015; Pandya *et al.*, 2011).

PAM are classified in two types (type I and type II) depending on their effects on the receptor desensitization (Chatzidaki *et al.*, 2015):

1. **Type I PAMs:** they increase the agonist-induced currents without effecting the receptors desensitization (Chatzidaki *et al.*, 2015; Hu *et al.*, 2009). Ivermectin, anthelminthic, Genistein, 5-hydroxyindole (5-HI), and NS-173, are well-known examples of Type I PAMs (Gronlien *et al.*, 2007; Krause *et al.*, 1998; Sattelle *et al.*, 2009).
 2. **Type II PAMs:** they can do both, increase the peak-currents amplitude and reduce the desensitization rate (Chatzidaki *et al.*, 2015). Examples of Type II PAMs are PNU-120596, TQS, A-867744, and JNJ-193094 (Dinklo *et al.*, 2010; Gronlien *et al.*, 2007; Hurst *et al.*, 2005; Malysz *et al.*, 2009).
- **Negative allosteric modulators (NAMs):** contrary to the PAMs, NAMs stabilize the non- conducting conformation of the receptor (T state), in other words, they reduce the average duration of channel opening without altering the agonist's binding to the receptor. NAMs are also frequently referred to as non-competitive antagonists (Millar *et al.*, 2015; Pandya *et al.*, 2011), and classified into two groups depending to their mechanisms of action:
 1. Antagonists that bind in the channel pore and block ions flow. This group is also known as open-channel blockers. Mecamylamine is a well-known example of this group (Crooks *et al.*, 2014; Papke *et al.*, 2001).
 2. Antagonists that modulate receptor properties such as gating or desensitization equilibrium like menthol, clozapine, zinc, and progesterone (Ashoor *et al.*, 2013; Chatzidaki *et al.*, 2015; Singhal *et al.*, 2007).
 - **Silent allosteric modulators (SAMs):** also known as neutral allosteric ligands, they bind to the receptor but have no effect on orthosteric agonist affinity or efficacy, they neither potentiate nor inhibit the responses to orthosteric agonists. On the other hand, they can competitively inhibit other allosteric compounds (PAMs, NAMs or allosteric agonists) by binding to the same allosteric site (Burford *et al.*, 2013; Chatzidaki *et al.*, 2015; Noblin *et al.*, 2012).

I.3 nAChRs pharmacological properties

I.3.1 The impact of subunits composition on nAChRs pharmacological properties

It has been shown that the nature of the subunits composing nAChRs determines the pharmacological properties of the nAChR subtypes. For instance, $\alpha 2$ - $\alpha 4$, $\alpha 6$, and $\alpha 7$ are involved in ligand binding, while $\alpha 5$, $\beta 3$, $\alpha 3$, $\alpha 6$, $\beta 2$, $\beta 4$ are accessory subunits, which means they do not directly participate in forming the binding site, but they can alter the receptor's pharmacological and biophysical properties (Crespi *et al.*, 2018; Jain *et al.*, 2016; Moroni *et al.*, 2008; Zhao *et al.*, 2020). As a result, each specific combination of nAChRs subunits form functional receptor that has unique pharmacological properties.

In heteromeric receptors, when mammals $\beta 2$ and $\beta 4$ subunits expressed with the $\alpha 3$ subunit in *Xenopus* oocytes, they formed pharmacologically distinct receptors. $\alpha 4\beta 2$ nAChRs had no affinity to the nAChRs agonist choline, while $\alpha 3\beta 4$ nAChRs were partially activated by choline. In addition, $\alpha 3\beta 2$ receptor exhibits high sensitivity to Dihydro- β -erythroidine (DH β E) antagonist, while $\alpha 3\beta 4$ receptors showed a very low sensitivity to it. These differences in sensitivity were attributed to the specific residue at position 59 in the $\beta 2$ subunit (threonine) compared to the $\beta 4$ subunit (lysine). Indeed, changing threonine 59 in $\beta 2$ to lysine, as found in $\beta 4$, leads to a substantial loss of sensitivity to DH β E (Alkondon *et al.*, 1993; Alkondon *et al.*, 1997; Harvey *et al.*, 1996; Papke *et al.*, 1996). Studies also demonstrated that nAChRs containing $\alpha 4$ subunit exhibits high affinity to nicotine, with $\alpha 4\beta 2$ nAChRs subtype having the highest affinity for nicotine in mammalian brain (Gotti *et al.*, 2013; Millar *et al.*, 2008).

Even homomeric nAChRs showed different pharmacological profile when they were expressed in *Xenopus* oocytes. Chicken $\alpha 8$ subtype exhibits greater sensitivity to agonists compared to the $\alpha 7$ subtypes. For example, 1,1-dimethyl-4-phenylpiperzinium iodide (DMPP) functions as a full agonist for the $\alpha 8$ subtype but acts as only a partial agonist for the $\alpha 7$ subtype (Bertrand *et al.*, 1992; Gerzanich *et al.*, 1994). Comparing to $\alpha 4\beta 2$ nAChRs, $\alpha 7$ homomeric nAChRs have low affinity to nicotine but they have the highest permeability to calcium ions and the highest rate of desensitization, suggesting their importance in regulating intracellular signaling and neurotransmitter release from neurons (Bertrand *et al.*, 1993; Seguela *et al.*, 1993; Zhao *et al.*, 2020).

Unlike mammalian receptors, expression of insect nAChRs in heterologous expression systems is very limited. To overcome this expression problem, insect α subunits are generally coupled with mammalian β subunits, forming a chimeric receptor. This coupling of insect α subunits and mammalian β subunits has allowed the study of certain receptor combinations in insects and their pharmacological properties. For example, the heterologous expression of the drosophila Dm α 2 subunit with the rat β 2 subunit shows a sensitivity to IMI that is 37 times greater than the rat α 4 β 2 receptors (Matsuda K *et al.*, 1998), suggesting that Dm α 2 subunit likely assembles with another subunit in drosophila to form a functional heteromeric receptor.

Studies also showed that subunits composition in drosophila play a fundamental role in nAChRs sensitivity to α -Bgt. Indeed, using immunoprecipitation tests and affinity chromatography for α -Bgt, it was found that three potential subunit combinations constitute an α -Bgt-sensitive receptors in drosophila, Dm α 3 β 1, Dm β 2 β 1, and Dm α 1 α 2D α 3 (Chamaon *et al.*, 2000; Chamaon *et al.*, 2002; Schulz *et al.*, 2000). On the other hand, expression of the Dm β 3 subunit with other drosophila α or β subunits formed functional nAChRs that was insensitive to α -Bgt (Lansdell *et al.*, 2002). Another example is the Dm α 1 β 2 and Dm α 3 β 2 chimeric receptors, which are sensitive to α -Bgt, whereas the Dm α 2 β 2 chimeric receptor is insensitive (Bertrand *et al.*, 2006; Lansdell *et al.*, 2000). The influence of subunit composition on pharmacological properties has also been observed in other insects. In *Ctenocephalides felis*, *Schistocerca gregaria*, and *Lucilia cuprina*, for example, the α 1 subunit exhibits sensitivity to α -Bgt, whereas the α 2 subunit is insensitive to α -Bgt, when they were co-expressed in *Xenopus* oocytes with chicken β 2 subunit (Dederer *et al.*, 2011), suggesting that these α subunits have distinct pharmacological properties. In addition, in the aphid *Myzus persicae*, the co-expression of Mper α 1, Mper α 2, and Mper α 3 subunits with a mammalian β 2 subunit showed that IMI acts identically on receptors formed from Mper α 2 and Mper α 3 subunits, whereas the Mper α 1 β 2 receptor was insensitive (Huang *et al.*, 1999). In honeybees, the first robust expression of nAChRs was by co-expressing of Am α 1, Am α 8, and Am β 1 subunits or Am α 1, Am α 2, Am α 8, and Am β 1 subunits with chaperons proteins like Am β RIC-3 in *Xenopus* oocytes (Ihara *et al.*, 2020). When Am α 1 subunit was co-expressed with chicken β 2 subunit in *Xenopus* oocytes, the Am α 1/ β 2 subunit ratio influenced the efficacy of IMI. For example, it has been shown that the agonist effect of IMI in terms of I_{max} was 2.6-fold larger for (Am α 1)₃(β 2)₂ nAChR than that for (Am α 1)₂(β 2)₃ nAChR (Matsuda *et al.*, 2020). While later studies showed that nAChR expression critically depended on the α 1 subunit N-terminal sequence (Brunello *et al.*, 2022).

I.3.2 α -Bgt sensitive and insensitive nAChRs

The snake venom protein α -bungarotoxin (α -Bgt) is a potent competitive blocker of nAChR in the neuromuscular junction. In vertebrates, it has no effect on most neuronal nAChRs, however, homomeric neuronal nAChRs like $\alpha 7$ receptors are considered an interesting exception (Bertrand *et al.*, 2006; Couturier *et al.*, 1990). Thus, depending on nAChRs sensitivity to α -Bgt, vertebrates nAChRs have been classified into two pharmacological subtypes: 1)- **α -Bgt-insensitive nAChRs**, which are heteromeric receptors composed of α subunits ($\alpha 2$ - $\alpha 6$) and β subunits ($\beta 2$ - $\beta 4$) and bind most ^3H -agonists with nM affinity; and 2)- **α -Bgt sensitive nAChRs**, which are primarily homomeric receptors made up of $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunits, and bind most agonists with μM affinity (Gotti *et al.*, 1997; Zoli *et al.*, 2015).

These two types of receptors can also be present in insect neurons. For example, in *Drosophila melanogaster*, it has been shown that as in vertebrates, $\alpha 7$ -like subunits, Dm $\alpha 5$, Dm $\alpha 6$ and Dm $\alpha 7$, can potentially form α -Bgt-sensitive receptors. However, Dm $\alpha 1$, Dm $\alpha 2$, Dm $\alpha 3$, Dm $\beta 1$ and Dm $\beta 2$ subunits can form heteromeric ACh receptors that can also bind to α -Bgt (Chamaon *et al.*, 2000; Fayyazuddin *et al.*, 2006; Lansdell *et al.*, 2004; Thany *et al.*, 2007; Wu *et al.*, 2005). In *P. americana*, by using patch-clamp technique and the application of different potentials and antagonists, it was possible to identify two subtypes of α -Bgt-insensitive nAChRs, nAChR1 and nAChR2, on DUM neurons from the last abdominal ganglion (Courjaret *et al.*, 2003; Courjaret *et al.*, 2001). Interestingly, these two subtypes differ in their voltage dependency, ion permeability, and sensitivity to various nicotinic antagonists. For instance, the application of nicotine to DUM neurons induces a bell-shaped current-voltage curve: the first phase of the curve corresponds to the nAChR1 subtype, from -100 mV to -50 mV, and is blocked by d-tubocurarine, a selective nAChR1 blocker, and the second phase, corresponds to the nAChR2 subtype, from -50 mV to +20 mV, and is blocked by mecamylamine (a selective nAChR2 blocker) as shown in (**Figure 17**) (Courjaret *et al.*, 2001).

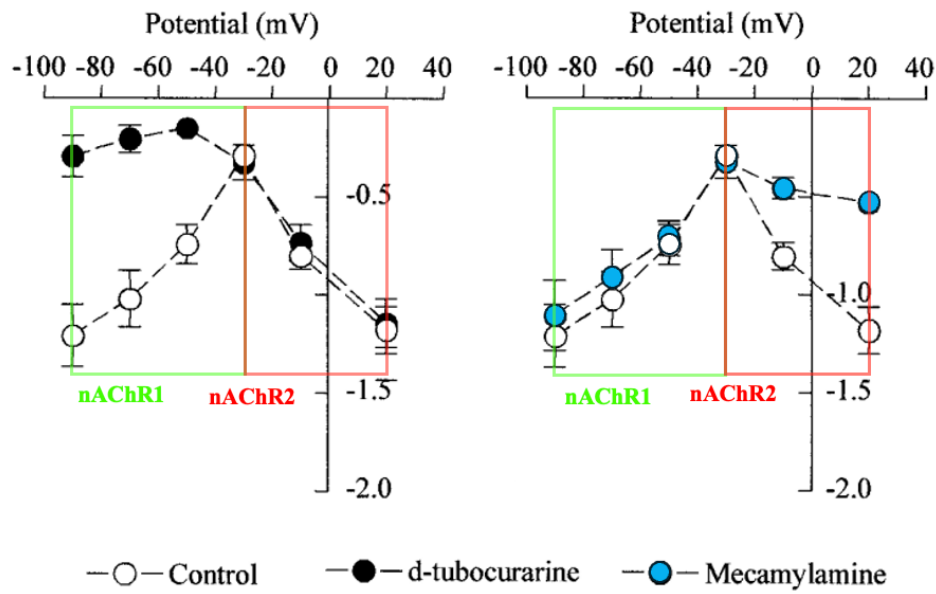


Figure 17: I/V curve induced by the application of 10mM NIC. The applied potential varies between -90 mV and -30 mV. A differentiation between the two nAChRs subtypes is made when the potential is -50mV. d-tubocurarine application induces a decrease in the nicotinic current between -90 mV and -50 mV. Therefore, nAChR1 is inhibited by this compound. While Mec application does not affect nAChR1 but inhibits nAChR2 (modified from Courjaret *et al.* 2001).

The pharmacological differences between the two subtypes have also been observed with the application of other compounds, such as non-competitive antagonists and neonicotinoids. For example, imidacloprid (IMI), which is a well-known neonicotinoid, acts as an agonist on the nAChR1 subtype, while it has no effect on the nAChR2 subtype.

More recent studies using RNA interference (RNAi) techniques, showed that these two subtypes are different in their subunit's composition, which may explain the difference in their pharmacological properties. Indeed, $\text{Pam}\alpha 3$, $\text{Pam}\alpha 8$, and $\text{Pam}\beta 1$ subunits were found to be involved in the composition of nAChR1, whereas $\text{Pam}\alpha 1$, $\text{Pam}\alpha 2$, and $\text{Pam}\beta 1$ subunits are part of nAChR2 (Sun *et al.*, 2017).

Additionally, even the α -bungarotoxin-sensitive nAChRs were found to correspond to two subtypes in DUM neurons. The first subtype, known as the desensitized nAChR (nAChD), desensitizes in the continuous presence of agonists, exhibits high binding affinity for agonists in the desensitized state, and it is inhibited by 100 nM IMI. While the second subtype is the

non-desensitized nAChR (nAChN), does not desensitize, is continuously activated by agonists, and it is selectively inhibited by 100 pM methyllycaconitine (MLA) (Salgado *et al.*, 2004).

II. Insecticides acting on insect nAChRs

II.1 History

The first pesticides to be ever used were arsenic, copper, sulfur, or botanical products such as pyrethrum and tobacco. In the 40s, DDT (dichloro-diphenyl-trichloroethane) was introduced as the first modern synthetic insecticide (Chang *et al.*, 1983). Neonicotinoids and other synthetic insecticides like carbamates and pyrethroids, were also developed along with DDT (Kollmeyer *et al.*, 1999; Gutiérrez *et al.*, 2021).

Neonicotinoids are a new class of synthetic insecticides with similar chemical structures to nicotine (Simon-Delso *et al.*, 2015). They became commercially available in the 1990s and rapidly emerged as one of the most widespread pesticides, playing a crucial role in controlling sucking insects and protecting the health of both plants and animals (Anadón *et al.*, 2020; Rohlman *et al.*, 2022). Nithiazine, 2-nitromethylene tetrahydro-1,3-thiazine, was the first neonicotinoid to be developed in the 1970s. Despite its initial promise as an effective insecticide, nithiazine exhibited low photostability. This instability significantly limited its practical application in agriculture and as a result, new neonicotinoids started to evolve (Seifert, 2014). Several heterocyclic aromatic compounds structurally similar to nithiazine were synthesized, with the distinctive feature of replacing the -nitromethylene group with -nitroguanidine or -cyanoamidine groups, making them more photostable (**Figure 18**) (Matsuda *et al.*, 2001; Tomizawa *et al.*, 2005).

Nowadays, neonicotinoids are classified into four groups based on their chemical structure and insecticidal properties:

1. First generation: consists of a group of compounds called chloronicotinyls or chloropyridyl. This group is best represented by imidacloprid, in addition to acetamiprid, thiacloprid, and nitenpyram (Seifert, 2014).
2. Second generation: also called thianicotinyls, with thiamethoxam being the first compound to be developed in this group. Clothianidin is a metabolite of thiamethoxam that also have an insecticidal activity (Hernández, 2023; Seifert, 2014).
3. Third generation: dinotefuran is the main neonicotinoid in this group (Hernández, 2023).
4. Fourth generation: consisting of sulfoxaflor and cycloxaprid (Cutler *et al.*, 2013;

Hernández, 2023). However, while some studies classify sulfoxaflor as a fourth-generation neonicotinoid, others studies categorize it as a novel insecticide belonging to the sulfoximines family (Ahmed *et al.*, 2016; Azpiazu *et al.*, 2021; Sparks *et al.*, 2013).

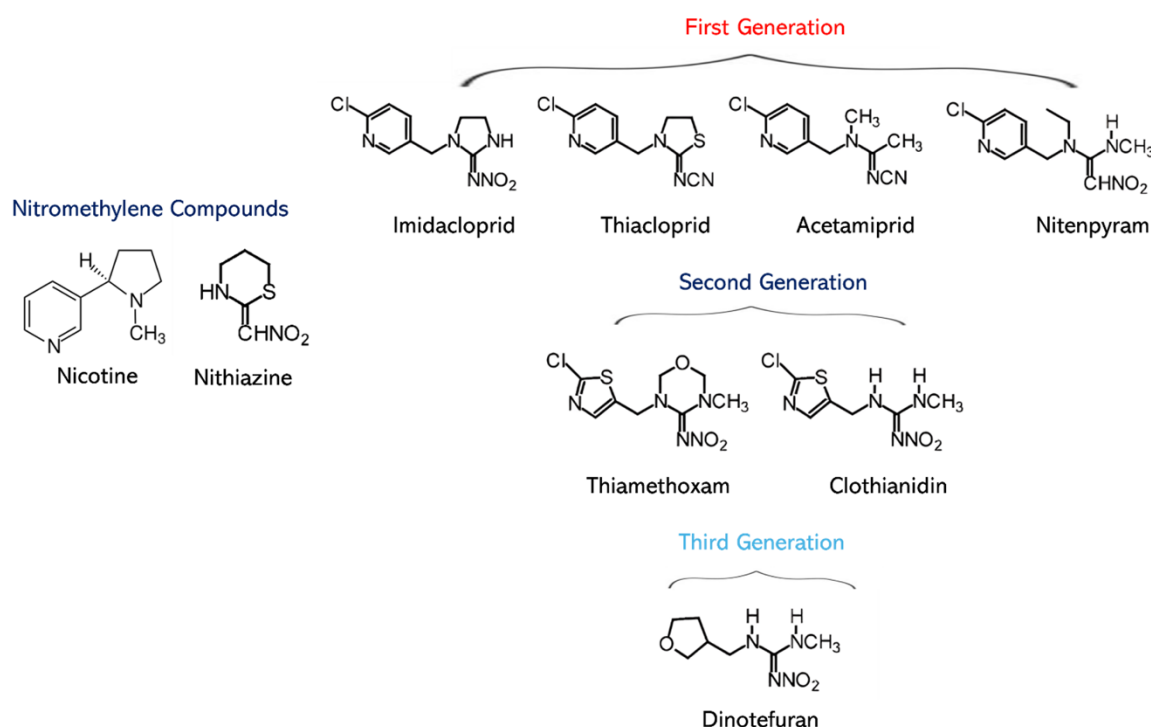


Figure 18: The molecular structures of nithiazine and its neonicotinoid derivatives, classified into three generations based on their chemical structure (modified from Matsuda *et al.* 2001, Tomizawa *et al.* 2005).

II.2 Neonicotinoids mode of actions

Using electrophysiological studies and radioligand binding assays it was found that neonicotinoids selectively target insect nAChRs. Neonicotinoids can act either as agonists (partial, full, or super) or antagonists on nAChRs. This depends mainly on two factors. The first one is neonicotinoid's structure. Indeed, the agonist actions of neonicotinoids can dramatically be affected by small structural variations. For example, studies on both *Drosophila* Dm α 2/Chicken β 2 hybrid nAChRs expressed in *Xenopus* oocytes and native neurons from the terminal abdominal ganglia of *P. americana*, showed that neonicotinoids with an acyclic guanidine moiety like clothianidin have stronger agonist efficacy than those with a cyclic guanidine moiety like imidacloprid (Ihara *et al.*, 2006). The open-chain structure of the acyclic

compounds might increase their flexibility, allowing them to bind more effectively and enhance agonist efficacy (Ihara *et al.*, 2006; Tan *et al.*, 2007). In *P. americana* DUM neurons, it was also shown that the affinity of nitromethylene-type neonicotinoids like IMI to nAChRs is higher than of nitroimine-type compounds like IMI derived, CH-IMI (Ihara *et al.*, 2006). Furthermore, on Dm α 2/Chicken β 2 hybrid nAChRs expressed in *Xenopus* oocytes, it was found that opening the imidazolidine ring of IMI led a larger amplitude response of clothianidin (CLT) comparing to IMI and even the ACh (Ihara, *et al.*, 2004).

Secondly, neonicotinoids' mode of action can also depend on the targeted nAChRs subtypes. For example, honeybee antennal lobe neurons and cultured Kenyon cells have different molecular composition of nAChRs (Simon-Delso *et al.*, 2015). As a result, IMI can be a full or a partial agonist on antennal lobe neurons, while it is acting only as a partial agonist on cultured Kenyon cells (Tan *et al.*, 2007; Toshima *et al.*, 2008). Same as in chicken nAChRs expressed in *Xenopus* oocytes, IMI has a partial agonist effect on α 7 receptors, while it has a very weak agonist action on α 4 β 2 receptors (Ihara *et al.*, 2003).

Additionally, it was found that the structural features of certain nAChRs α -subunits in drosophila play a crucial role in enhancing neonicotinoid affinity to drosophila nAChRs compared to vertebrate nAChRs. For example, the replacement of Rat α 4 subunit in α 4 β 2 receptor with either Dm α 1 or Dm α 2 subunit from drosophila increased the receptor sensitivity for IMI (Ihara *et al.*, 2003).

Thiamethoxam (TMX), which is a second-generation neonicotinoid (Maienfisch *et al.*, 2001; Thany, 2011), has a unique mode of action comparing to other neonicotinoids. Although it is a poor agonist for nAChRs, it can cause a strong depolarization in the cockroach cercus/giant interneuron synapse. This depolarization can be partially reduced by atropine, a muscarinic antagonist, concluding that TMX can bind to both nicotinic and muscarinic receptors (Lapied *et al.*, 1990).

Clothianidin (CLT) is a thiamethoxam metabolite that also has an insecticidal activity (Nauen *et al.*, 2003). It works as a super agonist for nAChRs (Brown *et al.*, 2006). In *P. americana* DUM neurons, application of CLT induces an inward current which was, contrary to TMX, unaffected by the presence of muscarinic antagonists like atropine. However, CLT-induced currents were reduced by α -Bgt and completely blocked by MLA, suggesting that CLT can act on α -Bgt-sensitive and insensitive subtypes (Courjaret *et al.*, 2001; Thany, 2009).

II.3 Key Residues in nAChRs critical for neonicotinoids selectivity and sensitivity

In general, all residues in the six loops (A, B, C, D, E, and G) of nAChRs are considered important for agonist binding. However, in insects, many residues in these loops are crucial for the receptor's selectivity towards neonicotinoids over ACh and other ligands. To identify these residues involved in electrostatic interactions with neonicotinoids, directed mutagenesis and molecular modeling experiments were conducted (Liu *et al.*, 2008).

On chicken homomeric $\alpha 7$ nAChRs expressed in *Xenopus* oocytes, when glycine residue in loop D is replaced by either aspartate G189D or glutamate G189E, it reduced the receptor's sensitivity to neonicotinoids. This decrease was attributed to the negative charge of both aspartate or glutamate which increases the negativity of loop D and alters the electrostatic environment of the binding site (Matsuda *et al.*, 2000). Neonicotinoids typically have a negatively charged nitro or cyano group that interacts with positively charged or neutral residues in the nAChRs binding pocket. Indeed, when desnitro-IMI (an IMI-derived molecule lacking the nitro group) tested on mutant receptors (G189N and G189Q), where glycine residue in loop D was replaced by asparagine and glutamine (both are neutral amino acids), the receptors' sensitivity to desnitro-IMI increased, indicating that the original decreased sensitivity to IMI was likely due to electrostatic repulsion between the nitro group in IMI and the negatively charged acidic residues (aspartate D and glutamate E) that had been muted (Matsuda *et al.*, 2000).

Besides the roles of loop D residues in $\alpha 7$ nAChRs, loop D also play an important role in the interaction between neonicotinoids and $\alpha 4\beta 2$ nAChRs. In their study, Toshima and colleagues investigated the impact of residue substitutions on chicken $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes. For example, $\alpha 4\beta 2$ nAChRs sensitivity to IMI was increased when threonine and glutamic acid in the loop D were replaced with arginine (T77R) and valine (E79V) respectively, in the $\beta 2$ subunit. Interestingly, this sensitivity was further enhanced by the E219P mutation in the loop C in $\alpha 4$ subunit, where glutamic acid was replaced with proline. This finding suggests that loops C and D of the nAChRs may play crucial roles in enhancing the receptor's interactions with neonicotinoids (Toshima *et al.*, 2009).

Similar results were found with drosophila Dm $\alpha 2$ /Chicken $\beta 2$ hybrid nAChRs expressed in *Xenopus* oocytes, when proline was replaced by glutamic acid (P242E) in the loop C of Dm $\alpha 2$ subunit. P242E mutation reduced the receptors sensitivity to IMI, confirming the role of loop

C from α subunit in the nAChRs-imidacloprid interaction (Shimomura *et al.*, 2004). Similarly, S221E mutation in the YXCC motif in loop C of Dm α 1 subunit also markedly reduced the agonist action of imidacloprid on D α 1 β 2 hybrid receptor expressed in *Xenopus* oocytes, pointing to a contribution of the serine221 to the selective action of the neonicotinoids tested (Shimada *et al.*, 2020).

Furthermore, some studies found that basic residues present only in insect α subunits may play a key role in selective interactions between insect nAChRs and neonicotinoid insecticides. For example, when α 4 subunit of the chicken α 4 β 2 was replaced with the Dm α 2 subunit from drosophila, the formed Dm α 2 β 2 hybrid receptor was more sensitive to IMI than the chicken α 4 β 2 nAChRs. This result suggests that the Dm α 2 subunit possesses structural characteristics that enhance its interaction with IMI (Matsuda *et al.*, 1998; Shimomura *et al.*, 2006). It was also found that insect α subunits have more amino acid residues in the interval between loops B and C compared with vertebrate nAChRs. Deletion of the loop B–C interval region from Dm α 2 subunit enhanced the effect of the P242E mutation on the Dm α 2 β 2 hybrid nAChRs. This result suggests important roles of the regions investigated in the nAChRs-imidacloprid interactions (Shimomura *et al.*, 2004).

In term of neonicotinoids binding sites, we should mention that native nAChRs from *Nilaparvata lugens* displayed two binding sites differentiate in their affinity to IMI, a very high affinity binding site (pM) that involves Nlug α 3, Nlug α 8, and Nlug β 1; and a secondary lower affinity (nM) site that involves Nlug α 1, Nlug α 2, and Nlug β 1 (Li *et al.*, 2010). However, in the majority of other insects like *P. americana* and *D. melanogaster*, binding experiments showed that only a single site of approximately nM affinity to IMI is thought to be present (Crossthwaite *et al.*, 2017; Lind *et al.*, 1998; Taillebois *et al.*, 2018). These findings could explain why IMI is particularly useful for controlling sap-feeding insects.

II.4 Neonicotinoids resistance

Although neonicotinoids effectively control numerous insect species, their excessive and unregulated application has led to significant resistance development among many insects. *Bemisia tabaci* resistance to IMI, was the first documented resistance to neonicotinoid, in 1996. It was found later that both target-site mutations and metabolic pathways are the key mechanisms behind the resistance development (Bass *et al.*, 2015).

II.4.1 Metabolic resistance

Metabolic resistance is considered the most prevalent resistance in insects. The most well-known mechanism is the overproduction of metabolic enzymes, such as esterases, glutathione transferases, and P450-dependent monooxygenases. Increasing enzymatic activity results in detoxification or sequestration of insecticides before they even reach their target site. Overexpression of CYP450s is frequently reported in insects' resistance strains (Bass *et al.*, 2015; Nikookar *et al.*, 2019; Puinean *et al.*, 2010; Wang *et al.*, 2022). In *Myzus persicae*, overexpression of both E4/FE4 esterase and CYP6CY3, a member of the cytochrome P450, led to a resistance towards organophosphates, carbamates, and pyrethroids, but not for neonicotinoids (de Little *et al.*, 2017). As a result, in the early 1990s, neonicotinoids such as imidacloprid, thiamethoxam, clothianidin, and acetamiprid, became the primary means of controlling pests such as *M. persicae* due to their effectiveness and lack of resistance. However, after two decades and due to the extensive and uncontrolled use, strains of *M. persicae* in Europe, the United States, and Japan become resistant to several neonicotinoids (Foster *et al.*, 2008; Margaritopoulos *et al.*, 2007; Nauen *et al.*, 2005). Indeed, overexpression of the gene encoding cytochrome P450, CYP6CY3, was observed in *M. persicae nicotianae* and was correlated with nicotine and neonicotinoids resistance (Peng *et al.*, 2016; Troczka *et al.*, 2021).

II.4.2 Target-site resistance related to nAChRs

Although the majority of resistance to neonicotinoids has been attributed to enhanced metabolism, a small number of hemipteran species have shown resistance linked to target-site mutations in nAChRs (Crossthwaite *et al.*, 2014). In the late 1990s, *Nilaparvata lugens*, emerged a resistance to IMI in China. The Y151S mutation, where tyrosine is replaced by serine at position 151 within loop B in both Nluga1 and Nluga3 subunits of the nAChR, has been identified as a key factor. Homozygotes with this mutation in both α subunits exhibit very high resistance levels, whereas heterozygotes display lower resistance, indicating that the Y151S mutation is necessary in both subunits for a high-level resistance (Crossthwaite *et al.*, 2014). Another mutation associated with resistance in *M. persicae* is V101I in the β 1 subunit, where valine is replaced by isoleucine at position 101 of the nAChR protein sequence (Xu *et al.*, 2022). In the aphid species, target-site resistance has been linked to the R81T mutation in the β 1 subunit, where arginine is substituted with threonine. This mutation in loop D of the β 1 subunit

has been shown to affect neonicotinoids efficacy and also the binding of other insecticides such as sulfoxamines (Cutler *et al.*, 2013; Homem *et al.*, 2020). These examples illustrate the complexity and variability of target-site resistance mechanisms among different insect species, highlighting the ongoing challenge of managing neonicotinoid resistance in agricultural pests.

II.5 Butenolide insecticides

Due to the emergence of resistance to neonicotinoid insecticides, new systemic insecticides, such as butenolide compounds, have been introduced to the market. Although these novel compounds differ in chemical structure from neonicotinoids, they exhibit a similar mode of action by targeting insects nAChRs (Nauen *et al.*, 2015). Flupyradifurone, the primary representative of the butenolide family, has been demonstrated to have significantly fewer adverse effects on honeybees (*Apis mellifera*) compared to other insecticides that also target nAChRs (Haas *et al.*, 2021). Furthermore, some insect species that have developed high levels of resistance to imidacloprid (IMI) have shown low or no resistance to flupyradifurone (FLU), highlighting the potential of butenolides as a promising alternative for effective insect management strategies (Caspary *et al.*, 2023). Contradictory, other studies described adverse effects of FLU on honeybee (Scheibli *et al.*, 2023) This discrepancy highlights the need to better understand the mode of action of this insecticide.

III. Regulation of nAChRs by intracellular calcium pathways

III.1 The role of calcium ions in neuronal signaling and function

Calcium (Ca^{2+}) ions are vital second messenger involved in both intra- and extracellular signaling cascades, regulating essential activities in both vertebrates' and invertebrates' cells. In neurons, extensive Ca^{2+} signaling pathways have been developed to maintain neuronal functions, including synaptic transmission, synaptic plasticity, neuronal excitability, and gene expression. These pathways involve a precise coordination of calcium influx through various channels, calcium release from intracellular stores, and the activation of calcium-dependent signaling molecules such as kinases and phosphatases.

Voltage-gated calcium channels (VGCCs) are the primary type of calcium channels involved in neuronal synaptic transmission. They regulate Ca^{2+} influx into neurons in response to changes in membrane potential. Membrane depolarization activates VGCCs allowing calcium ions to flow into the neuron (Benquet *et al.*, 2002; Berridge, 1998; Catterall, 2011; Correa *et al.*, 2023).

In vertebrates, neuronal VGCC can be classified into P-/Q-, N-, L-, T-, and R-subtypes, encoded by $\alpha 1$ A-E genes, according to their electrophysiological and pharmacological properties (Lang, 2007). The $\alpha 1$ subunit has a crucial role in determining the pharmacological and biophysical characteristics of calcium currents. Current subtypes can be categorized based on their activation thresholds, either low voltage activation (LVA) or high voltage activation (HVA). They are also distinguished by their inactivation kinetics, with long-lasting (L-type) versus transient (T-type) responses, and by their sensitivity to dihydropyridines (DHP) and various toxins. The diversity of these calcium currents is driven by ten different genes that encode distinct $\alpha 1$ subunits, which can be categorized into three main families: CaV1, CaV2, and CaV3, based on their amino acid sequence similarity (Ertel *et al.*, 2000).

Each type of VGCC is expressed in specific regions of the nervous system and plays distinct roles in neuronal excitability, neurotransmitter release, and synaptic plasticity. For example, N-type calcium channels are predominantly found in presynaptic terminals where they regulate neurotransmitter release (Kang *et al.*, 2013), while L-type calcium channels are involved in dendritic integration and synaptic plasticity (Humeau *et al.*, 2007).

In *Drosophila melanogaster*, HVA calcium channels in motoneurons are made up of multiple subunits, including the $\alpha 1$ subunit and various $\alpha 2\delta$ subunits like Dm $\alpha 2\delta 1$ and Dm $\alpha 2\delta 3$. The specific combination of these subunits determines the channel's properties and its role in drosophila neuronal functions (Heinrich & Ryglewski, 2019, 2020). Among the different HVA calcium channels, the cacophony (cac) channels are a well-studied type in drosophila. They are encoded by the *cac* gene and have the $\alpha 1$ subunit forming the channel pore. The cac channels play a major role in neuronal Ca^{2+} currents and trigger homeostatic regulation of K^+ channels, impacting neuronal excitability through Ca^{2+} and K^+ current interactions (Peng *et al.*, 2007). Furthermore, studies showed that intracellular Ca^{2+} homeostasis plays a crucial role in drosophila neural circuit formation and neuronal function and physiology. For example, in drosophila pupal neurons, flight circuit maturation can be modulated through inositol 1,4,5-

trisphosphate receptors (IP₃R) and store-operated Ca²⁺ entry (SOCE) mechanisms (Chakraborty *et al.*, 2017; Venkiteswaran *et al.*, 2009).

In *Periplaneta americana*, both HVA and LVA calcium channels have been identified in DUM neurons. Although the activation and thresholds of these channels are similar to those found in vertebrates, the pharmacological properties of their Ca²⁺ currents differ significantly. For example, LVA currents in vertebrates are more sensitive to Ni²⁺ than to Cd²⁺, but the opposite is true in DUM neurons. Additionally, while L-type HVA currents in vertebrates are sensitive to both dihydropyridines and phenylalkylamines, HVA currents in *P. americana* DUM neurons are only sensitive to phenylalkylamines (Wicher & Penzlin, 1994, 1997).

In addition, TRP or transient receptor potential channels, have been identified in insects. These Ca²⁺ permeable channels were first identified in *Drosophila* photoreceptors, playing a key role in the sensory processes by translating a variety of visual, mechanical, and chemical stimuli into neuronal signals. However, while *Drosophila* TRP (dTRP) were only expressed in the photoreceptors, *P. americana* TRP (pTRP) were expressed throughout the nervous system, particularly, in DUM neurons, where they play a key role in modulating the pacemaker conductance and adjusting the neuronal activity. Furthermore, it was found that pTRP is downregulated by cAMP, while dTRP was not affected by this pathway.

Interestingly, in *P. americana* DUM neurons, both HVA and TPR γ have been shown to affect neonicotinoids mode of action. Indeed, calcium influx through HVA and TPR γ reduced acetamiprid induced currents through nAChR2 (Dubois *et al.*, 2012).

In addition to Ca²⁺ entry through VGCCs, neurotransmitters can also induce an influx of calcium using receptor-operated channels. For example, NMDA receptors, which are glutamate-gated ion channels, are well-known for their high permeability to Ca²⁺. Ca²⁺ influx through NMDA is essential for several neuronal functions, like long-term potentiation (LTP), and cognitive functions such as learning and memory (Berridge, 1998; Schiller *et al.*, 1998). Beside Ca²⁺ influx into the neurons, intracellular Ca²⁺ release from endoplasmic reticulum (ER) serves as an alternative mechanism for increasing intracellular calcium concentration. Ca²⁺ release from ER occurs mainly through inositol 1,4,5-trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs) (Berridge, 1998). Ca²⁺ release through these receptors can regulate both short- and long-term plasticity at the pre- and postsynaptic terminals of central synapses. These Ca²⁺ signaling and plasticity were well-studied on hippocampal CA3-CA1 synapses (Padamsey *et al.*, 2018). In addition, Ca²⁺ released from ryanodine receptors has an important

role in calcium dependent inactivation (CDI) which is a fundamental autoregulatory mechanism of VGCCs in neurons (Ali *et al.*, 2023).

In neuronal nAChRs, it was discovered that extracellular Ca^{2+} can modulate currents induced by ACh. This suggests that calcium acts as an allosteric modulator of these receptors and indicates the presence of calcium binding sites (Mulle *et al.*, 1992; Vernino *et al.*, 1992). In $\alpha 7$ nAChRs receptors, Ca^{2+} binds at the periphery of the ligand-binding sites and is stabilized by pairs of negatively charged (anionic) residues. These anionic residues come from both the principal face and the complementary face of each binding site. After binding Ca^{2+} can act as a powerful potentiator of $\alpha 7$ activated by low concentrations of ACh (Galzil *et al.*, 1996; Natarajan *et al.*, 2020). In specific, these Ca^{2+} binding sites are located in the N-terminal domain of the receptor. Indeed, when combining the N-terminal domain of chicken $\alpha 7$ nAChRs and C-terminal part of the 5-Hydroxytryptamine type 3 (5-HT₃) receptor, the $\alpha 7$ -V201-5-HT₃ chimera was potentiated by calcium ions, similar to the $\alpha 7$ receptor but unlike the 5-HT₃ receptor (Galzil *et al.*, 1996). Furthermore, mutations in the 12 amino acid canonical domain or $\alpha 7$ 161-172 domain within the N-terminal, which is involved in the ligand binding and allosteric modulation, significantly alter calcium's potentiation effects of nAChRs. Mutations at Aspartate 163 (D163) and Serine 169 (S169) enhance potentiation, mutations at Glutamate 161 (E161), Serine 165 (S165), and Tyrosine 167 (Y167) reduce it, while mutation at Glutamate 172 (E172) completely abolishes these calcium effects (Galzil *et al.*, 1996).

This crucial role of the 161-172 domain was further illustrated using the 5-HT₃ receptor, which is also a ligand gated ion-channel, but typically insensitive to Ca^{2+} variations. Indeed, incorporating the 161-172 domain into 5-HT₃ receptor made it responsive to Ca^{2+} , which subsequently potentiated its activity (Galzil *et al.*, 1996). These observations suggest that the 161-172 domain is involved in Ca^{2+} binding, with Glu172 playing a crucial role.

In insects' neurons, nAChRs, like those in vertebrates, serve as significant pathways for Ca^{2+} entry into the cell. Application of ACh or nAChRs agonists results in a substantial increase in intracellular Ca^{2+} levels in Kenyon cells (Bicker, 1996; Campusano *et al.*, 2007; Dupuis *et al.*, 2012). Cognitive functions involving neuronal nAChRs, such as learning and long-term memory in bees, can therefore depend on intracellular calcium levels (Dupuis *et al.*, 2012).

III.2 Intracellular calcium pathways in neuronal levels

Cytoplasmic Ca^{2+} increase can affect several downstream enzymes like Ca^{2+} dependent protein kinases and phosphatases. These calcium binding proteins serve as calcium sensors, detecting changes in calcium levels within the cell and initiating downstream cellular responses that influence neuronal function and synaptic plasticity (Ahmad *et al.*, 2019; Bagur *et al.*, 2017; Mattson *et al.*, 1991). Among the key players, Ca^{2+} /calmodulin-dependent protein kinases (CaMKs), Protein kinase A (PKA), and protein kinase C (PKC), that have significant roles. For example, CaMKs phosphorylate target proteins, affecting neurotransmitter release, gene expression, and long-term potentiation, all vital for learning and memory. PKA and PKC, which are primarily regulated by cyclic AMP (cAMP) and diacylglycerol (DAG) respectively, can also be modulated by calcium ions. While AMP-activated protein kinase (AMPK), mainly related to cellular energy status, can also have a role in calcium signaling pathways. This kinase acts as a metabolic regulator, maintaining cellular energy homeostasis and responding to stress conditions (Chu *et al.*, 2014; Hell *et al.*, 1995; Marinangeli *et al.*, 2018; Sadeghian *et al.*, 2022; Solà *et al.*, 1999) (**Figure 19**).

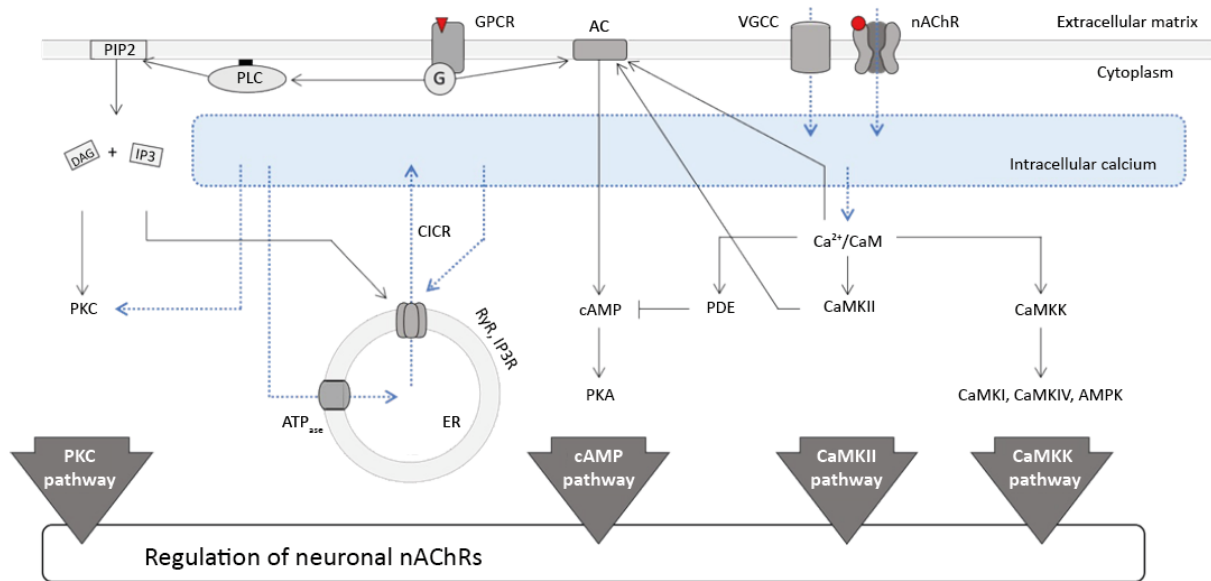


Figure 19: A diagram illustrating the regulation of neuronal nAChRs via calcium-dependent intracellular pathways. PIP2, phosphatidylinositol-4,5-biphosphate; PLC, phospholipase C; GPCR, G protein-coupled receptor; AC, adenylate cyclase; VGCC, voltage-gated calcium channel; DAG, diacylglycerol; IP3, inositol triphosphate; CICR, calcium-induced calcium release; PKC, protein kinase C; RyR, ryanodine receptor; IP3R, IP3 receptor; PKA, protein kinase A; cAMP, cyclic AMP; PDE, phosphodiesterase; CaM, calmodulin; CaMK, CaM-kinase; CaMKK, CaMK-kinase; AMPK, AMP-activated protein kinase; nAChR, nicotinic acetylcholine receptor.

III.2.1 The Calcium-Calmodulin dependent protein kinase (CaMK) pathway

➤ CaM and CaMK structure and activation

Calmodulin (CaM) is a small protein with a molecular weight of about 16.7 kDa, highly abundant in the central nervous system of both vertebrates and invertebrates. Ca^{2+} binding to CaM is a key mechanism by which neurons respond to changes in intracellular Ca^{2+} levels and regulate a wide range of neuronal processes, mainly synaptic function and neurotransmitter release (Solà, *et al.*, 2001). Each CaM molecule contains four calcium-binding sites. After Ca^{2+} binding, CaM undergoes a conformational change that facilitates its interaction with various target protein and regulates their activity (Halder *et al.*, 2024; Solà *et al.*, 2001). Each CaM molecule consists of:

1. Two globular domains called "lobes" at the N-terminal and C-terminal ends.
2. Flexible central linker region binds the two loops.

Each lobe contains two calcium (Ca^{2+})-binding EF motifs, (EF-1 and EF-2) in the N-terminal, and (EF-3 and EF-4) in the C-terminal (Ikura *et al.*, 2006; Lakowski *et al.*, 2007; Piazza, *et al.*, 2017). Each EF motif consists of α -helix E, α -helix F (index finger and thumb) and a conserved Ca^{2+} binding loop between them (**Figure 20**) (clenched middle finger). After binding with Ca^{2+} , the helix F would be pushed away from helix E, leading to significant conformation changes of EF-hand domain, as well as the protein. This will expose EF-hand proteins' active site and facilitate its interaction with downstream targets to exhibit functions like enzymatic activities or signaling transduction (Zuo *et al.*, 2023). Twelve residues in the EF motif loop, including aspartates, asparagine, and glutamates, are involved in binding to Ca^{2+} (Bentley *et al.*, 2000).

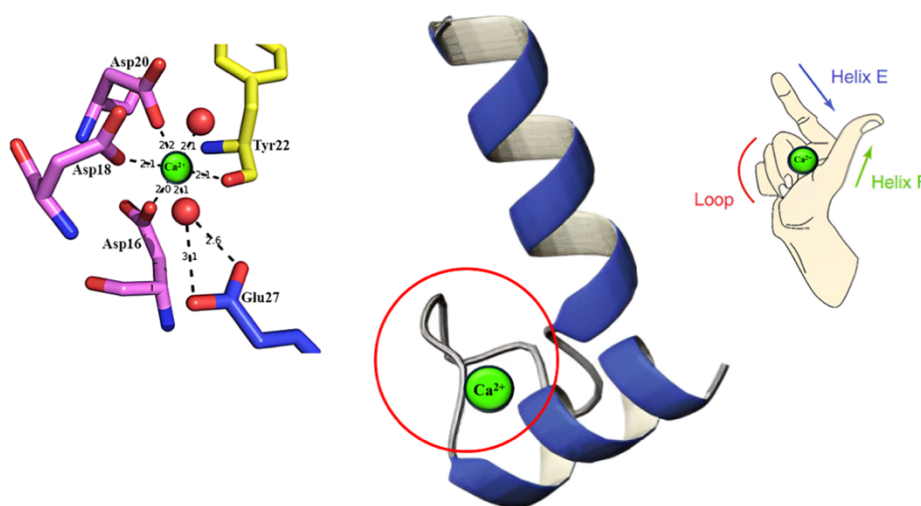


Figure 20: The EF-hand calcium-binding motif resembles a right hand; the index finger and thumb represent the E- and F-helices, respectively, the middle finger mimics the Ca^{2+} chelating loop (modified from Ishida *et al.* 2013, Kumar *et al.* 2014).

➤ [Ca²⁺ /CaM-dependent protein kinase \(CaMK\) family](#)

Among the targeted proteins regulated by Ca^{2+} /CaM is the Ca^{2+} /CaM-dependent protein kinase (CaMKs) family. They are Ser/Thr protein kinases that play crucial roles in neuronal signaling pathways by phosphorylating serine and threonine residues on target proteins. This family

includes: CaMKI, CaMKII, CaMKIII, CaMKIV, CaMKV, and two CaMKK (CaMKK α and CaMKK β) (Brzozowski *et al.*, 2019; Wayman *et al.*, 2008).

In terms of function and specificity, all CaMKs are multifunctional kinases with a broad range of substrates and roles in various cellular processes. However, CaMKIII is also known as the elongation factor-2 kinase (eEF-2K), that can phosphorylate the elongation factor 2 (EF-2), inhibiting peptide chain elongation during protein translation (Adaikkan *et al.*, 2018; Zhang *et al.*, 2021).

Despite the substrate specificity of CaMKs, their molecular structure is similar. An amino terminal catalytic domain followed by a regulatory domain containing an autoinhibitory segment overlapping with the CaM binding sequence (**Figure 21**) (Griffith, 2004; Tokumitsu & Sakagami, 2022).

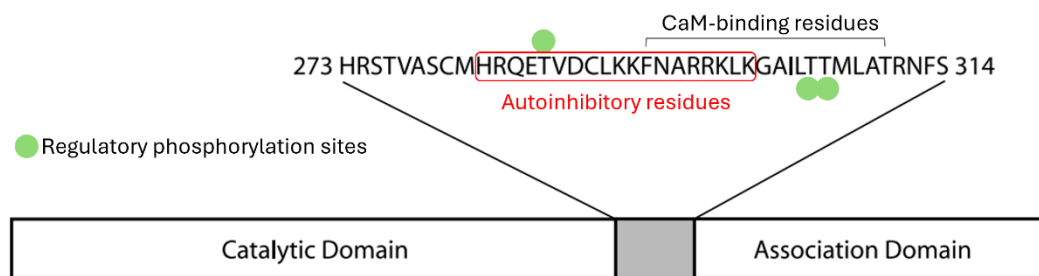


Figure 21: Schematic diagram of CaMKII domain structure. All CaMKII isozymes contain an N-terminal catalytic domain, an internal regulatory domain, and a C terminal that mediates holoenzyme formation. The regulatory domain, whose sequence is shown above the diagram, is bipartite. The proximal end (aa 282-300) contains residues that interact with the catalytic domain to inhibit phosphotransferase activity. The distal portion of this domain (aa 293-310) binds to Ca²⁺/CaM. Regulatory phosphorylation sites at Thr286, Thr305, and Thr306 are indicated by green dots (modified from Griffith *et al.* 2004).

Basically, under low intracellular Ca²⁺ concentrations, the autoregulatory domain interacts with the catalytic domain to inhibit its activity. Specific residues within the autoregulatory domain mimic a protein substrate, which prevents the binding of actual substrates to the catalytic domain. When intracellular Ca²⁺ concentrations increase, CaM binds Ca²⁺ ions, which induces conformational changes to interact with the regulatory domain of CaMKs. Ca²⁺/CaM, binding allosterically changes the autoinhibition state of the CaMK catalytic domain enabling them to become active and phosphorylate downstream substrates (Griffith, 2004; Hudmon *et al.*, 2002; Tokumitsu *et al.*, 2022). In addition to Ca²⁺/CaM binding, some CaMKs are activated by

phosphorylation, either autophosphorylation or trans-phosphorylation, by activating kinases (Griffith, 2004; Hudmon *et al.*, 2002). In CaMKII the binding of Ca^{2+} /CaM also initiates the autophosphorylation of CaMKII at sites Thr286, Thr305, and Thr306 which can further sustain its activity even after Ca^{2+} levels drop (Griffith, 2004).

Similar to CaMKII, CaMKI and CaMKIV are also autoinhibited in their basal state and are activated by Ca^{2+} /CaM binding. However, these kinases require phosphorylation by CaMKK at specific sites to achieve full activation (Goldberg, *et al.*, 1996). In rats, both CaMKK α and CaMKK β undergo intramolecular autophosphorylation at multiple sites. For CaMKK α , Thr93 and Ser179 are key sites, while CaMKK β autophosphorylates at Ser22, Thr215, Thr482, and Thr517. These phosphorylation events disrupt autoinhibitory mechanisms and enhance kinase activity, ensuring that CaMKKs can effectively regulate their downstream targets and participate in essential cellular functions (Tokumitsu *et al.*, 2022).

➤ [CaMKs roles in neuronal levels](#)

In mammals, CaMKII is recognized for its essential role in regulating synaptic plasticity, nicotine withdrawal or anxiety, and many degenerative diseases. For example, CaM-CaMKII-CREB signaling pathway, which regulates Ca^{2+} concentration in neuronal cells, plays an important role in synaptic degeneration and memory loss in Alzheimer's disease (AD), and it is also a potential therapeutic target for treating AD. In addition, it was demonstrated that spinally located CaMKII is involved in the modulation of nicotine-induced antinociception (Cho *et al.*, 1998; Damaj, 2000; Jackson *et al.*, 2009; Pchitskaya *et al.*, 2018). The neuroprotective role of CaMKK β in stroke among aged mice has been confirmed. Targeting CaMKK β could be a potential strategy for reducing long-term disability following a stroke (Liu *et al.*, 2016). In addition, CAMKK2-AMPK kinase pathway represents a target for therapeutic approaches to treat Alzheimer's disease (Coello *et al.*, 2013).

Furthermore, CaM plays a pivotal role in regulating neuronal excitability and synaptic transmission by mediating the activity of L-type voltage-gated Ca^{2+} channels (CaV1.2 and CaV1.3). These channels are crucial for initiating calcium influx during neuronal activity, but prolonged intracellular Ca^{2+} elevation can be cytotoxic. To prevent this, CaM facilitates a protective mechanism known as Ca^{2+} -dependent inactivation (CDI). Upon channel opening and the subsequent increase in intracellular Ca^{2+} levels, Ca^{2+} -bound CaM binds to the IQ-motif in

the C-terminal domain of CaV channels, inducing a conformational change that leads to rapid inactivation of the channels (Ames, 2021).

It is also worth mentioning that CaMKK2 controls insulin signaling, metabolic homeostasis, inflammation and cancer cell growth highlighting its potential as a therapeutic target for a variety of diseases. For example, CaMKK2 inhibition with STO-609 impairs tumorigenicity of liver cancer cells and reduces cell growth and viability in ovarian cancer (Anderson *et al.*, 2008; Mukherjee *et al.*, 2023; Tomaszewski *et al.*, 2022; York *et al.*, 2017).

In honeybees, studies have confirmed that CaMKII is crucial for olfactory learning and memory formation. Indeed, researchers have demonstrated that CaMKII plays a significant role in the processes underlying long-term memory (LTM) (Scholl *et al.*, 2015). In drosophila motor nerve terminals, it was found that CaMKII can influence the synaptic plasticity and neuronal activity by contributing to phosphoinositide 3-kinase (PI3K) activation by metabotropic glutamate receptor (mGluR) (Lin *et al.*, 2011). Furthermore, CaMK plays a crucial role in the modulation of GABA receptor-gated Cl⁻ currents in *Periplaneta americana* cockroach DUM neurons (Alix *et al.*, 2002).

III.2.2 The AMP-activated protein kinase (AMPK) pathway

5' AMP-activated protein kinase (AMPK) is considered a crucial metabolic regulator in all eukaryotes. It maintains cellular energy homeostasis in a variety of tissues, including the brain. In neurons, AMPK was shown to be implicated in neuronal proliferation, neuronal differentiation, synaptic connectivity, and neuroprotection. For example, it was found that AMPK is hyperactivated in degenerating neurons in several neurodegenerative diseases, including Alzheimer's disease (AD) (Domise *et al.*, 2019; Rosso *et al.*, 2016).

AMPK is allosterically activated by AMP, which binds to the γ -subunit of AMPK, promoting phosphorylation of the enzyme and increasing its activity. Importantly, AMPK can also be phosphorylated on a threonine residue (Thr172) in response to calcium flux via CaMKK β (Mihaylova *et al.*, 2011). Thus, elevated calcium concentrations can stimulate AMPK activation via CaMKK β , providing a link between calcium signaling and AMPK activity.

In addition, a previous study showed that the drug Resveratrol increases cytosolic Ca²⁺ levels and activates AMPK through the Ca²⁺/CaMKK pathway, resulting in decreased levels of amyloid- β (A β), which contributes to the development of AD (Vingtdeux *et al.*, 2010).

In invertebrates, studies on *D. melanogaster* and *C. elegans* suggest pivotal role of the AMPK molecule in energy stores and organization of behavioral and physiological responses to nutrient-limitations (Greer *et al.*, 2007; Johnson *et al.*, 2010). In addition, mutations in *Loechnig* (*loe*) gene which plays a role in regulating AMPK- γ expression in the *D. melanogaster* nervous system, found to affect energy regulation and neuronal health, leading to brain pathology in adult flies (Tschäpe *et al.*, 2002).

III.2.3 The cAMP-dependent protein kinase (PKA) pathway

In neurons, PKA is involved in the regulation of synaptic plasticity by phosphorylating and modulating various target proteins, including ion channels, neurotransmitter receptors, and synaptic proteins. Calcium influx into neurons can directly or indirectly activate PKA. One direct mechanism involves the calcium/calmodulin complex, which can activate adenylyl cyclases (ACs) to increase intracellular levels of cAMP, consequently activating PKA. It was found that PKA activation through Ca^{2+} is an essential biochemical pathway for synaptic plasticity, and long-term potentiation (LTP) (Ohadi *et al.*, 2019; Wang *et al.*, 2013).

PKA can either enhance or inhibit the activity of VGCCs depending on the specific subtype of VGCC and the phosphorylation site (Hell *et al.*, 1995). In addition, PKA is involved of the peptidergic modulation of VGCCs in DUM neurons from *P. americana* cockroaches, playing a critical role in synaptic plasticity mechanisms in insect neurons (Wicher, 2001). In drosophila, it was demonstrated that PKA can also modulate the activity of ligand-gated channels, such as the glutamate receptor DGluRIIA (Davis *et al.*, 1998).

Additionally, several studies have confirmed the crucial role of both PKA and PKA regulators in learning and memory within the drosophila mushroom body (MB). For example, transgenic elevation of PKA activity in the drosophila MB negatively affected their olfactory learning (Crittenden *et al.*, 1998; Drain *et al.*, 1991; Nighorn *et al.*, 1991).

III.2.4 The cGMP-dependent protein kinase (PKG) pathway

In mammals, cGMP-dependent protein kinase (PKG) pathways have been widely implicated in long-term synaptic, memory formation, and neurotransmission release (Bollen *et al.*, 2014; Chien *et al.*, 2003; Costa *et al.*, 2011; Nugent *et al.*, 2009; Zhuo *et al.*, 1994). Indeed, PKG is a

crucial substrate for nitric oxide (NO), which have a variety of pre- and postsynaptic effects (Hawkins *et al.*, 1998; Schuman *et al.*, 1991).

Once calcium enters the cell, it forms $\text{Ca}^{2+}/\text{CaM}$ complex, which activates NO production from L-Arginine. Later, NO binds to and activates NO-guanylyl cyclase (NO-GC) in the nearby cells, which in turn increases synthesis of cyclic GMP (cGMP) from guanosine triphosphate (GTP) and results in PKG activation (**Figure 22**) (Francis *et al.*, 2010).

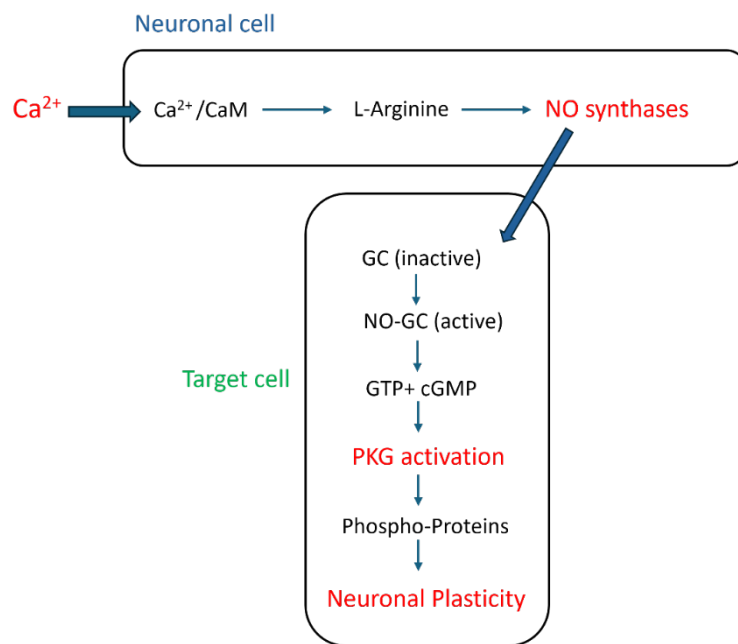


Figure 22: The role of calcium ions in NO-cGMP-PKG pathway in neurons. Ca^{2+} , calcium ions; CaM, calmodulin; NO, nitric oxide; GC, guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic GMP; PKG, cGMP-dependent protein kinase.

Several electrophysiological studies demonstrated the role of NO-cGMP-PKG in synaptic plasticity. For example, it was found that inhibition of PKG in rat lateral amygdala can negatively affects LTP (Ota *et al.*, 2008). NO-cGMP-PKG signaling pathway was also found to promotes synaptic plasticity and fear memory consolidation via activation of ERK/MAPK pathway (Ota *et al.*, 2008).

Both cGMP and PKG have several roles in insect nervous system (Caplan *et al.*, 2013; Kaun *et al.*, 2007; Scheiner *et al.*, 2014). In *D. melanogaster*, and similar to mammals, cGMP can modulate learning and memory processes. For example, it was found that high PKG activity can enhance memory acquisition and retention, while lower PKG activity can negatively

modulate these functions (Kaun *et al.*, 2007). Furthermore, in nociceptive sensory neurons of *Aplysia californica*, NO-cGMP-PKG pathway was found to be crucial for the induction of long-term hyper-excitability after a noxious stimulus (Lewin *et al.*, 1999).

[III.2.5 The protein kinase \(PKC\) pathway](#)

One of the major downstream effectors of calcium signaling is protein kinase C (PKC). PKC consists of a family of closely related enzymes highly concentrated in the CNS.

In mammals' neurons, PKC activation serves as a key molecular mechanism underlying synaptic transmission and several neuronal functions (Huang, 1989; Matsumura *et al.*, 1997; Seo *et al.*, 2004; Wang *et al.*, 2001). In *Drosophila melanogaster*, PKC inhibition was found to significantly affects self-learning (Brembs *et al.*, 2008; Colomb *et al.*, 2016). In addition, drosophila olfactory sensory neurons (OSNs) responses to odorant stimulation were shown to be regulated by PKC. Indeed, *in vivo* injection of PKC activators enhanced drosophila odor response (Sargsyan *et al.*, 2011).

PKC can be activated by various signals including its binding to Ca^{2+} and diacylglycerol (DAG). DAG is produced through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) in the cell membrane by phospholipase C (PLC) that can be activated by certain receptor types, including G protein-coupled receptors (Farah *et al.*, 2012; Giorgione *et al.*, 2006; Huang, 1989; Liu *et al.*, 2017). PKC isoenzymes are categorized into calcium-dependent or conventional forms (α , β I, β II, γ), novel types (δ , ϵ , η , θ), and atypical variants (ν / λ , ζ). Moreover, two recently identified members (μ , ν) have been added to this classification. Conventional PKCs (cPKCs) activation require calcium ions and diacylglycerol (DAG), whereas novel PKCs (nPKCs) activation only require DAG, and atypical PKCs (aPKCs) are activated independently of calcium and DAG (Ananthanarayanan *et al.*, 2003; Battaini, 2001; Kamiya *et al.*, 2016).

[III.2.6 The cyclic AMP \(cAMP\) pathway](#)

Cyclic AMP (cAMP) serves as an intracellular second messenger for numerous extracellular signals in the nervous system of both vertebrates and invertebrates. Studies on the sea slug *Aplysia* showed that cAMP signaling mediates both short- and long-term facilitation at sensorimotor synapses. Similarly, in the rodent hippocampus, cAMP plays a role in facilitating

long-term potentiation (LTP) at excitatory glutamatergic synapses (Brunelli *et al.*, 1976; Frey *et al.*, 1993; Schacher *et al.*, 1988; Weisskopf *et al.*, 1994). In drosophila, numerous studies demonstrated the role of cAMP in neurons excitability, synaptic plasticity and memory. As a result, drosophila became an excellent model to investigate cAMP signaling role in synaptic plasticity (Cheung *et al.*, 1999; Lee *et al.*, 2000; Zhong *et al.*, 1991).

When a ligand binds to a G protein-coupled receptor, it activates the G α s subunit of cAMP. This activation involves the conversion of GTP to GDP, which subsequently activates the membrane enzyme adenylyl cyclase (AC). AC then catalyzes the production of cAMP from ATP (Serezani *et al.*, 2008). cAMP acts on cAMP-dependent protein kinase (PKA). PKA activation can in turn phosphorylates other downstream proteins. cAMP-activating G protein is composed of 3 subunits, G α , G β and G γ . When the protein binds a GTP, it dissociates into two parts: G α is isolated from the other two, which form a G $\beta\gamma$ dimer (Hildebrandt, 1997). Depending on the subfamily to which G α belongs, its interaction with the AC varies: it can be stimulatory (G α s), inhibitory (G α i), or have an indeterminate action (G α q). The G $\beta\gamma$ dimer is either inhibitory or stimulatory, depending on the CA isoform under consideration. This constitutes a first criterion for differentiating the different isoforms of the AC family (Hanoune *et al.*, 2001). Nine isoforms (AC1-9) encoded by different genes have been characterized in several mammalian tissues and respond differently to G $\beta\gamma$. AC1, AC5 and AC6 are stimulated, while AC2, AC4 and AC7 are inhibited by the dimer. A tenth, cytoplasmic, so-called soluble isoform (sAC) has been purified in rat testes and is not regulated by G proteins (Buck *et al.*, 1999). AC3 is weakly stimulated by PKC, inhibited by CaMKII and stimulated by CaM, while AC8 is stimulated only by CaM (Sunahara *et al.*, 2002), AC9 is the only isoform stimulated by CaMKII via a G α q dimer (Cumbay & Watts, 2005). Additionally, CaM and CaMKs can directly influence cAMP levels. Ca²⁺ activates CaM and CaMKs, which then modulate cAMP production by regulating the activity of AC and phosphodiesterase (PDE) (Corsi, 2012). PDEs are characterized by a highly variable N-terminal regulatory domain, a conserved catalytic domain, and a C-terminal domain (Conti, 2000; Jeon *et al.*, 2005). The regulatory domain contains binding sites for various molecules, such as CaM, cAMP, or cGMP, and these sites vary among PDEs (Bolger, 1994; Conti, 2000). In mammals, eleven PDE families (PDE1-11) have been identified. While multiple PDE families are selective for cAMP, only PDE1 is stimulated by CaM. Consequently, when CaM activates PDE1, it leads to the conversion of cAMP to AMP, reducing cytoplasmic cAMP levels (Keravis *et al.*, 2012).

III.2.7 Ryanodine receptors (RyRs) and inositol triphosphate receptors (IP₃Rs)

As we have mentioned before, both external Ca²⁺ influx into the cytoplasm, and Ca²⁺ release from internal stores, are critical to maintain Ca²⁺ homeostasis. The main internal Ca²⁺ stores are the endoplasmic reticulum (ER) in neurons, and the sarcoplasmic reticulum (SR) in muscle cells. Ca²⁺ release from these stores is mediated by two classes of transmembrane receptors, inositol 1,4,5-trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs), both are members of the voltage-sensitive ion channel (VIC) superfamily (Heine *et al.*, 1998; Lümnen 2013; Sattelle *et al.*, 2008; Toprak *et al.*, 2021).

Both receptors are essential in insects' physiology and development. For example, RyRs play key roles in muscle contraction and hormone secretion, while IP₃Rs are mainly involved in events related to learning, memory, neuronal signalling, and lipid metabolism (Toprak *et al.*, 2021).

IP₃Rs can be activated by both inositol 1,4,5-trisphosphate (IP₃) and low concentrations of cytoplasmic Ca²⁺, while higher concentrations (above 300 nM) inhibit channel activity. Three IP₃R isoforms have been identified in mammals, IP₃R1, IP₃R2, and IP₃R3. *IP3R* genes are expressed in most cells, particularly in the ER of neurons, adipocytes, and oocytes (Decuyper *et al.*, 2011; Toprak *et al.*, 2021).

In insects, they only possess one *IP3R* gene. The first IP₃R was identified from *D. melanogaster*, that has approximately 60% amino acid identity with the three mammalian IP₃Rs. Insects IP₃Rs are commonly composed of 2724–2833 residue. However, and contrary to insects RyR, the crystal structures of insects IP₃R is still to be examined (Toprak *et al.*, 2021).

IP₃Rs play a pivotal role in IP₃-induced calcium release (IICR) from ER, an essential cellular signaling process for various physiological functions, including muscle contraction, neurotransmitter release (Berridge, 2009; Demydenko *et al.*, 2022). In this process, G-protein-coupled receptors (GPCRs) play a key role. Indeed, it is known that GPCRs can stimulate phospholipase C (PLC) that hydrolyses the phosphorylated plasma membrane glycolipid, phosphatidylinositol 4,5-bisphosphate (PIP₂), into secondary messengers, diacylglycerol (DAG) and IP₃. IP₃ binds to IP₃-binding sites in the N-terminus of the tetrameric IP₃R to initiate conformational changes leading to opening of the Ca²⁺ permeable pore and Ca²⁺ release from the ER (Demydenko *et al.*, 2022).

In mammals, RyRs are found in three isoforms, RyR1, RyR2, and RyR3, showing 65% homology at the amino acid level. The localization of the three mammalian isoforms is tissue-

dependent. For example, RyR1 is mainly found in skeletal muscle, while RyR2 is the major isoform in cardiac muscle. In the brain, RyR3 is the mainly expressed isoform (Lanner *et al.*, 2010).

Interestingly, it has been found that the transmembrane regions of the three isoforms contain three sites of diversity (D1, D2, and D3) that account for several of the functional differences between receptor subtypes. For example, D2 is implicated in the coupling of RyR1 to Cav1.1, something not seen for RyR2. While D1 influences RyR1 sensitivity to Ca^{2+} and caffeine (Sattelle *et al.*, 2008). Indeed, it is well established that activation of CaV1.1 in skeletal muscle triggers RyR1 gating through direct conformational coupling, without the need for calcium entry *via* VGCCs. While in cardiac myocytes, RyR2-mediated calcium release depends on a depolarization-induced calcium influx through CaV channels, a process known as calcium-induced calcium release (CICR) (Lümmen, 2013). In neurons, and similar to CICR seen in cardiac myocytes, neurons rely on calcium influx through voltage-gated calcium channels (such as CaV2.1 and CaV2.2) to trigger CICR. In addition, calcium influx through nAChRs, especially, $\alpha 7$ nAChR, that have a high permeability to Ca^{2+} , can activate downstream pathways like CICR through RyRs, amplifying the calcium signal within the neurons (Shen *et al.*, 2009). Furthermore, it was found that both RyR1 and RyR2 isoforms can be regulated by different factors, like CaM. For example, Ca^{2+} -free apo-CaM activates RyR1, whereas Ca^{2+} -bound CaM inhibits the channel (Lümmen, 2013).

In insects, only one RyR isoform has been identified. In drosophila, the RyR gene encodes a protein consisting of 5,216 amino acid residues, sharing only 45–47% sequence identity with the three mammalian RyR genes (Sattelle *et al.*, 2008). In the muscle and nervous tissues from different insect species like houseflies (*M. domestica*), cockroaches (*P. americana*), and the corn earworm (*Helicoverpa zea*), radioligand binding data revealed the pharmacology of the high-affinity ryanodine binding sites. In insects, ryanodine, which is a toxic natural alkaloid, has historically been used as an insecticide, but its toxicity to mammals has limited its continued application. Today, two primary classes of chemical insecticides are in use: phthalic acid diamides and anthranilic diamides. Both of these groups act as potent activators of insect RyRs, effectively disrupting calcium homeostasis and leading to paralysis and death in targeted pests (Cordova *et al.*, 2006; Kadala *et al.*, 2019; Sattelle *et al.*, 2008).

Interestingly, it was found that American cockroaches DUM neurons exhibit a Ca^{2+} resting current ($I_{\text{Ca,R}}$), that helps in regulating $[\text{Ca}^{2+}]_i$, as well as the pacemaker depolarization during neuronal spiking. While the neurohormone D (NHD) was found to enhance $I_{\text{Ca,R}}$, leading to

increased $[Ca^{2+}]_i$. The ryanodine receptor antagonist, dantrolene, reduces the NHD-induced calcium transients, indicating that NHD operates via CICR mechanisms. Notably, the CICR effect significantly diminishes when the NHD-induced potentiation of $I_{Ca,R}$ is blocked by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and mefenamic acid, showing that enhanced Ca^{2+} resting currents can activate CICR channels in DUM neurons (Heine *et al.*, 1998).

III.3 Regulation of nAChRs by kinases and calcium binding proteins

nAChRs regulation by calcium pathways is considered a complex process that influences receptor function, desensitization, trafficking, and internalization. Although insects and mammals share many structural and functional similarities in nAChRs, there are significant differences in how these receptors are regulated and how they respond to various intracellular factors like calcium and its downstream proteins, like PKC, PKA, PKG, cAMP, and CaMKs (Table 2).

Table 2: A table summarizing the various effects of PKC, PKA, cAMP, and CaMKII on nAChRs in different species and receptor subtypes.

Enzyme	Receptors (specie)	Effect on nAChRs	References
PKC	Mammals $\alpha 4\beta 2$ (Human)	- Phosphorylation sites: Ser550 and Ser362 - Enhances the recovery from desensitization rate - Its blocking cause receptors deactivation - Modulates receptors trafficking and localization	(Downing <i>et al.</i> , 1987; Eilers <i>et al.</i> , 1997; Fenster <i>et al.</i> , 1999; Lee <i>et al.</i> , 2015; Miller <i>et al.</i> , 2018; Pollock <i>et al.</i> , 2007; Simmons <i>et al.</i> , 1990)
PKC	Mammals $\alpha 7$ (Chicken)	- Unclear effect - Potential serine/threonine phosphorylation sites	(Ahmad <i>et al.</i> , 2019; Moss <i>et al.</i> , 1996)
PKC	<i>Periplaneta americana</i> nAChR1	- Positively regulated by PKC1 - Negatively regulated by PKC2	(Courjaret <i>et al.</i> , 2003)

PKA	Mammals $\alpha 7$ (Human)	• Phosphorylation sites: Ser365 • Enhances the desensitization rate • Reduces receptors surface expression and activity	(Komal <i>et al.</i> , 2015)
PKA	Mammals $\alpha 4\beta 2$ (Human)	• Phosphorylation sites: Ser362 and Ser467 • Modulates surface expression via 14-3-3 proteins	(Jeanclos <i>et al.</i> , 2001; Begley <i>et al.</i> , 2016; Begley <i>et al.</i> , 2013; Pollock <i>et al.</i> , 2007)
PKA	<i>Locusta migratoria</i> $\alpha 8$	• Phosphorylation sites: Ser337 • Increase agonists efficacy	(Sun <i>et al.</i> , 2023)
PKA	<i>Periplaneta americana</i> nAChR1	• Its inhibition reduces NIC-induced currents	(Courjaret <i>et al.</i> , 2001)
PKA	<i>Drosophila melanogaster</i>	• cAMP/PKA cascade enhances NIC effects	(Hou <i>et al.</i> , 2004)
cAMP	Mammals $\alpha 3$ (Chicken) $\alpha 7$ (Human)	• Enhances sensitivity to ACh in cultured neurons • 8-Br-cAMP decrease $\alpha 7$ nAChRs response • Putative phosphorylation sites on $\alpha 3$ subunits	(Higgins <i>et al.</i> , 1988; Margiotta <i>et al.</i> , 1987) (Komal <i>et al.</i> , 2015; Vijayaraghavan <i>et al.</i> , 1990)
cAMP	<i>Periplaneta americana</i> nAChR1	• High cAMP levels decrease nicotinic currents • Low cAMP levels increase nicotinic currents via PKA	(Dubois <i>et al.</i> , 2012; Courjaret <i>et al.</i> , 2001)
cAMP	<i>Periplaneta americana</i> nAChR2	• High cAMP levels increase the sensitivity to ACT and CLT via TRPγ	(Dubois <i>et al.</i> , 2012)
CaMKII	Mammals $\alpha 4\beta 2$ / $\alpha 7$ (Human)	• Modulates receptor localization, activity, neurotransmitter release, and plasticity • Possible indirect phosphorylation via PKA	(Bagur <i>et al.</i> , 2017; Fisher <i>et al.</i> , 2000; Kutlu <i>et al.</i> , 2016; Begley <i>et al.</i> , 2016; Begley <i>et al.</i> , 2013; Miller <i>et al.</i> , 2018; Wang <i>et al.</i> , 2006)
CaMKII	<i>Periplaneta americana</i> nAChR1	• Ca²⁺/CaM / CaMKII modulate NIC and CLT currents • CaMKII-E inhibition reduces calcium increase after NIC application	(Calas-List <i>et al.</i> , 2013; Courjaret <i>et al.</i> , 2001; Houchat <i>et al.</i> , 2022)

PKG	<i>Periplaneta americana</i> nAChR2	• cGMP application increases NIC-induced currents • PKG inhibition reduces NIC-induced currents • Its effects linked to the intracellular Ca^{2+} levels	(Mannai <i>et al.</i> , 2016)
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III.3.1 Regulation of nAChRs by PKC

Several studies have demonstrated that the activation of PKC enhances the rate of nAChRs desensitization in various neuronal preparations (Downing & Role, 1987; Simmons *et al.*, 1990). The precise effects of PKC on nAChRs can vary depending on the cell type, receptor subtype, and the specific phosphorylation sites targeted by PKC. In addition, the interplay between PKC and other signaling pathways, such as cAMP/PKA pathway, can further modulate the regulation of nAChRs by PKC.

In mammals, PKC activation can control $\alpha 4\beta 2$ nAChRs function through the phosphorylation of specific serine/threonine residues on $\alpha 4$ subunits. For example, *in vivo* study using human nAChRs $\alpha 4$ subunits expressed in SHEP1 cells, it was found that Ser550 and possibly Ser362 are phosphorylated by PKC. This phosphorylation directly influences the activity, desensitization, trafficking, and localization of nAChRs. In addition, it has been found that blocking of PKC activity by either downregulation or direct inhibition can cause deactivation of the $\alpha 4\beta 2$ receptors (Eilers *et al.*, 1997; Fenster *et al.*, 1999; Lee *et al.*, 2015; Miller *et al.*, 2018; Pollock *et al.*, 2007).

While several studies support the specific role for PKC in regulating the activity of the mammalian $\alpha 4\beta 2$ nAChRs (Eilers *et al.*, 1997), the direct link between mammals $\alpha 7$ nAChRs activity and PKC is still to be investigated. For example, Moss *et al.* 1998 demonstrate that the major cytoplasmic loop of both the rat and the chicken nAChRs $\alpha 7$ subunit has a direct phosphorylation site of PKA, but they do not seem to constitute a potential substrate for direct PKC phosphorylation (Moss *et al.*, 1996). However, a recent study of Ahmad *et al.* 2019, demonstrated that human nAChRs $\alpha 7$ contains serine/threonine phosphorylation sites of both PKA and PKC (Ahmad *et al.*, 2019).

In the context of *Periplaneta americana* cockroach's dorsal unpaired median (DUM) neurons, it has been shown that nAChR1 receptor experiences both up-regulation and down-regulation mediated by two types of PKC:

- 1- The conventional PKC (cPKC) or PKC1: positively regulates nAChR1, can respond to PMA and changes in intracellular calcium levels, but does not react to the PKC2 activator DiC8.
- 2- The novel PKC (nPKC) or PKC2: negatively regulates nAChR1, can be activated by DiC8 but remains unaffected by increases in intracellular calcium levels. Indeed, DiC8, the PKC2 activator, was able to reduce both CLT and NIC- induced currents through DUM neurons nAChRs (Courjaret *et al.*, 2003; Houchat *et al.*, 2020a).

It was demonstrated that neuronal nAChR1 can be modulated through PKC1 and PKC2 after ACh binding to muscarinic receptors in *Periplaneta americana* cockroach's DUM neurons (**Figure 23**) (Courjaret *et al.*, 2003).

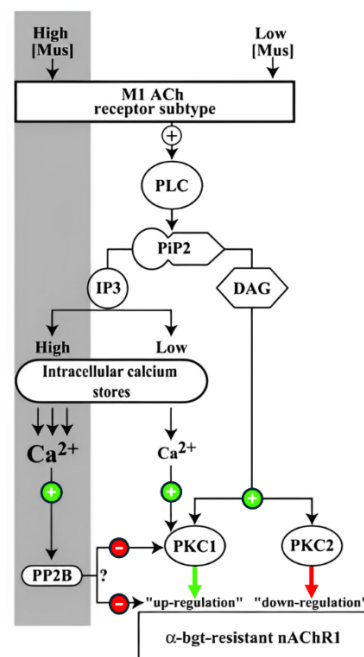


Figure 23: nAChR1 regulation through PKC pathway in DUM neurons of the cockroach *P. americana*. Activation of a G protein-coupled receptor (muscarinic receptor M1) induces production of IP3 and DAG. DAG activates both PKC1 and PKC2. PKC1 positively regulates nAChR1, while PKC2 negatively regulates it. Only PKC1 is regulated by intracellular calcium, particularly released from intracellular stores. Mus, muscarine; PLC, phospholipase C; PIP2, phosphoinositol biphosphate; IP3, inositol triphosphate; DAG, diacylglycerol; PP2B, protein phosphatase 2B; PKC, protein kinase C.

III.3.2 Regulation of nAChRs by PKA

While PKC has been shown to facilitate recovery from the desensitized states in human nAChRs, research has indicated that PKA can enhance the desensitization of nAChRs (Ahmad *et al.*, 2019). In mammals, PKA can phosphorylate specific serine residues sites on $\alpha 7$ and $\alpha 4$ nAChRs subunits, resulting in modulation of receptor activity and surface expression (Komal *et al.*, 2015; Pollock *et al.*, 2007).

Phosphorylation of serine 365 in the M3–M4 cytoplasmic loop of the $\alpha 7$ subunit by PKA can negatively modulate $\alpha 7$ nAChRs activity, as demonstrated by (Komal *et al.*, 2015). This reduction in $\alpha 7$ nAChRs activity after the endogenous activation of PKA is due to a reduction in nAChRs surface expression without any changes in single-channel conductance or gating kinetics, contrary to insects, where the reduction in nAChRs activity was associated with a decrease in current amplitude (Courjaret *et al.*, 2001; Komal *et al.*, 2015).

In mammals, PKA can also impact $\alpha 4\beta 2$ nAChRs subtypes. Initial studies demonstrated that rat $\alpha 4\beta 2$ receptors, either isolated from brain or expressed in *Xenopus* oocytes, were phosphorylated *in vitro* by PKA. PKA phosphorylates Ser362 and Ser467 on the M3-M4 cytoplasmic domain of the $\alpha 4$ subunit. Studies also showed that the chaperone 14-3-3 proteins can modulate *in vitro* cell surface expression of mammalian $\alpha 4\beta 2$ nAChRs by PKA-driven interactions between 14-3-3 proteins and phosphoserine motif RSLSVQ of $\alpha 4$ subunit (Jeanclos *et al.*, 2001; Begley *et al.*, 2016; Begley *et al.*, 2013; Pollock *et al.*, 2007).

In most insect's species, there is a binding motif for 14-3-3 proteins in the cytoplasmic loop of $\alpha 8$ nAChR subunits. A novel study identified a putative PKA phosphorylation site within Ser337 of $\alpha 8$ subunit. PKA-mediated phosphorylation of Loc $\alpha 8$ S337 in *Locusta migratoria* enhanced interaction with Loc14-3-3 ϵ which increased agonist efficacy on hybrid Loc $\alpha 8/\beta 2$ nAChRs expressed in *Xenopus* oocytes. This effect was abolished by PKA inhibitors, highlighting the role of PKA in modulating insects' nAChR pharmacological properties (Sun *et al.*, 2023).

The impact of PKA on nicotine currents in insect neurons has also been investigated. It was demonstrated that inhibiting PKA activity using H89 resulted in reduced nicotine current amplitudes in DUM neurons from *P. americana* cockroaches (Courjaret *et al.*, 2001). Additionally, forskolin activation of AC and direct intracellular cAMP increases reduce nicotinic currents via nAChR1, suggesting modulation by the cAMP/PKA (Courjaret *et al.*,

2001). Other mechanisms involving insect nAChRs, like addiction, are modulated by PKA. For example, in *D. melanogaster*, wild-type flies treated with a PKA inhibitor and a strain expressing defective PKA are less receptive to NIC than wild-type flies. Repeated exposure of wild-type flies to NIC increases cAMP levels and responses, thus, the effect of NIC is enhanced by a mechanism involving the cAMP/PKA cascade (Hou *et al.*, 2004).

III.3.3 Regulation of nAChRs by cAMP

Although the effects of cAMP-dependent phosphorylation on muscle nAChRs have been extensively explored in vertebrates, cAMP effect on neuronal nAChRs still to be investigated. However, many studies demonstrated crucial roles of cAMP in synaptic plasticity. For example, in cultured chicken ciliary ganglion neurons, cAMP dependent mechanism enhanced nAChRs sensitivity to ACh, but interestingly, without any change in receptor properties like desensitization, or nAChRs total number (Margiotta *et al.*, 1987). Same results were demonstrated in bovine adrenal chromaffin cells. cAMP analogues application enhanced the nAChRs response, but with a small effect on agonist-induced desensitization and nAChRs total surface number. These findings were explained as cAMP-dependent process converts nAChRs from a “silent” state on the neurons to a “functionally available” one (Higgins *et al.*, 1988; Margiotta *et al.*, 1987). However, later study of Vijayaraghavan, showed that chick ciliary ganglion nAChRs can be phosphorylated *in situ* in a cAMP-dependent manner on putative $\alpha 3$ subunits (Vijayaraghavan *et al.*, 1990). More recent studies using HEK 293T cells, found that 8-Br-cAMP, which is the cell membrane-permeable analogue of cAMP can decrease $\alpha 7$ nAChRs response without changing the desensitization kinetics. This was explained by cAMP activating PKA which in turn activates D1/D5 dopamine receptors (Komal *et al.*, 2015). Furthermore, the increased sensitivity to NIC after repeated exposure is regulated by the cAMP/PKA signaling pathway. This pathway involves the transcription of CREB (cAMP-response element-binding protein)-dependent genes, that play a crucial role in synaptic plasticity (Carlezon *et al.*, 2005; Silva *et al.*, 1992; Trifilieff *et al.*, 2006).

cAMP involvement in mechanisms underlying neural plasticity was also demonstrated in drosophila neurons. In drosophila mushroom bodies, it was found that cAMP can modulate NIC-induced Ca^{2+} influxes in the in a PKA-dependent or independent manner (Pavot *et al.*, 2015). In addition, cAMP levels elevation in drosophila neurons can modulate the frequency of

miniature excitatory postsynaptic potentials (mEPSPs) at a cholinergic central synapse (Lee *et al.*, 2000).

In DUM neurons from cockroach *P. americana*, it was found that adenylyl cyclase (AC) can regulate nAChR1 via a cAMP/PKA pathway. After AC activation by Ca^{2+} /CaM, it increases cAMP generation, cAMP in turn activates PKA, which ultimately phosphorylates and positively regulates nAChR1 (Bodereau-Dubois *et al.*, 2012; Courjaret *et al.*, 2001). However, these results were obtained only when low cAMP concentration (0.1mM) were added to the intrapipette solution. While internal cAMP concentrations higher than 0.1mM reduced NIC-induced currents (Courjaret *et al.*, 2001).

Furthermore, cAMP can modulate clothianidin action on nAChRs expressed in DUM neurons. At high CLT concentration (1mM), the intrapipette application of cAMP increases currents induced by CLT. In this condition, cAMP effect is associated to a strong increase of intracellular Ca^{2+} concentration leading to an increase of CLT-induced currents. While low CLT concentrations do not induce sufficient calcium influx to produce these effects (Calas-List *et al.*, 2013).

In addition, cAMP can alter other ion channels' function, modulating intracellular calcium concentration which in turn influences nAChRs sensitivity to neonicotinoids. For example, high levels of cAMP were found to activate TRP γ , reducing calcium influx, and as a result increasing nAChR2 sensitivity to both acetamiprid and clothianidin (Dubois *et al.*, 2012).

III.3.4 Regulation of nAChRs by CaMKs

In mammals, nAChRs activation by nicotine could activate associated CaMKII directly, which in turn leads to phosphorylation of nAChRs subunits or of downstream targets.(Miller *et al.*, 2018). Although a quantitative interaction between $\alpha 4\beta 2$ nAChRs and multiple isoforms of CaMKII has been identified in mouse and the human brain, research are still going to define a direct phosphorylation site of this kinase in nAChRs (Bagur *et al.*, 2017; Begley *et al.*, 2016; Begley *et al.*, 2013; Sałaciak *et al.*, 2021; Swulius *et al.*, 2008). Studies showed that even if nAChRs may not be a major substrate for CaMKII in the mouse brain but an increase in $\alpha 4\beta 2$ nAChRs phosphorylation was demonstrated when they co-transfected with CaMKII in HEK cells. However, this increase in nAChRs phosphorylation is potentially indirect by PKA and another unidentified kinase. This link between $\alpha 4\beta 2$ nAChRs and CaMKII can affect receptors

localization to intracellular compartments or the plasma membrane (Miller *et al.*, 2018). Numerous studies on vertebrate neurons have demonstrated that Ca^{2+} influx through nAChRs can regulate these receptors *via* intracellular pathways involving CaM and CaMK. In the majority of cases, this regulation involves $\alpha 7$ receptors. For example, in rat hippocampal neurons, it was found that fluphenazine, a calmodulin antagonist, prevented NMDA receptor current inhibition by nAChR activity. (Fisher *et al.*, 2000) In addition, inhibition of CaMKII by KN-62 (an isoquinolinesulfonamide derivative) prevents NIC from potentiating glutamate release induced by 4-aminopyridine (Wang *et al.*, 2006). As a result, CaM-dependent regulatory pathways can play a role in neurotransmitter release regulated by nAChRs activation. In rat hippocampal, it was also found that CAMKs direct or indirect activation by nicotine may modulate hippocampal plasticity and in parallel hippocampus-dependent learning and memory (Kutlu *et al.*, 2016).

In insects such as *P. americana* and *D. melanogaster*, various CaMKII isoforms have been identified (Griffith, 1997; Taillebois *et al.*, 2013). In *P. americana*, CaMKII cloning revealed 5 isoforms (PaCaMKII A to E), with only CaMKII-E isoform expressed in DUM neurons. Later, it was found that Ca^{2+} /CaM / CaMKII pathway is involved in the modulation of both nicotine and clothianidin current through nAChRs in DUM neurons. In specific, CaMKII-E inhibition using antisense oligonucleotides reduced the intracellular calcium levels increase after nicotine pulse application (Calas-List *et al.*, 2013; Courjaret *et al.*, 2001; Houchat *et al.*, 2022).

Beside the involvement of CaMKII, calmodulin was also found to regulate insects nAChRs. This was demonstrated by using both CaM inhibitor, W7 and CaM antagonist, calmidazolium (CdZ) (Courjaret *et al.*, 2001; Houchat *et al.*, 2022).

III.3.5 Regulation of nAChRs by PKG

In mammals, while several studies demonstrated the role of PKG pathways in regulating mAChRs, the direct link between PKG and neuronal nAChRs modulation is still to be investigated (Liu *et al.*, 1997; Wang *et al.*, 1995).

In contrast, studies on insects' neurons showed that PKG pathways play an important role in nAChRs regulation. For example, in DUM neurons from *P. americana*, it was found that both nAChR1 and nAChR2 can be regulated by a pathway involving cGMP and PKG (Mannai *et*

al., 2016). Indeed, the intrapipette application of 100 μ M cGMP increases NIC-induced currents amplitude, but only on the second part of the biphasic current-voltage curve corresponding to nAChR2. Additionally, the inhibition of PKG using KT5823 (KT) reduces the amplitude of nicotinic currents via nAChR2, and this reduction was not affected by Mec. Furthermore, the modulation of nicotine effects on nAChR2 by PKG is linked to the intracellular calcium. Indeed, when intracellular calcium increase is inhibited using Cadmium chloride (CdCl_2), the inhibitory effect of KT5823 was abolished. However, when NO-GC was specifically blocked by ODQ, nicotinic currents on both parts of the biphasic curve were reduced. These results indicate that both nAChR1 and nAChR2 subtypes could be regulated by the cGMP/PKG pathway (**Figure 24**). The direct increase in cGMP causes an increase in nicotinic currents through nAChR2 via PKG activation, while the increase in cGMP via GTP degradation by GC would have a strong modulatory effect on both nAChR1 and nAChR2 (Mannai *et al.*, 2016).

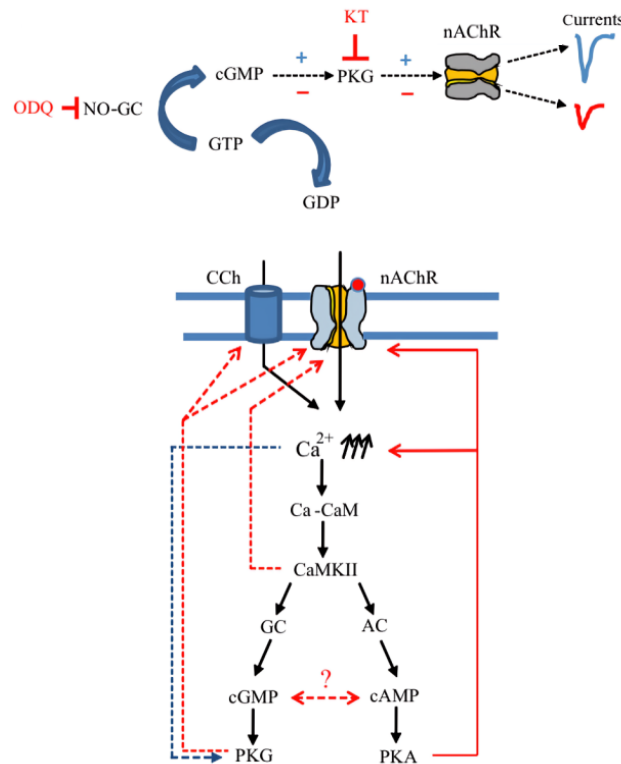


Figure 24: Diagram of the regulation of nAChRs by the cGMP/PKG pathway in *P. americana* DUM neurons. The red dashed lines represent the pathways involved in this process. CaM: calmodulin; CCh: calcium channel; GC: guanylyl cyclase; PKA: protein kinase A; PKG: cGMP-dependent protein kinase.

Furthermore, in *Manduca sexta* isolated neurons, it has been shown that nAChRs activation by ACh can control cGMP levels via NO production. Indeed, using both nicotinic antagonists like Mec, and NO inhibitors like ODQ, prevented cGMP levels increase (Zayas *et al.*, 2002). Since NO production requires Ca^{2+} , the link between calcium increase through nAChRs was also investigated. The findings indicated that nAChRs activation is linked to NOS production, potentially by directly facilitating Ca^{2+} influx (Zayas *et al.*, 2002).

IV. Research team problematic and thesis objective

This thesis was conducted at the Physiologie, Ecologie et Environnement (P2E) laboratory within the Neurobiologie et Neuropharmacologie des Canaux Ioniques (NNCI) team.

The main objective of our team is to study both insect and mammalian ion channel receptors, particularly nAChRs. Since nAChRs are involved in many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, and in insects, they are the main target of neonicotinoids and their derivatives, like sulfoximines and butenolides.

This team is interested in the functional and pharmacological properties of nAChRs using different approaches such as electrophysiology, site-directed mutagenesis, and toxicology to better understand nAChRs structure and their intracellular regulation mechanisms.

Using electrophysiological, molecular biology, and recently, calcium imaging techniques, we have been investigating how the functional and pharmacological properties of insect neuronal nAChRs can alter their responses to neonicotinoids.

One of the team's hypotheses is that different regulatory pathways can modulate the mode of action of neonicotinoids and the sensitivity of nAChRs to these compounds. Specifically, in *Periplaneta americana* cockroaches. Several signaling pathways regulating nicotine and neonicotinoids mode of actions have been studied (Calas-List *et al.*, 2013; Courjaret *et al.*, 2003; Courjaret *et al.*, 2001).

The objective of this thesis is to better understand the involvement of intracellular calcium regulatory pathways in modulating the activity of insect nAChRs and their response to insecticides. The main idea is that, manipulating the intracellular regulatory processes of nAChRs could impact insect sensitivity to neonicotinoids, and can explain the resistance mechanisms.

To achieve this goal, experiments focused on nAChRs expressed in the DUM neurons of the last abdominal ganglion of *Periplaneta americana* cockroach were conducted. We used whole-cell patch-clamp techniques and ratiometric calcium imaging with fura-2-AM. These techniques are detailed in the "Materials and Methods" section.

Within the context of my thesis, my work was organized as follow:

- 1- The first objective was to study the involvement of CaMKK/AMPK pathway in regulating nAChRs expressed in *P. americana* cockroaches DUM neurons.
- 2- The second objective was to study in parallel the role of intracellular calcium release from endoplasmic reticulum through RyRs and IP₃R in modulating FLU mode of action, which is a novel systemic butenolide insecticide.

The first chapter of this thesis focuses on deeper understanding of CaM role in regulating α -Bgt-sensitive and -insensitive nAChRs, nAChR1 and nAChR2, using specific inhibitors and activators of protein kinases and other CaM-dependent molecules like AMPK. In addition, selective nAChR2 agonists, like LMA10203, were used to specifically define the type of nAChRs affected by CaMKK/AMPK pathway.

The second chapter studies the FLU mode of action on native DUM neurons for the first time. We focused on the role of calcium release from ER through RyRs and IP₃Rs on inward currents and calcium increase induced by FLU. This was achieved by using dantrolene and ryanodine, both selective RyRs blockers, and 2-APB, a selective IP₃Rs antagonist.

My results are presented in the form of two published manuscripts in the "results" section. The "Conclusion and Perspectives" section synthesizes and combines all the obtained results.

Chapter II: Materials and methods

I. Biological model: American cockroaches, *Periplaneta americana*

American cockroaches *Periplaneta americana* are a common specie of cockroaches found in the United States and other parts of the world. They are considered excellent models for neurobiological studies. They belong to the *Blattidae* family and rank among the largest cockroach species, measuring on average 4 cm in length. They thrive in warm, moist environments, but can adapt to a variety of conditions.

Males and females can be easily differentiated, the males surpass females in length as their wings extend 4 to 8 mm beyond the abdomen's tip. Both genders possess a pair of slender, jointed cerci at the abdomen's end. Male cockroaches feature cerci comprising 18 to 19 segments, whereas females' cerci typically consist of 13 to 14 segments. Additionally, male American cockroaches sport a pair of styli between the cerci, a feature absent in females.

American cockroaches undergo three developmental stages: egg, nymph, and adult. Immature cockroaches emerge within 6 to 8 weeks and undergo 7 successive molts before becoming adults in 6 to 12 months depending on environmental conditions.

Like other insects, *P. americana* cockroaches have a central nervous system consisting of a brain and a ventral nerve cord. They also possess sensory organs, such as antennae and compound eyes. The nerve cord of *P. americana* includes the suboesophageal ganglion that plays a crucial role in controlling the muscles of the abdomen, especially during locomotion, three thoracic ganglia, and six abdominal ganglia. These ganglia are distributed along the ventral nerve cord and easily observable with a magnifying glass. They play vital roles in processing sensory information and coordinating motor functions throughout the cockroach's body (**Figure 25**).

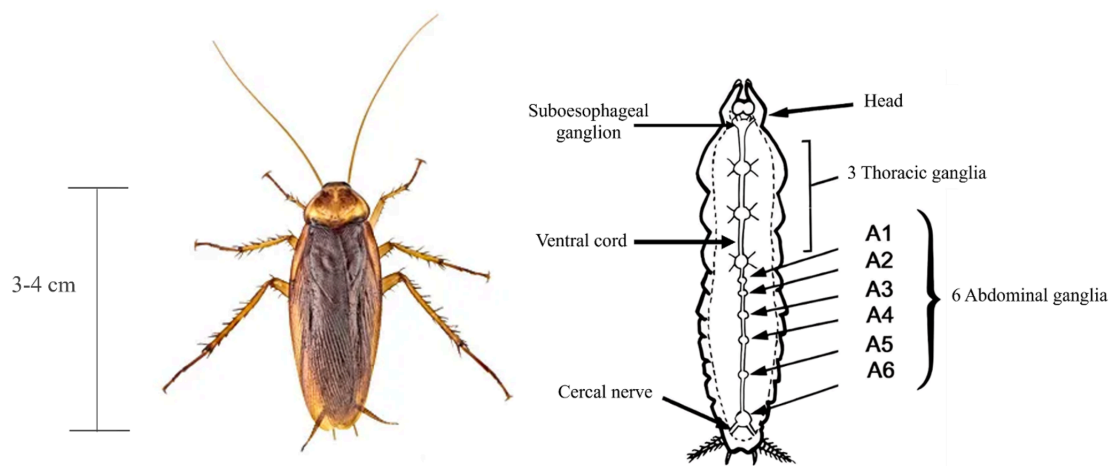


Figure 25: The cockroach *Periplaneta americana* and its nervous system (modified from Titlow *et al.* 2013)

I.1 DUM neurons

DUM neurons, also known as dorsal unpaired median neurons, are relatively large neurosecretory cells found in various insects, including American cockroaches in the last abdominal ganglion in and in thoracic ganglia. They typically have a diameter ranging from 45 to 60 μm , and are organized in three clusters along the dorsal midline, with 36 octopamine-immunoreactive neurons identified in both sexes. While both male and female cockroaches possess these neurons, males are often used for practical reasons such as consistent anatomical accessibility or hormonal stability, which facilitates comparative studies. Octopamine, a biogenic amine analogous to norepinephrine in vertebrates, is a key player in modulating these neurons, influencing processes such as flight metabolism, visual dishabituation, and other critical behaviors (Dacks *et al.*, 2005; Sinakevitchs *et al.*, 1995).

In addition, these neurons exhibit an endogenous pacemaker activity and contain a variety of receptors like nAChRs and GABAergic that can regulate the neurons' spontaneous activity and neurosecretory function (Lapied *et al.*, 1990; Sinakevitch, *et al.*, 1996; Stankiewicz *et al.*, 2012). Electrophysiological studies, such as patch-clamp recordings, have revealed a rich diversity of ion channels in these neurons, including potassium channels, and multiple calcium channel subtypes. These channels are crucial not only for maintaining the resting membrane potential but also for shaping the firing patterns of the neurons (Grolleau *et al.*, 2000).

DUM neurons' large size and accessibility make them particularly suitable for electrophysiological experiments, allowing researchers to investigate the activity of ion channels, such as nAChRs, and the mode of action of neonicotinoid insecticides. All experiments are conducted on DUM neurons from male individuals. The insects are kept in the laboratory at 29°C, away from any insecticides, with a 12-hour day: 12-hour night photoperiod.

I.2 DUM neurons preparation

The cockroach terminal abdominal ganglion (TAG) was isolated by dissection along the longitudinal dorsal-median line. Forceps were used to remove the alimentary canal as well as the overlying muscle and trachea. For the experiments evaluating the effect of STO injection on the TAG, we used a Hamilton syringe. Thus, 15 µl of STO was injected into the central part of the TAG. The TAG was then incubated for 3 hours at 29°C, after which the DUM neurons were isolated using a standard saline extracellular solution (in mM: 200 NaCl, 3.1 KCl, 5 CaCl₂, 4 MgCl₂, 10 HEPES, 10 mM; pH 7.4 adjusted with NaOH) with sucrose (50 mM).

DUM neurons were isolated from the cockroach TAG. After being carefully dissected, the TAG was placed in the standard saline extracellular solution. Each compound in this solution was purchased from Sigma Aldrich, France. The TAG was incubated (35 min, 29°C) in the saline supplemented with collagenase (type I, 1.5 mg/ml, GIBCO), rinsed twice and placed in the saline supplemented with foetal calf serum (5% by volume), 50 UI/ml penicillin and 50 µg/ml streptomycin (Sigma Aldrich, France). They were then mechanically dissociated by gentle trituration using fire-polished Pasteur pipettes. Before adding the cell culture, the isolated DUM neurons were left for 10 minutes to settle and adhere to Petri dishes (35/09 mm, NEST, Grosseron, France) coated with poly-D-lysine hydrobromide (MW, 70,000-150,000; Sigma Aldrich, France) for patch-clamp experiments, or to coverslips (22 x 40 x 0.17 mm, HIRSCHMANN, Verrerie Villeurbannaise, France) for calcium imaging experiments. Cell cultures were incubated overnight at 29°C. All preparations were made under semi sterile conditions *i.e.*, all equipment was sterilized with ethanol and solutions were filtered (**Figure 26**).

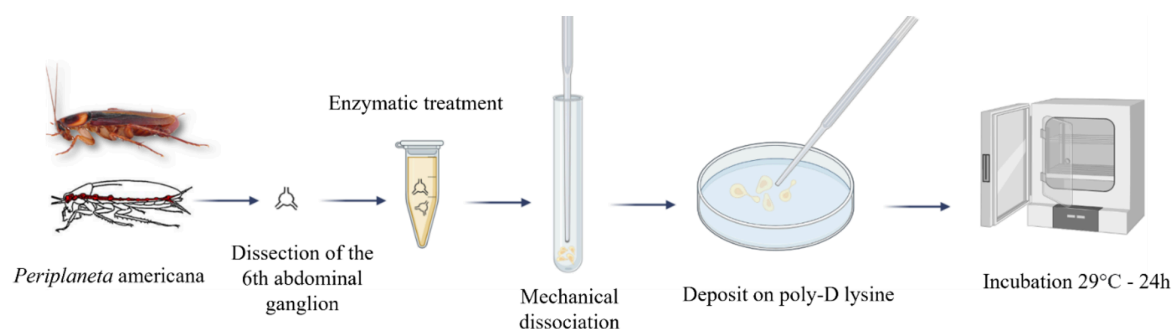


Figure 26: Detailed steps of DUM neurons isolation process.

II. Patch clamp technique (whole cell configuration)

Signals were recorded using the patch-clamp technique in the whole-cell recording configuration in voltage-clamp mode using an Axopatch 200B (Patch-clamp amplifier, Axon Instruments, Foster City, CA). They were digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments) and Axoscope 10.2 software (Axon Instruments). Signals were low pass-filtered at 5 kHz. The Petri dishes containing isolated DUM neurons (**Figure 27**) were placed onto an inverted microscope (AE 2000, Motic), and continuously bathed with the standard saline using a gravity perfusion system positioned within 100 μm from the cell body. In all experiments DUM neurons capacitance ranged from 200 to 250 pF to ensure reproducible current amplitudes. Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with a standard internal solution (in mM: 160 K-D gluconic acid, 10 K-fluoride, 10 NaCl, 1 MgCl_2 , 0.5 CaCl_2 , 20 HEPES, 10 EGTA, 3 ATP; pH 7.4 adjusted with KOH). Their resistances ranged from 1.5 to 2 M Ω . Agonists were applied via pneumatic pressure ejection (15 psig, 300 ms; Miniframe, Medical System Corporation, USA). The pressure ejection was made using a glass micropipette, with resistance ranging from 1.5 to 2 M Ω , positioned in a solution within 100 μm of the isolated cell body. All compounds were purchased from Sigma Aldrich, France.

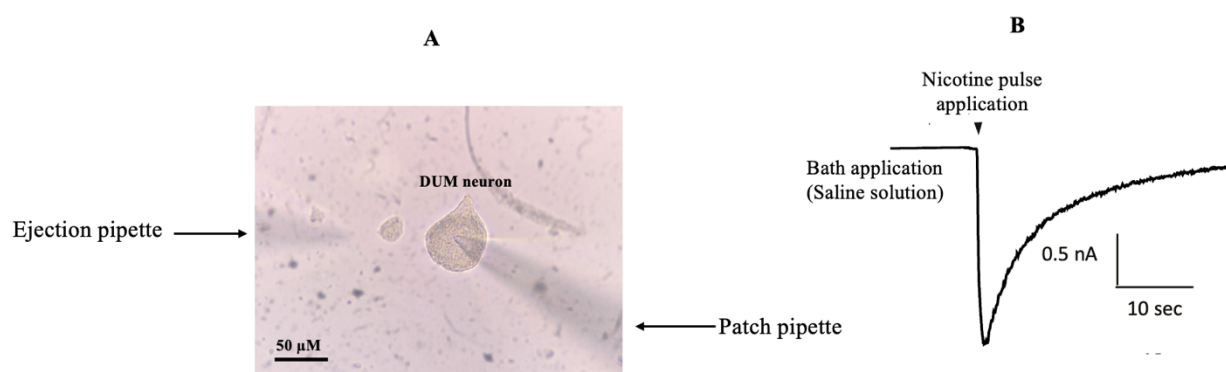


Figure 27: Patch clamp recording. (A) A single DUM neuron placed onto an inverted microscope (AE 2000, Motic); magnification 40x. (B) Inward current induced by the pulse application of 1mM nicotine using ejection pipette.

III. Intracellular calcium imaging

III.1 The principle of the technique

Calcium imaging is a technique used to measure the changes in calcium ion concentrations in active cells using a dye or a genetically encoded calcium indicator and is considered a powerful tool to explore neuronal activity.

These dyes have two components: a chelating part that selectively binds calcium and a fluorescent part that, when excited by a specific ultraviolet wavelength, emits light of a specific wavelength. Calcium binding alters the fluorescence properties of the dye and visually reflects changes in calcium concentration within the cell.

In the laboratory, the dye used on cockroach DUM neurons is fura-2-acetoxymethyl ester, or fura-2-AM. This dye emits fluorescence at two different excitation wavelengths depending on whether calcium is bound or free. When excited at 340 nm, it emits fluorescence corresponding to bound calcium, while at 380 nm, it emits fluorescence corresponding to free calcium. Calculating a ratio between the fluorescence emitted at the two excitation wavelengths ($F_{340\text{nm}}/F_{380\text{nm}}$) highlights the variations in intracellular calcium concentration over time and/or under different conditions, such as the application of pharmacological agents. Therefore,

fura-2-AM is classified as a ratio-metric dye. Additionally, compared to a single dye, it has the advantage of being much less sensitive to photobleaching.

Fura-2 has different fluorescence properties depending on whether it is bound to calcium or not. When Fura-2 is free from calcium ions, it has a different excitation/emission spectrum compared to when it is bound to calcium. Fura-2 exhibits dual-wavelength excitation properties. It absorbs light at around 340 nm when it is not bound to calcium ions and around 380 nm when it is bound to calcium ions.

III.2 The applied protocol

Intracellular calcium levels were recorded using the ratiometric fluorescent dye fura-2-AM (**Figure 28**). Coverslips containing isolated cell bodies were rinsed twice times and incubated in the dark (45 min, 37°C) in the saline supplemented with sucrose (50 mM), Pluronic F-127 (0.1 %) and fura-2-AM (10 µM). All compounds were purchased from Sigma Aldrich, France.

They were rinsed twice more and mounted on a perfusing chamber (PM-1, SA-OLY/2-AL, Warner Instruments) under an inverted microscope (IX73, Olympus) installed in a light-tight box. Neurones were bathed using a gravity perfusion system from the outside of the box, with tubes entering the box through 1 mm holes. This allowed us to control the levels of saline without exposing the samples to exterior light. Pressure ejection of nicotine (15 psig, 300 ms; PLI 100, Medical System Corporation, USA) was carried out using the same glass micropipettes as those used for the patch-clamp. Control cells were subjected to a pulse application of 1 mM nicotine, or LMA10203 in saline solution, whereas tested cells were pre-treated for 10 min with STO, α -Bgt, or Mec before pulse application of nicotine or LMA10203. Dye uptake was measured by incubating cells in cell culture containing the indicated concentration of Fura-2AM. After 10 min incubation time, the medium was removed by spinning the cells with saline solution. For all recordings, the following parameters were applied: exposure time: 100-200 ms, gain: 0 dB, recording time: 2 min, and acquisition interval: 1.5 s. Calcium intracellular levels were measured using a brightfield/340/380 nm LED camera (CoolLED pE-340^{fura}) and the *cellSens Dimension* acquisition software (Olympus).

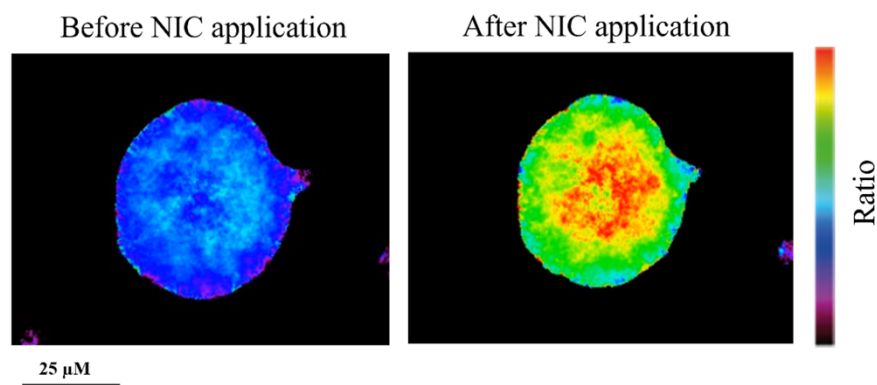


Figure 28: Calcium imaging recording of a single DUM neuron before and after application of 1mM nicotine (NIC).

IV. Drugs and solutions

In all experiments (patch clamp and calcium imaging), nicotine, LMA10203, and flupyradifurone (FLU) were diluted in saline solution, then applied using a pulse application of 300 ms (15 psig) at different concentrations (Houchat *et al.*, 2022; Houchat *et al.*, 2019; Houchat *et al.*, 2020a; Mannai *et al.*, 2016). α -Bgt was diluted in saline solution to a final concentration of 0.5 μ M (Courjaret *et al.*, 2001; Thany *et al.*, 2008). The compounds A-76 (AMPK activator), DM, d-tubocurarine (d-TC, nAChR1 antagonist), mecamylamine (Mec, nAChR2 antagonist), and STO (CaMKK selective blocker), dantrolene (RyR antagonist), ryanodine (RyR blocker), and 2-aminoethoxydiphenyl borate (2-APB, IP₃R inhibitor), were dissolved in DMSO to make a stock solution of 0.01% DMSO, and then used in the saline solution for all experiments. α -Bgt, STO, Mec, d-TC, CdCl₂, caffeine and dorsomorphin (DM), were applied using a gravity bath perfusion system positioned within 100 μ m from the cell body (VC-6 valve controller, Warner Instrument, USA). Only A-76 and dantrolene were diluted in the intrapipette solution.

Chapter III: Identification of CaMKK/AMPK pathway as a new regulator of nicotinic currents through nAChRs

I. Presentation of the article

Context: Due to the extensive and uncontrolled use of neonicotinoids, many insects' species developed high levels of resistance, posing significant challenges to pest management strategies. Therefore, exploring the resistance mechanisms towards neonicotinoids is of great importance. Intracellular Ca^{2+} pathways that regulate nAChRs can affect how insects respond to neonicotinoid insecticides, potentially contributing to the development of resistance mechanisms. Understanding these pathways is essential for developing strategies to manage and overcome these challenges.

Objective: Recent electrophysiological studies performed on insect neurosecretory cells found that calcium/calmodulin-dependent protein kinases (CaMKs) family can modulate nAChRs phosphorylation state and change their sensitivity to neonicotinoids. For example, in a previous study we demonstrated that CaMKII inhibition can reduce nAChRs respond to the neonicotinoid, clothianidin (Houchat *et al.*, 2020b). To explore more how can intracellular calcium/Calmodulin pathways affect nAChRs activity, we used cockroaches' *Periplaneta americana* dorsal unpaired median (DUM) neurons. These neurons are a class of insect neurosecretory cells that express two different α -bungarotoxin- insensitive nAChRs subtypes, nAChR1 and nAChR2, that have different pharmacological properties and sensitivity to neonicotinoid.

Results: We found that STO-609 reduced NIC-induced currents, particularly when co-applied with α -Bgt. This reduction was counterbalanced after the intracellular application of the AMPK activator, A-76. A-76 was also able to prevent the reduction in NIC-induced currents observed with the AMPK inhibitor, dorsomorphin. When the selective nAChR2 activator, LMA10203, was applied, currents induced by LMA10203 were also reduced under the presence of STO-609. Using calcium imaging we found that pre-treatment of DUM neurons with STO prevented the increase in intracellular Ca^{2+} levels, which was independent on α -Bgt. In addition, Mec inhibited the increase in intracellular calcium induced by LMA10203.

Conclusion: In this study we revealed that a potentially distinct CaMKK/AMPK pathway could be involved in the intracellular regulation of nAChR2 expressed in *P. americana* DUM neurons.

II. Synthèse de l'article

Contexte : En raison de l'utilisation extensive et incontrôlée des néonicotinoïdes, de nombreuses espèces d'insectes ont développé des niveaux élevés de résistance, ce qui pose des défis importants pour la lutte contre les ravageurs. Il est donc très important d'explorer les mécanismes de résistance aux néonicotinoïdes. Les voies intracellulaires du Ca^{2+} qui régulent les nAChRs peuvent affecter la façon dont les insectes répondent aux insecticides néonicotinoïdes, contribuant potentiellement au développement de mécanismes de résistance. Il est essentiel de comprendre ces voies pour développer des stratégies permettant de gérer et de surmonter ces défis.

Objectif : Des études électrophysiologiques récentes réalisées sur des cellules neurosécrétrices d'insectes ont montré que la famille des protéines kinases dépendantes du calcium et de la calmoduline (CaMKs) peut moduler l'état de phosphorylation des nAChRs et modifier leur sensibilité aux néonicotinoïdes. Par exemple, dans une étude précédente, nous avons démontré que l'inhibition de CaMKII peut réduire la réponse des nAChRs à un néonicotinoïde, la clothianidine (Houchat *et al.*, 2020b). Pour explorer davantage comment les voies intracellulaires calcium/Calmoduline peuvent affecter l'activité des nAChRs, nous avons utilisé les neurones dorsal unpaired median (DUM) de la blatte *P. americana*. Ces neurones sont une classe de cellules neurosécrétrices d'insectes qui expriment deux sous-types différents de nAChRs insensibles à l' α -Bgt, nAChR1 et nAChR2, qui ont des propriétés pharmacologiques et une sensibilité aux néonicotinoïdes différentes.

Résultats : Nous avons constaté que le STO-609 réduisait les courants induits par la NIC, en particulier lorsqu'il était co-appliqué avec l' α -Bgt. Cette réduction était contrebalancée par l'application intracellulaire de l'activateur de l'AMPK, l'A-76. L'A-76 a également été capable d'empêcher la réduction des courants induits par la NIC observée avec l'inhibiteur de l'AMPK, la dorsomorphine. Lorsque l'activateur sélectif du nAChR2, LMA10203, a été appliqué, les courants induits par LMA10203 ont également été réduits en présence de STO-609. En utilisant l'imagerie calcique, nous avons constaté que le prétraitement des neurones DUM avec STO empêchait l'augmentation des niveaux de Ca^{2+} intracellulaire, qui était indépendante de l' α -Bgt. De plus, la Mec inhibait l'augmentation du calcium intracellulaire induite par LMA10203.

Conclusion: Dans cette étude, nous avons démontré qu'une voie de signalisation CAMKK/AMPK potentiellement distincte, pourrait être impliquée dans la régulation intracellulaire de l'activité des nAChR2 exprimés dans les neurones DUM de la blatte *P. americana*.

III. Publication n°1 : Taha *et al.*, 2024

ORIGINAL ARTICLE

The calcium–calmodulin-dependent protein kinase kinase inhibitor, STO-609, inhibits nicotine-induced currents and intracellular calcium increase in insect neurosecretory cells

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Abstract

Insect neuronal nicotinic acetylcholine receptors (nAChRs) are transmembrane receptors that play a key role in the development and synaptic plasticity of both vertebrates and invertebrates and are considered to be major targets of neonicotinoid insecticides. We used dorsal unpaired median (DUM) neurons, which are insect neurosecretory cells, in order to explore the intracellular mechanisms leading to the regulation of insect neuronal nAChRs in more detail. Using whole-cell patch-clamp and fura-2AM calcium imaging techniques, we found that a novel CaMKK/AMPK pathway could be involved in the intracellular regulation of DUM neuron nAChRs. The CaMKK selective inhibitor, STO, reduced nicotinic current amplitudes, and strongly when co-applied with α -Bgt. Interestingly, intracellular application of the AMPK activator, A-76, prevented the reduction in nicotine-induced currents observed in the presence of the AMPK inhibitor, dorsomorphin. STO prevented the increase in intracellular calcium induced by nicotine, which was not dependent on α -Bgt. Currents induced by 1 mM LMA, a selective activator of nAChR2, were reduced under bath application of STO, and mecamylamine, which blocked nAChR2 subtype, inhibited the increase in intracellular calcium induced by LMA. These findings provide insight into potential complex mechanisms linked to the modulation of the DUM neuron nAChRs and CaMKK pathway.

KEYWORDS

calcium, calmodulin, calmodulin kinase, nicotine, nicotinic receptors, STO-609

1 | INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast synaptic transmission in the insect nervous system and are major targets of neonicotinoid insecticides. The

functional properties of neuronal nAChRs can be modulated by different mechanisms including intracellular calcium pathways. Calcium ions (Ca^{2+}) are critical intracellular secondary messengers that play a key role in the development and plasticity of the nervous system (Berridge et al., 2000; Dajas-Bailador & Wonnacott, 2004; Evans

Abbreviations: A-76, A-769662; AC, adenylyl cyclase; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; Ca^{2+} , calcium ions; CaM, calmodulin; CaMKII, calcium–calmodulin-dependent protein kinase II; CaMKK, CaM kinase kinase; cAMP, cyclic adenosine monophosphate; CdCl₂, cadmium chloride; Cdz, calmidazolium; cGMP, cyclic guanosine monophosphate; CLT, clothianidin; DAG, diacylglycerol; DM, dorsomorphin; d-TC, d-tubocurarine; DUM, dorsal unpaired median; HVA, high voltage activated; LMA, LMA10203; Mec, mecamylamine; nAChRs, nicotinic acetylcholine receptors; NIC, nicotine; NO, nitric oxide; PaCaMKII-E, *Periplaneta americana* CaMKII isoform E; PDE, phosphodiesterase; PKC, protein kinase C; PKG, protein kinase G; STO, STO-609 7-oxo-7H-benzimidazo[2,1-a] benz[de]isoquinoline-3-carboxylic acid; TAG, terminal abdominal ganglia; TRP γ , transient receptor potential γ ; VDCCs, voltage-dependent Ca^{2+} channels; VGCCs, voltage-gated Ca^{2+} channels.; α -Bgt, α -bungarotoxin.

Maria Taha and Jean-Noël Houchat contributed equally to this work.



& Zamponi, 2006; Iftinca & Zamponi, 2009). Although intracellular regulation of nAChRs through Ca^{2+} pathways can play a fundamental role in modulating the pharmacology of receptors (Deshayes et al., 2018), these pathways have been little studied in insects contrary to mammalian neuronal nAChRs (Bagur & Hajnoczky, 2017; Damaj, 2000; McKay et al., 2007). Previous studies performed on cockroaches demonstrated that application of acetylcholine (ACh) in the accessory medulla caused dose-dependent increases in intracellular Ca^{2+} via nAChRs (Baz el et al., 2013). Recent electrophysiological recordings on dorsal unpaired median (DUM) neurons, which are the insect neurosecretory cells, provided evidence that Ca^{2+} -dependent pathways are involved in the regulation of nicotine-induced currents (Houchat et al., 2022; Mannai et al., 2016). DUM neurons are pacemaker neurons that have the intrinsic ability to generate spontaneous rhythmic activity. They expressed two α -bungarotoxin (α -Bgt)-insensitive nAChR subtypes, nAChR1 and nAChR2, which are differently regulated by intracellular Ca^{2+} pathways following the activation of the Ca^{2+} -calmodulin (CaM) complex (Courjaret et al., 2003; Courjaret & Lapied, 2001). These results allowed us to generate two main hypotheses through the activation of nAChR1 and nAChR2, respectively. The first hypothesis suggests that nAChR1 subtype regulation was sensitive to two distinct types of protein kinase-C (PKC) and CaM activation. The CaM inhibitor W7 completely blocked the increase in nicotinic currents caused by the adenylyl cyclase (AC) activator, forskolin, suggesting that the Ca^{2+} -CaM complex was a major factor accounting for the regulation of the cyclic adenosine monophosphate (cAMP) levels induced by AC activation (Courjaret & Lapied, 2001). To confirm the involvement of cAMP- and phosphodiesterase (PDE)-activating pathways, we recently demonstrated that the PDE inhibitor, calmidazolium (CdZ), decreased nicotine-evoked current amplitudes when applied to DUM neurons. This decrease in nicotine current amplitudes was also associated with a decrease in intracellular Ca^{2+} levels in the DUM neurons (Houchat et al., 2022). In this context, blocking PDE results in cAMP activation which could explain this decrease in nicotine currents. Similar mechanisms involving cAMP were also found using the neonicotinoid insecticide, clothianidin (CLT). At low CLT concentrations, changes to intracellular cAMP levels result in the modulation of CLT-induced current amplitudes. However, at high CLT concentrations, the increase in cAMP levels had no effect on CLT-evoked currents (Calas-List et al., 2013). The second hypothesis suggested that the Ca^{2+} -CaM complex activates a particular Ca^{2+} -CaM-dependent protein kinase (CaMKII) isoform, called PaCaMKII-E (List et al., 2014; Taillebois et al., 2013). Indeed, cockroach nervous systems encoded five CaMKII isoforms which are highly expressed in the nerve cord, the muscle, gut, head, accessory glands, and thoracic ganglia. Their sequences were identical to one another, except for the presence of 2 peptides of 21 amino acids (Taillebois et al., 2013). Interestingly, we found that only the PaCaMKII-E isoform was expressed in the DUM neurons (Taillebois et al., 2013), and was involved in the intracellular regulation of α -Bgt-insensitive nAChRs (List et al., 2014). Inhibition of PaCaMKII-E using antisense oligonucleotides reduced nicotine and CLT-evoked current amplitudes, and this effect was also

associated with a decrease in intracellular Ca^{2+} levels in the DUM neurons (List et al., 2014).

In the present study, we aim to further explore how intracellular Ca^{2+} pathways modulate DUM neuron nAChRs in insects. We propose that a novel pathway involving CaM kinase kinase (CaMKK) could participate in the modulation of DUM neuron nAChRs through the activation of α -Bgt sensitive and insensitive, including nAChR2 subtype. In particular, we found that bath application of the CaMKK inhibitor STO-609 (STO) induced and inhibition of nicotine-induced currents, and in intracellular Ca^{2+} levels. Both LMA10203 (LMA)-induced currents and the Ca^{2+} response were blocked by mecaminylamine, which suggests that nAChR2 could be considered a mediated receptor. Overall, this study suggested the existence of a CaMKK/AMPK pathway involved in the intracellular modulation of DUM neuron nAChRs.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments were performed using adult male cockroaches *Periplaneta americana* reared at 29°C on a 12:12h light/dark cycle. Cockroaches were purchased from Arbiotech Company (Saint Gilles, France).

2.2 | Dissection of cockroach terminal abdominal ganglion

The cockroach terminal abdominal ganglion (TAG) was isolated by dissection along the longitudinal dorsal-median line (Lapied et al., 1993). Forceps were used to remove the alimentary canal as well as the overlying muscle and trachea. For the experiments evaluating the effect of STO on the TAG, we used a Hamilton syringe. Thus, 15 μL of STO was injected into the central part of the TAG (Figure 1A). The TAG was then incubated for 3h at 29°C, following which the DUM neurons were isolated using a standard saline extracellular solution (in mM: 200 NaCl, 3.1 KCl, 5 CaCl_2 , 4 MgCl_2 , 10 HEPES, and 10mM; pH 7.4 adjusted with NaOH) with sucrose (50mM). DUM neurons from different cockroaches (around 500 cockroaches were used for the study) were pooled for patch-clamp recordings.

2.3 | DUM neuron isolation

DUM neurons were isolated from the cockroach TAG (Lapied et al., 1993; Sinakevitch et al., 1996). After being carefully dissected, the TAG was placed in the standard saline extracellular solution. Each compound in this solution was purchased from Sigma Aldrich, France. The TAG was incubated (35min, 29°C) in the saline supplemented with collagenase (type I, 1.5 mg/mL,

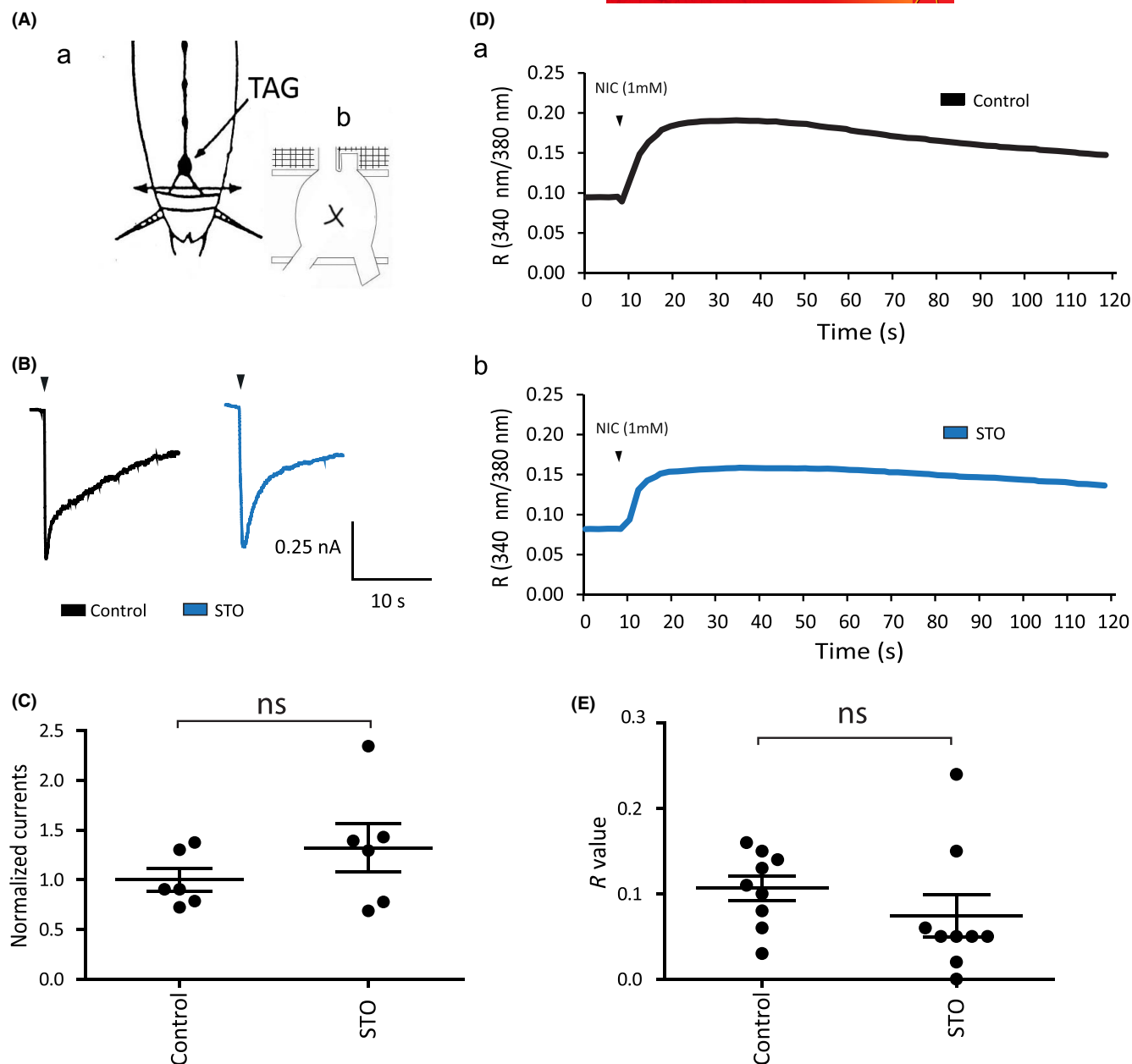


FIGURE 1 Effect of microinjecting the ganglia with STO on both current amplitude and intracellular calcium increase after 1 mM NIC pulse applications. (A) The terminal abdominal ganglion was injected with both saline solution (control condition) and 15 μ M STO, and incubated in cell culture for 3 h, before DUM neurons dissociation for patch-clamp and calcium imaging recordings. TAG, Terminal abdominal ganglion. (a) Scheme showing a part of the nerve cord and terminal abdominal ganglion. (b) The terminal abdominal ganglion showing the injection area. The cross indicates the injection area in the terminal abdominal ganglion. (B) Examples of NIC-induced currents in both control cells and cells from STO-injected ganglia. Currents were recorded at -50 mV holding potential. Arrowheads represent pulse application (300 ms, 15 psig) of 1 mM NIC. (C) Scatter dot plots illustrating the effects of 1 mM NIC under control conditions ($n=6$ cells), and in STO-injected conditions ($n=6$ cells). Unpaired t -test, $p=0.26$, $t=1.205$, $df=10$. (D) Similarly, no significant difference in intracellular calcium increases after pulse application of 1 mM NIC (15 psig, 300 ms) was found between control DUM neurons (a) and DUM neurons from the STO-injected ganglia (b). (E) Scatter dot plots illustrating NIC-induced calcium peak (R value) in control cells ($n=10$ cells), and cells from STO-injected ganglia ($n=9$ cells). Unpaired t -test, $p=0.28$, $t=1.12$, $df=16$. ns, not significant.

GIBCO), rinsed twice, and placed in the saline supplemented with fetal calf serum (5% by volume), 50 UI/mL penicillin, and 50 μ g/mL streptomycin (Sigma Aldrich, France). They were then mechanically dissociated by gentle trituration using fire-polished Pasteur pipettes. The isolated DUM neurons were then left to settle

and adhere to Petri dishes (35/09 mm, NEST, Grosseron, France) coated with poly-D-lysine hydrobromide (MW, 70 000–150 000; Sigma Aldrich, France) for patch-clamp experiments, or to coverslips (22 $\times$ 40 $\times$ 0.17 mm, HIRSCHMANN, Verrerie Villeurbannaise, France) for calcium imaging. Cell cultures were incubated 24 h at



29°C before patch-clamp or calcium imaging experiments were carried out (Lapied et al., 1989). All preparations were made under semi-sterile conditions, that is, all equipment was sterilized with ethanol and solutions were filtered.

2.4 | Electrophysiological recordings

Signals were recorded using the patch-clamp technique in the whole-cell recording configuration in voltage-clamp mode using an Axopatch 200B (Patch-Clamp Amplifier, Axon Instruments, Foster City, CA). They were digitized and acquired using a Digidata 1440 analog-digital converter (Axon Instruments) and Axoscope 10.2 software (Axon Instruments). Signals were low pass filtered at 5 kHz. The Petri dishes containing isolated DUM neurons were placed onto an inverted microscope (AE 2000, Motic), and continuously bathed with the standard saline using a gravity perfusion system positioned within 100 μ m from the cell body (Calas-List et al., 2013). In all experiments, DUM neurons' capacitance ranged from 200 to 250 pF to ensure reproducible current amplitudes. Patch pipettes (borosilicate glass capillary tubes: GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with a standard internal solution (in mM: 160 K-D gluconic acid, 10 K-fluoride, 10 NaCl, 1 MgCl₂, 0.5 CaCl₂, 20 HEPES, 10 EGTA, and 3 ATP; pH 7.4 adjusted with KOH). Their resistances ranged from 1.5 to 2 M Ω . Nicotine was applied via pneumatic pressure ejection (15 psig, 300 ms; Miniframe, Medical System Corporation, USA). The pressure ejection was made using a glass micropipette, with resistance ranging from 1.5 to 2 M Ω , positioned in a solution within 100 μ m of the isolated cell body. All compounds were purchased from Sigma Aldrich, France.

2.5 | Intracellular calcium recordings

Intracellular calcium levels were recorded using the ratiometric fluorescent dye fura-2AM (Malgaroli et al., 1987). Coverslips containing isolated cell bodies were rinsed twice times and incubated in the dark (45 min, 37°C) in the saline supplemented with sucrose (50 mM), pluronic F-127 (0.1%), and fura-2AM (10 μ M). All compounds were purchased from Sigma Aldrich, France. They were rinsed twice more and mounted on a perfusing chamber (PM-1, SA-OLY/2-AL, Warner Instruments) under an inverted microscope (IX73, Olympus) installed in a light-tight box. Neurons were bathed using a gravity perfusion system from the outside of the box, with tubes entering the box through 1 mm holes. This allowed us to control the levels of saline without exposing the samples to exterior light. Pressure ejection of nicotine (15 psig, 300 ms; PLI 100, Medical System Corporation, USA) was carried out using the same glass micropipettes as those used for the patch clamp. Control cells were subjected to a pulse application of 1 mM nicotine, or LMA in saline solution, whereas tested cells were pre-treated for 10 min with STO,

α -Bgt, or Mec before pulse application of nicotine or LMA. Dye uptake was measured by incubating cells in cell culture containing the indicated concentration of fura-2AM. After 10 min incubation time, the medium was removed by spinning the cells with saline solution. For all recordings, the following parameters were applied: exposure time: 100–200 ms, gain: 0 dB, recording time: 2 min, and acquisition interval: 1.5 s. Calcium intracellular levels were measured using a brightfield/340/380 nm LED camera (CoolLED pE-340^{fura}) and the *cellSens Dimension* acquisition software (Olympus). The release of the dye from loaded cells into the extracellular medium may impact measurements in cell suspension, and this occurrence was observed to vary with the cell type. In DUM neurons, the application of pluronic acid, a non-ionic surfactant employed, as a cell membrane stabilizer, protected the cell membranes and enhanced the absorption of fura-2AM (Malgaroli et al., 1987; Moreau et al., 2020).

2.6 | Drugs and solutions

In all experiments (patch-clamp and calcium imaging), nicotine and LMA were diluted in saline solution, and then applied using a pulse application of 300 ms (15 psig) at a concentration of 1 mM (Houchat et al., 2019, 2020, 2022; Mannai et al., 2016). α -Bgt was diluted in saline solution to a final concentration of 0.5 μ M (Courjaret & Lapied, 2001; Thany et al., 2008). The compounds A-769662 (A-76, AMPK activator), DM, d-tubocurarine (d-TC, nAChR1 antagonist), mecamlamine (Mec, nAChR2 antagonist), and STO (CaMKK selective blocker) were first dissolved in DMSO (stock solution), and then diluted in saline solution to make a final solution of 0.01% DMSO (working solutions). α -Bgt, STO, Mec, d-TC, CdCl₂, and DM were applied using a gravity bath perfusion system positioned within 100 μ m from the cell body (VC-6 valve controller, Warner Instrument, USA). Only A-76 was diluted in the intrapipette solution. All currents were recorded at –50 mV holding potential, except in Figures 6 and 10 for LMA.

2.7 | Statistical analyses

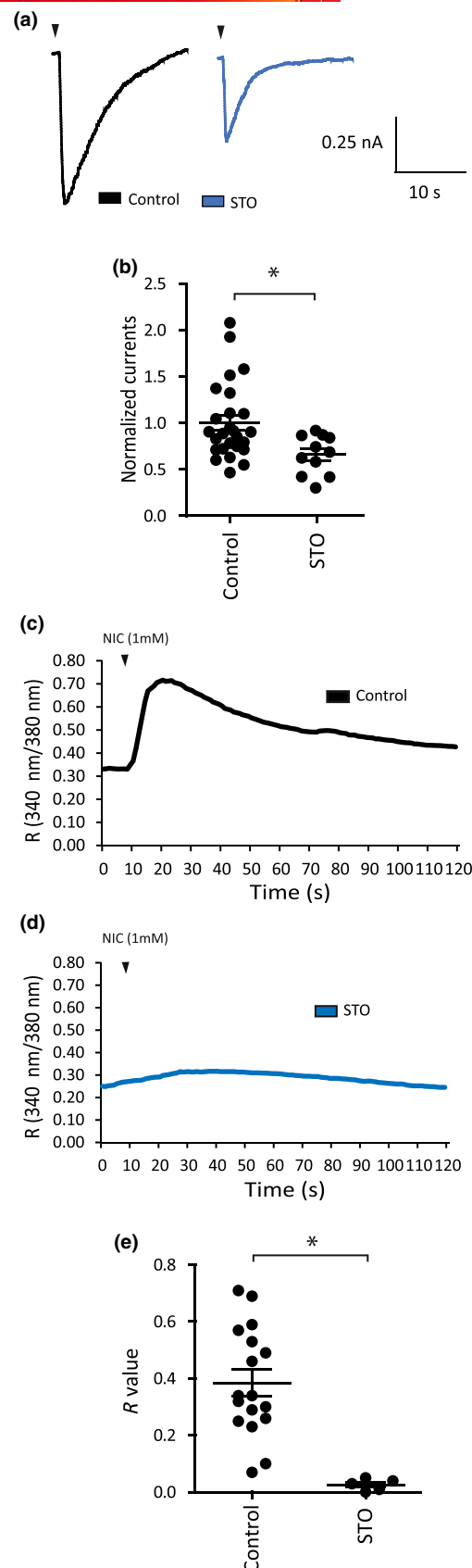
Ionic currents were normalized according to the mean amplitude in control conditions (I/I_{mean}), and intracellular levels of calcium were expressed as *R* values (*R* is the 340 nm/380 nm ratio value). R-studio 8.0 software and Prism 9 (GraphPad Software Inc) were used to compare mean current amplitudes and mean calcium peaks. Data were analyzed using one-way ANOVA, Kruskal-Wallis test, or Student *t*-test, depending on the size of the group and whether the data were parametric or non-parametric. Normal distribution (Gaussian distribution) of the data was tested using a Shapiro-Wilk test. All tests used were two-tailed. All data points were included in each assay. The difference was considered significant when the *p* value was <0.05. For all scatter dot-plot graphs, a dot represents one cell and error bars represent mean with SD. *n* = number of cells.

FIGURE 2 Effects of STO on NIC-induced currents and intracellular calcium levels. (a) Examples of NIC-induced currents under control condition, and 10 min bath application of 15 μ M STO. (b) Scatter dot plots illustrating the mean currents induced by 1 mM NIC under control condition ($n=26$ cells) and 15 μ M STO ($n=11$ cells), $*p<0.05$, Kruskal–Wallis with Holm post-hoc test. (c) Increase in intracellular Ca^{2+} levels after pulse application of 1 mM NIC (300ms, 15 psig), and (d) under 10 min bath application of 15 μ M STO. (e) Scatter dot plots representing calcium peak (R value) induced by 1 mM NIC in control condition ($n=17$ cells) and STO bath application ($n=5$ cells). $*p<0.05$, Kruskal–Wallis with Holm post-hoc test. In all cases, arrowheads represent pulse application (300ms, 15 psig) of 1 mM NIC.

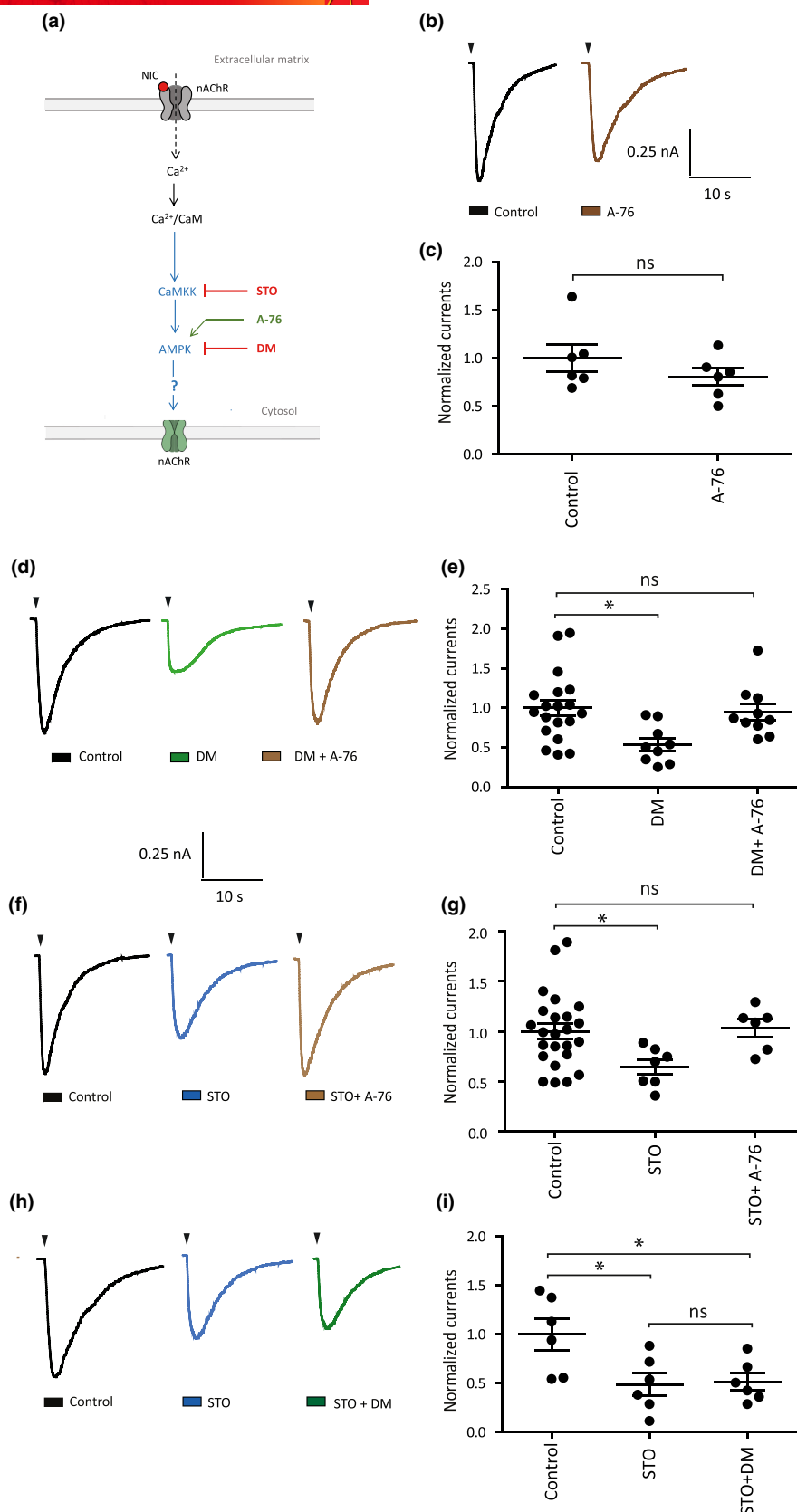
3 | RESULTS

3.1 | Effects of STO-609 on nicotine-induced currents and intracellular calcium level

To further understand the involvement of Ca^{2+} pathways in the modulation of nicotine-induced currents, we used the CaMKK inhibitor, 7-oxo-7H-benzimidazo[2,1-a] benz[de]isoquinoline-3-carboxylic acid (STO) (Tokumitsu et al., 2002, 2003). Since we knew that the sixth abdominal ganglion controls the release of cockroach sexual glands, we wanted to know whether a direct microinjection of STO into this ganglion could affect the nicotinic currents recorded in the DUM neurons. Thus, the sixth abdominal ganglion was isolated, then microinjected with 15 μ M STO (Li et al., 2023; Rajakyla et al., 2020), and incubated for 3h at 29°C before isolating the DUM neurons. Using patch clamp, we found that nicotine-evoked current amplitudes were not affected following STO microinjection (Figure 1B,C). In addition, no significant difference in the intracellular Ca^{2+} levels was found after pulse application of 1 mM nicotine (Figure 1D,E). Thus, DUM neurons were incubated in STO for all experiments as the cell permeability of STO and its inhibitory effect on CaMKK activity had been demonstrated in a previous study (Tokumitsu et al., 2002). In line with our previous data (Houchat et al., 2022; List et al., 2014), 15 μ M STO was bath applied for 10min, before pulse application of 1 mM nicotine, and DUM neurons recorded at -50 mV holding potential (DUM neuron-holding potential). Ten minutes after STO bath application, nicotine-evoked currents were reduced, with peak amplitudes averaging between -1.00 ± 0.08 nA and -0.66 ± 0.06 nA ($n=11$ cells, $p<0.05$, Figure 2a,b). Having found that application of 1 mM nicotine induced an increase in intracellular Ca^{2+} levels (Houchat et al., 2022; List et al., 2014), here we studied how STO could modulate the intracellular Ca^{2+} levels. We found that bath application of 15 μ M STO prevented the increase in intracellular Ca^{2+} levels after 300ms pulse application of 1 mM nicotine ($n=5$ cells, $p<0.05$, Figure 2c–e). In line with previous studies (Courjaret et al., 2003; Courjaret & Lapied, 2001; List et al., 2014; Mannai et al., 2016; Thany et al., 2007, 2008), we suggested that a new pathway involving CaMKK could play an important role in the modulation of cockroach neuronal nAChRs (Figure 3a). To explore our hypothesis, we evaluated the effect of adding the adenosine monophosphate (AMP)-activated protein kinase (AMPK) inhibitor, DM, to the



extracellular solution, as well as the intrapipette application of the AMPK activator, A-769662 (A-76). As described in Figure 3b,c, patch pipette application of 10 μ M A-76 had no significant difference in



nicotine current amplitudes ($n=6$ cells, $p>0.05$). Bath application of $10\mu\text{M}$ DM significantly reduced nicotine-induced current amplitudes from $-1.00\pm0.10\text{nA}$ to $-0.54\pm0.08\text{nA}$, when $10\mu\text{M}$ A-76 was added to the intrapipette solution, this reduction was

counterbalanced, the effects of DM were abolished, and the mean nicotine-evoked current amplitudes was $-0.95\pm0.10\text{nA}$ ($n=9-10$ cells, $p<0.05$, Figure 3d,e). In order to ensure there was a direct link between CaMKK and AMPK activation, DM and STO were

FIGURE 3 Effects of intrapipette application of A-76 and bath application of DM on NIC-induced currents. (a) A diagram illustrating the potential CaMKK/AMPK pathway involved in the regulation of nAChRs expressed in cockroach DUM neurons. (b) Examples of 1 mM NIC-induced currents (300 ms, 15 psig) in control condition and under intrapipette application of 10 μ M A-76. (c) Scatter dot plots summarizing the effect of 1 mM NIC under control condition ($n=6$ cells) and under the intrapipette application of 10 μ M A-76 ($n=6$ cells). Unpaired t -test, $p=0.27$, $t=1.168$, $df=10$. ns, not significant. (d) Examples of NIC-induced currents in saline solution (control condition), bath application of 10 μ M DM, and co-application of 10 μ M DM with 10 μ M A-76. (e) Scatter dot plots illustrating the effect of 1 mM NIC under control conditions ($n=19$ cells), bath application of 10 μ M DM ($n=9$ cells), and co-application of 10 μ M DM with 10 μ M A-76 ($n=10$ cells). * $p<0.05$ (Kruskal–Wallis with Holm post-hoc test). ns, not significant. Effects of STO co-application with A-76 (f, g) and DM (h, i) on NIC-induced currents. (f) Examples of NIC-induced currents under control conditions, bath application of 15 μ M STO, and co-application of 15 μ M STO with 10 μ M A-76. (g) Scatter dot plots representing the effects of 1 mM NIC under control conditions ($n=24$ cells), bath application of 15 μ M STO ($n=7$ cells), and co-application of 15 μ M STO with 10 μ M A-76 ($n=6$ cells). ANOVA with Tukey post-hoc test, $p=0.012$, $F=4.5$, and $df=37$. (h) Examples of NIC-induced currents under control condition and during a 10 min bath application of STO, and co-application of STO with DM. (i) Scatter dot plots illustrating the effects of 1 mM NIC under control conditions ($n=6$ cells), 10 min bath application of 15 μ M STO ($n=6$ cells), and 10 min co-application of 15 μ M STO with 10 μ M DM ($n=6$ cells). Student's t test, $p=0.008$, $t=4.16$, and $df=5$ between control and STO; $p=0.02$, $t=3.2$, and $df=5$ between control and co-application of STO with DM; unpaired t -test, $p=0.8$, $t=0.2$, and $df=10$ between STO and co-application of STO with DM. * indicates a significant difference ($p<0.05$). ns, not significant.

co-applied with A-76. In this condition, A-76 inhibited the decrease in nicotine current amplitudes induced in the presence of STO. Currents were reversed from -0.65 ± 0.07 nA to -1.03 ± 0.09 nA ($n=9$ –10 cells, $p<0.05$, Figure 3d,e). Moreover, when we co-applied STO with A-76, the nicotinic-induced current amplitudes were reduced from -1.38 ± 0.22 nA to -0.72 ± 0.12 nA ($n=6$ cells, $p<0.05$, Figure 3f,g). However, no statistically significant difference was observed compared to the application of STO alone ($n=6$ cells, $p>0.05$, Figure 3h,i). In other words, DM did not induce an additional effect when co-applied with STO.

3.2 | Effect of STO-609 involved the activation of the α -Bgt-insensitive nAChR2 subtype

The major question regarding these results was the involvement of both α -Bgt-sensitive and -insensitive nAChRs, and in particular nAChR1 and nAChR2 subtypes, which were identified in previous studies (Courjaret et al., 2003; Courjaret & Lapied, 2001; Thany et al., 2008). As it was expected, bath application of 0.5 μ M α -Bgt partially reduced nicotine-evoked currents from -1.00 ± 0.08 nA to 0.44 ± 0.12 nA ($n=6$ cells, Figure 4a,b, $p<0.05$), and co-application of STO and 0.5 μ M α -Bgt strongly reduced nicotine-induced currents to -0.13 ± 0.04 nA ($n=6$ cells, $p<0.05$, Figure 4a,b). We hypothesized that part of the STO effect on nicotine-evoked currents could involve the activation of both α -Bgt-sensitive and -insensitive nAChRs, including nAChR1 and nAChR2 subtypes. This hypothesis was studied through the co-application of STO with both 20 μ M d-TC and 20 μ M Mec, which are selective blockers of nAChR1 and nAChR2 subtypes, respectively (Courjaret et al., 2003; Courjaret & Lapied, 2001). Firstly, we found that nicotinic currents were reduced to -0.3 ± 0.05 nA under bath application of 20 μ M d-TC ($p<0.05$, $n=6$ cells, Figure 4c,d), and to -0.35 ± 0.06 nA under 20 μ M Mec bath application ($n=6$ cells, $p<0.05$, Figure 4c,d). When they were co-applied with 0.5 μ M α -Bgt, currents were reduced to -0.13 ± 0.02 nA under bath application of d-TC ($p<0.05$, $n=6$ cells, Figure 4e,f), and completely blocked with Mec (-0.02 ± 0.01 nA, $n=6$ cells,

Figure 4e,f). Our first assumption was to suggest that the activation of the CaMKK pathway is linked to (1) the α -Bgt-sensitive receptors and (2) to the α -Bgt-insensitive nAChR2 subtype as a result of the effect of Mec. In this case, α -Bgt must affect intracellular calcium levels in the same way as found with STO. Interestingly, the intracellular calcium increase induced by 1 mM nicotine was completely blocked under bath application of 0.5 μ M α -Bgt ($n=6$ cells, $p<0.05$, Figure 5a,b), and co-application with STO did not present significant difference ($n=6$ cells, $p>0.05$, Figure 5c,d). Thus, we aimed to understand how the α -Bgt-insensitive nAChR2 subtype could be associated with the STO effect and CaMKK pathway. In a previous study, we demonstrated that the benzamide compound LMA acted as a specific activator of nAChR2 which is expressed on cockroach DUM neurons (Mathe-Allainmat et al., 2012). In this context, all currents were recorded at -10 mV holding potential, where LMA was able to induce inward currents (Mathe-Allainmat et al., 2012; Thany et al., 2008). Thus, it was used to further investigate the link between nAChR2 activation and the CaMKK pathway. We first confirmed that 0.5 μ M α -Bgt did not decrease LMA-evoked current amplitudes, demonstrating that α -Bgt-sensitive receptors were not involved in the effect of LMA. Currents were -0.29 ± 0.03 nA under control conditions and -0.24 ± 0.01 nA under 0.5 μ M α -Bgt bath application ($p>0.05$, $n=6$ cells, Figure 6a,b). The next step was to evaluate the effect of 1 mM LMA under bath application of 15 μ M STO. We found that LMA-evoked current amplitudes were reduced from -0.34 ± 0.02 nA under control conditions to -0.20 ± 0.01 nA with STO ($n=15$ cells, $p<0.05$, Figure 6c,d). As we demonstrated with NIC, we also assessed the effect of DM and A-76, on LMA-induced currents through nAChR2. Bath application of 10 μ M DM significantly reduced LMA-induced currents to -0.16 ± 0.01 nA ($n=8$ cells, $p<0.05$, Figure 6e,f), whereas the decrease in LMA-evoked currents was reversed in the presence of A-76 ($n=8$ cells, $p>0.05$, Figure 6g,h). We found also that 1 mM LMA induced a low increase in intracellular Ca^{2+} levels compared to 1 mM nicotine ($n=7$ cells, $p<0.05$, Figure 7). It should be noted that when DUM neurons were bathed in 0.5 μ M α -Bgt, this did not prevent the LMA-induced Ca^{2+} increase ($n=5$ cells, $p>0.05$, Figure 8). Given these results, we

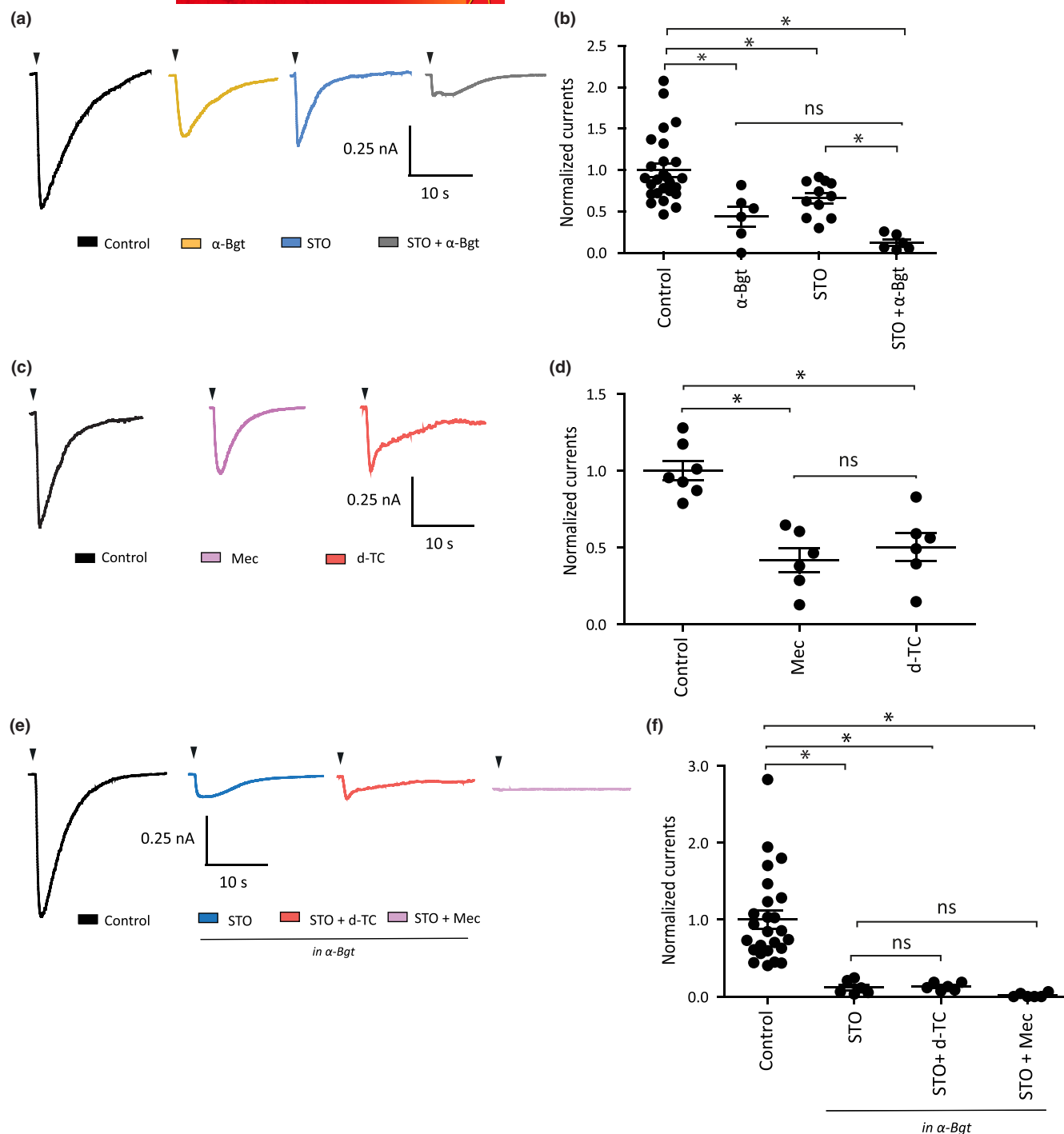


FIGURE 4 Effects of bath application of STO on NIC-induced currents through α -Bgt-insensitive nAChRs using d-TC and Mec, nAChR1, and nAChR2 selective antagonists, respectively. (a) NIC-induced currents under control condition, bath application of $0.5 \mu\text{M}$ α -Bgt and co-application of $0.5 \mu\text{M}$ α -Bgt and $15 \mu\text{M}$ STO. (b) Scatter dot plots summarizing NIC-induced currents under control condition ($n=26$ cells), $0.5 \mu\text{M}$ α -Bgt ($n=6$ cells), and co-application of $0.5 \mu\text{M}$ α -Bgt and $15 \mu\text{M}$ STO ($n=6$ cells). * $p < 0.05$ (Kruskal–Wallis with Holm post-hoc test). (c) Examples of nicotine-induced currents under control conditions, and bath application of $20 \mu\text{M}$ d-TC and $20 \mu\text{M}$ Mec. (d) Scatter dot plots illustrating the effects of 1 mM NIC under control condition ($n=7$ cells), bath application of $20 \mu\text{M}$ Mec ($n=6$ cells), and under bath application of $20 \mu\text{M}$ d-TC ($n=6$ cells). * $p > 0.05$ Kruskal–Wallis with Holm post-hoc test. ns, not significant. Examples of NIC-induced currents (e) and scatter dot plots (f), under control conditions ($n=25$ cells), bath application of $15 \mu\text{M}$ STO ($n=6$ cells), and co-application of $15 \mu\text{M}$ STO with both $20 \mu\text{M}$ d-TC ($n=6$ cells) and $20 \mu\text{M}$ Mec ($n=6$ cells). All currents were recorded during permanent perfusion of $0.5 \mu\text{M}$ α -Bgt to block α -Bgt-sensitive receptors. * $p < 0.05$ (Kruskal–Wallis with Holm post-hoc test). In all experiments, arrowheads represent pulse application (300 ms, 15 psig) of 1 mM NIC. Currents are recorded at -50 mV holding potential. ns, not significant.

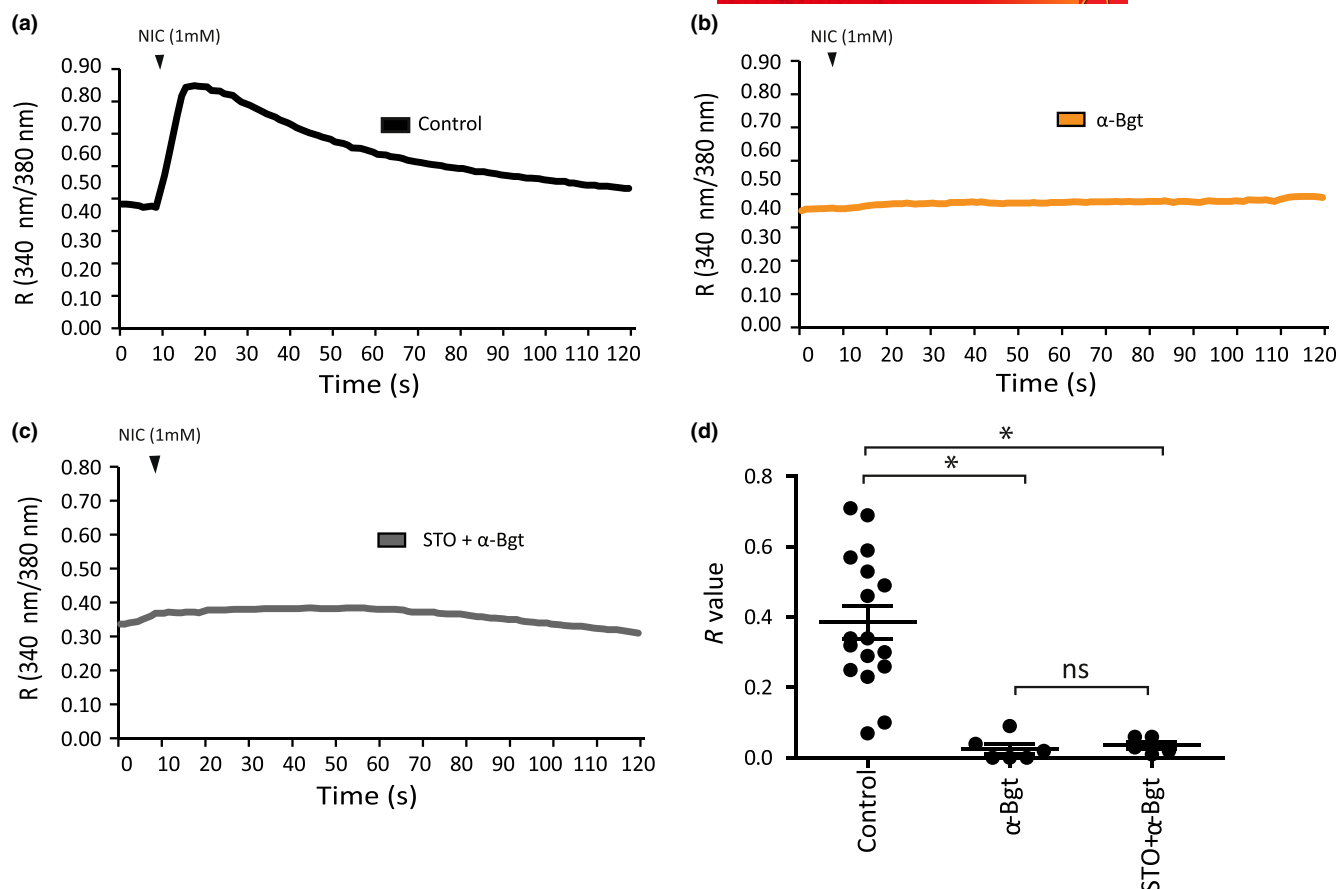


FIGURE 5 Effects of $0.5\text{ }\mu\text{M}$ $\alpha\text{-Bgt}$ on nicotine-induced increase in intracellular Ca^{2+} . (a) Intracellular calcium increase induced by 1 mM NIC (control condition) was completely blocked by bath application of $0.5\text{ }\mu\text{M}$ $\alpha\text{-Bgt}$ (b). (c) Co-application of $15\text{ }\mu\text{M}$ STO and $0.5\text{ }\mu\text{M}$ $\alpha\text{-Bgt}$. (d) Scatter dot plots showing control conditions ($n=17$ cells), and the inhibitory effect of $\alpha\text{-Bgt}$ ($n=6$ cells) and co-application of $15\text{ }\mu\text{M}$ STO with $0.5\text{ }\mu\text{M}$ $\alpha\text{-Bgt}$ ($n=5$ cells) on NIC-induced increase in intracellular calcium levels. $*p<0.05$, Kruskal–Wallis with Holm post-hoc test. ns, not significant. Arrowheads indicate pulse application (300 ms, 15 psig) of 1 mM NIC.

studied the effect of Mec, which specifically blocked the nAChR2 subtype (Courjaret et al., 2003). The increase in intracellular Ca^{2+} levels induced by pulse application of 1 mM LMA was blocked with $20\text{ }\mu\text{M}$ Mec ($n=16$ cells, $p<0.05$, Figure 9), confirming that the nAChR2 subtype was involved in the intracellular effect of STO.

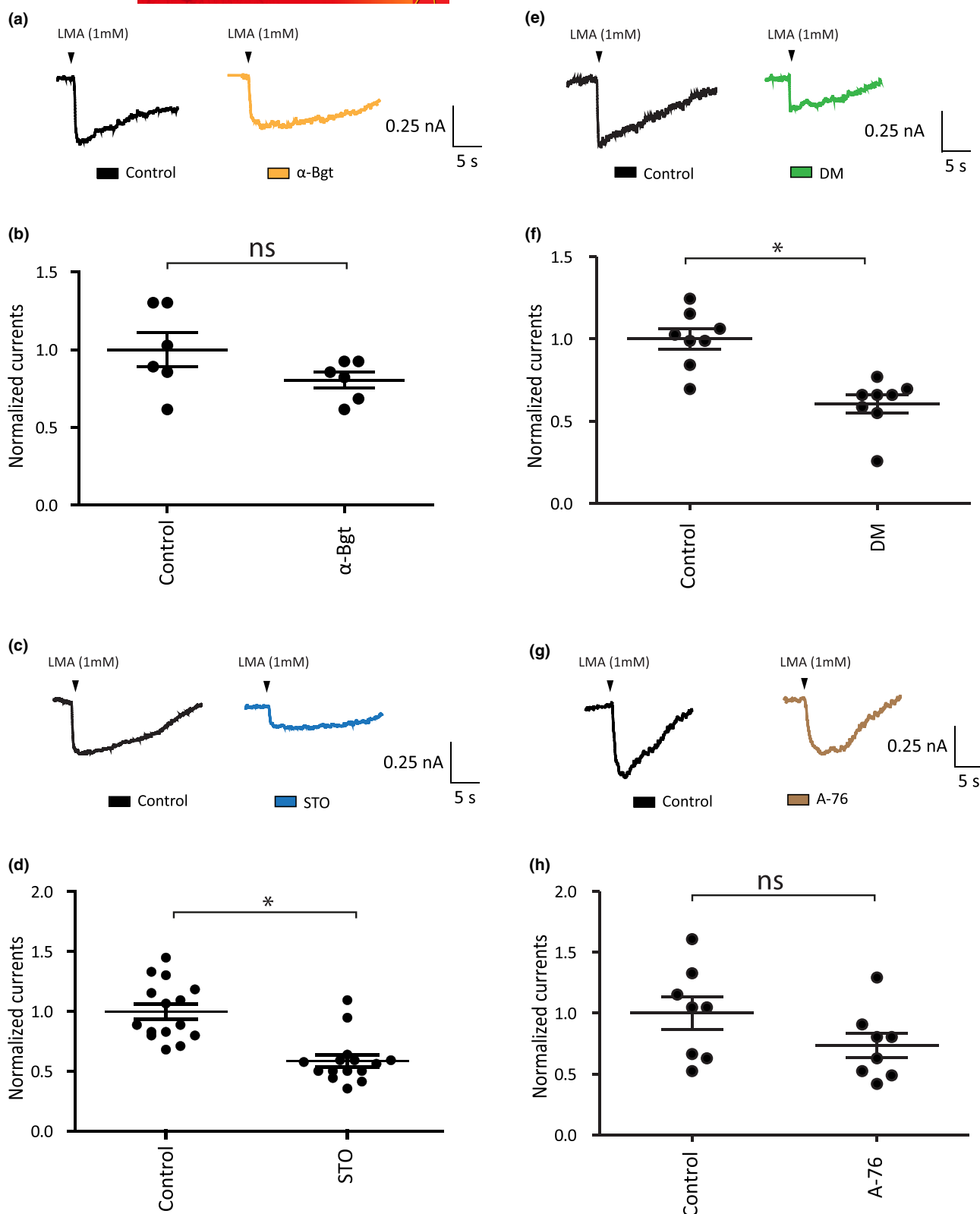
3.3 | Cadmium chloride does not affect nicotinic-evoked currents when CaMKK is inhibited by STO

For comparison, we also examined responses to cadmium chloride (CdCl). Indeed, previous studies demonstrated a link between high voltage-activated (HVA) calcium channels, transient receptor potential γ (TRP γ), which were activated at depolarized and hyperpolarized membrane potentials, and the increase in intracellular Ca^{2+} levels (Wicher et al., 1994; Wicher & Penzlin, 1994). Moreover, bath application of $500\text{ }\mu\text{M}$ CdCl affected the amplitude of NIC-evoked currents (Thany et al., 2008), and variation of the membrane potential which affected the sensitivity of nAChR2 subtype (Bodereau-Dubois et al., 2012). To examine the effect of STO on NIC-evoked

currents at both hyperpolarized and depolarized holding potentials, it was co-applied with CdCl, a calcium channel blocker (Thany et al., 2008). In the present condition, we found no significant effect when $500\text{ }\mu\text{M}$ CdCl was added to the extracellular solution, recorded at -10 mV ($-0.23\pm0.02\text{ nA}$ under STO and $-0.22\pm0.03\text{ nA}$ under STO with CdCl, $p>0.05$, $n=8$ cells, Figure 10a), -50 mV (-0.55 ± 0.11 under STO and -0.33 ± 0.07 under STO with CdCl, $p>0.05$, $n=7$ cells, Figure 10b), and -90 mV ($-0.53\pm0.13\text{ nA}$ under STO and $-0.27\pm0.03\text{ nA}$ under STO with CdCl, $n=7$ cells, Figure 10c) holding potentials. The effects of STO were independent to the holding potentials.

4 | DISCUSSION

Studies had previously shown that several pathways including specific targets proteins such PKA, two distinct PKC, PKG and CaMK, were associated with the up-regulation and down-regulation of insect nAChRs, and in particular, in neurosecretory cells such as DUM neurons (Courjaret et al., 2003; Courjaret & Lapied, 2001; Mannai et al., 2016; Thany et al., 2007). Recent studies confirmed an increase in



intracellular Ca^{2+} following nicotine activation of DUM neuron nAChRs (Houchat et al., 2022; List et al., 2014). The increase in intracellular Ca^{2+} levels suggested an activation of both α -Bgt-insensitive nAChR1 and nAChR2 subtypes. Here, we suspected a complex involvement of CaMKK when DUM neuron nAChRs were activated by nicotine in the presence of STO. The involvement of CaMKK pathways was also

confirmed using DM, which blocks AMPK, a cell-autonomous sensor to energetic changes (Oakhill et al., 2011; Riek et al., 2008). CaMKK is known to activate AMPK in response to a Ca^{2+} increase (Mihaylova & Shaw, 2012). We found that DM reduced nicotine current amplitudes and its effect was inhibited by A-76, which was also able to restore the inhibitory effect of STO. Because nicotine currents were significantly

FIGURE 6 Effects of α -Bgt, STO, DM, and A-76 on LMA-induced currents. (a) Examples of LMA-induced currents under control condition and bath application of $0.5\ \mu\text{M}$ α -Bgt. (b) Scatter dot plots representing the effect of $1\ \text{mM}$ LMA under control conditions ($n=6$ cells) and bath application of $0.5\ \mu\text{M}$ α -Bgt ($n=6$ cells, unpaired t -test, $p=0.1416$, $t=1.596$, and $df=10$). (c) Examples of currents induced by LMA under control condition and bath application of $15\ \mu\text{M}$ STO. (d) Scatter dot plots illustrating the effect of $1\ \text{mM}$ LMA under control conditions ($n=16$ cells) and bath application of $15\ \mu\text{M}$ STO ($n=16$ cells, unpaired t -test, $p=0.0001$, $t=5.143$, and $df=28$). (e) Examples of LMA-induced currents under control condition and bath application of $10\ \mu\text{M}$ DM. (f) Scatter dot plots displaying the effects of $1\ \text{mM}$ LMA under control conditions ($n=8$ cells) and bath application of DM ($n=8$ cells, unpaired t -test, $p=0.0003$, $t=4.840$, and $df=14$). (g) Examples of LMA-induced currents under control condition and after intrapipette application of $10\ \mu\text{M}$ A-76. (h) Scatter dot plots illustrating the effects of $1\ \text{mM}$ LMA under control conditions ($n=8$ cells), and after intrapipette application of $10\ \mu\text{M}$ A-76 ($n=8$ cells, unpaired t -test, $p=0.1314$, $t=1.602$, and $df=14$). In all cases, arrowheads indicate pulse application (300ms, 15 psig) of $1\ \text{mM}$ LMA. Currents are recorded at $-10\ \text{mV}$ holding potential. * $p<0.05$.

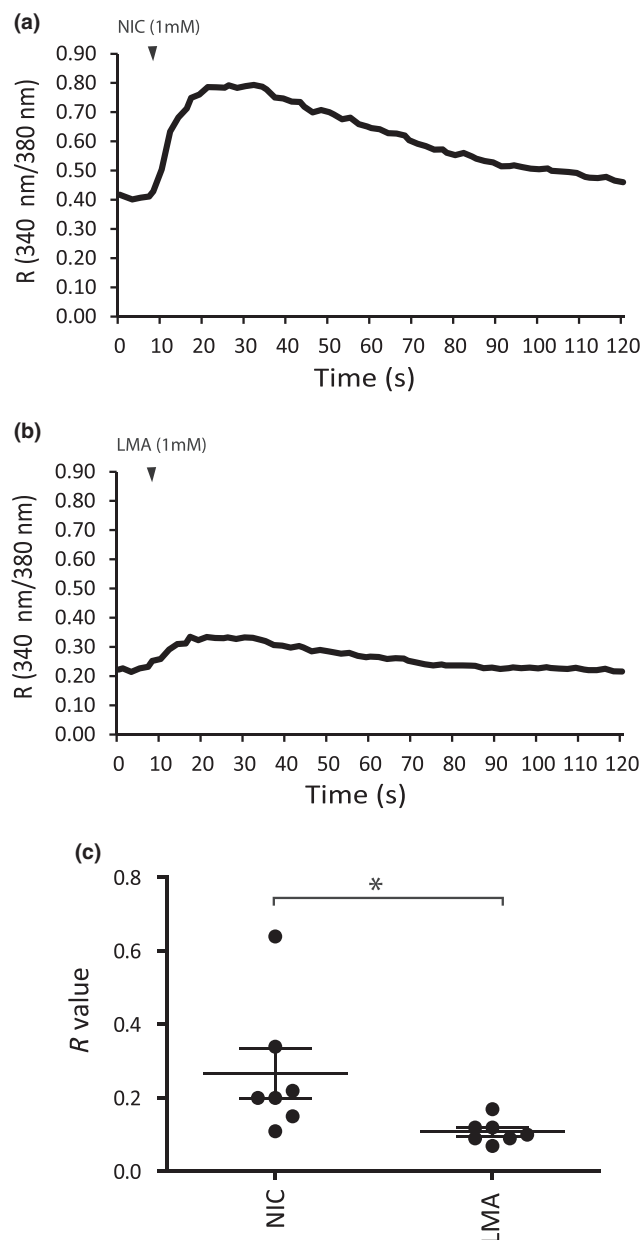


FIGURE 7 Intracellular calcium increase induced by $1\ \text{mM}$ NIC (a) and $1\ \text{mM}$ LMA (b). (c) Scatter dot plots illustrating calcium peak (R value) induced by $1\ \text{mM}$ NIC ($n=7$ cells) and LMA ($n=7$ cells, unpaired t -test, $p=0.042$, $t=2.276$, $df=12$). Arrowheads indicate 300ms pulse application (15 psig) of each compound. * $p<0.05$.

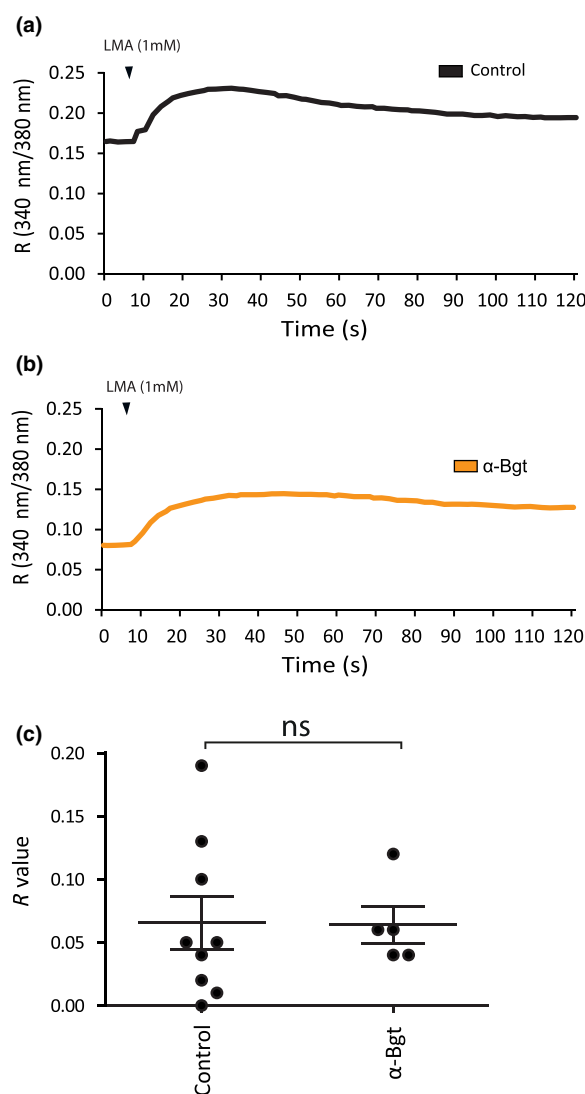


FIGURE 8 Effects of bath application of α -Bgt on intracellular calcium increase induced by LMA. Pulse application of $1\ \text{mM}$ LMA evoked an increase in intracellular calcium levels (a) which was not affected by bath application of $0.5\ \mu\text{M}$ α -Bgt (b). Cells were incubated for 15 min with $0.5\ \mu\text{M}$ α -Bgt. Arrowheads indicate pulse application of $1\ \text{mM}$ LMA (300ms pulse duration, 15 psig). (c) Scatter dot plots representing LMA-induced calcium peak in control conditions ($n=9$ cells), and under bath application of $0.5\ \mu\text{M}$ α -Bgt ($n=5$ cells). Unpaired t -test, $p=0.96$, $t=0.05$, and $df=12$. ns, not significant.

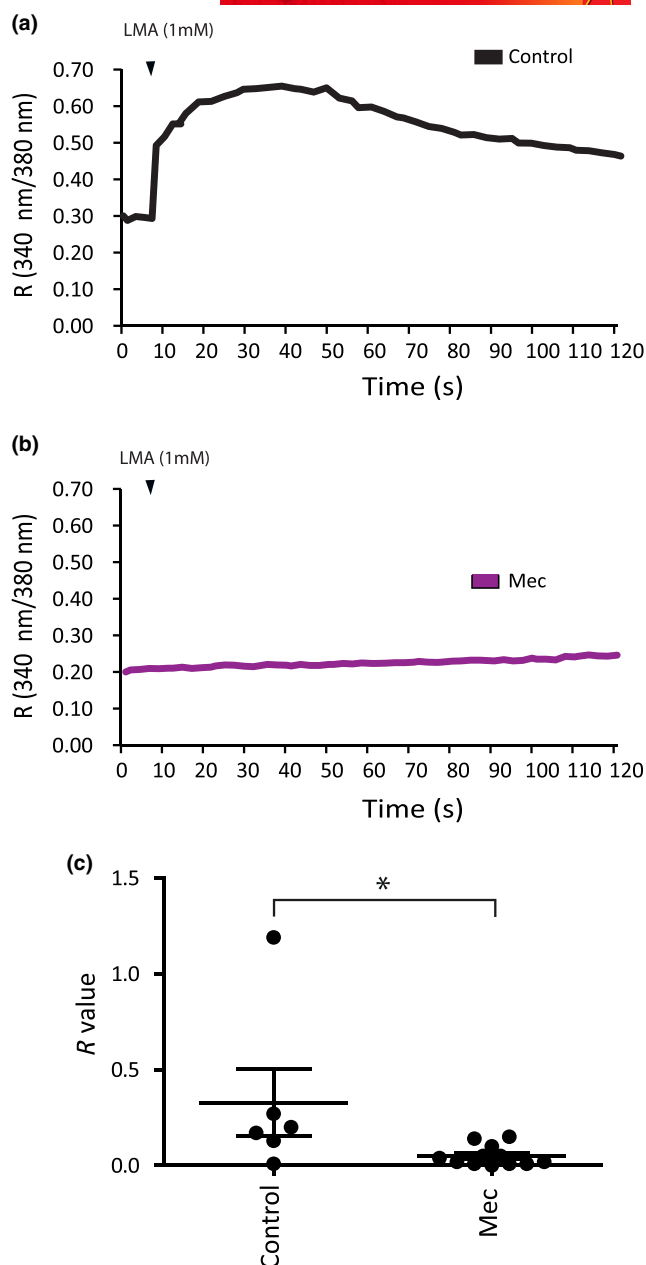


FIGURE 9 Effects of Mec bath application on the increase in intracellular calcium levels induced by LMA. Pulse application of 1 mM LMA evoked an increase in intracellular calcium levels (a) which was strongly decreased by bath application of Mec (b). Cells were incubated for 10 min with 20 μM Mec. Arrowheads indicate pulse application of 1 mM LMA (300 ms pulse duration, 15 psig). (c) Scatter dot plots illustrating LMA-induced calcium peak (R value) in control conditions (n=6 cells), and after bath application of Mec (n=16 cells). Unpaired t-test, $p=0.0108$, $t=2.810$, $df=20$. * $p<0.05$.

reduced when we co-applied α -Bgt and STO, we first proposed that the STO effect revealed a strong activation of α -Bgt-sensitive nAChR subtypes. Indeed, as previously demonstrated (Grolleau et al., 1996), α -Bgt completely blocked the intracellular calcium increase induced by NIC. We proposed that the majority of calcium influx in DUM neurons was through α -Bgt-sensitive nAChRs which have a high permeability to calcium. In general, nicotinic receptors with relatively high

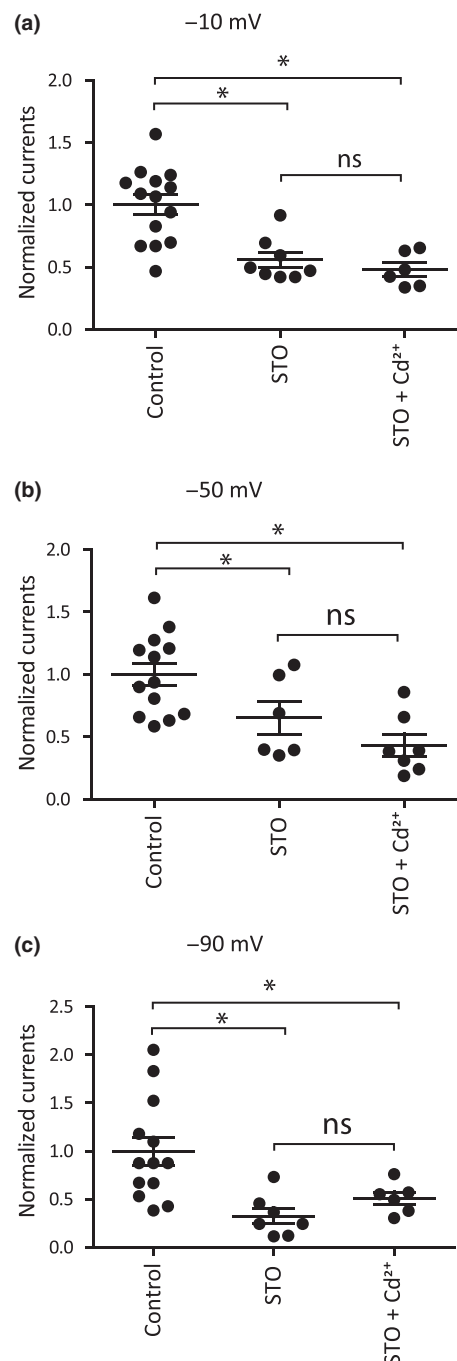


FIGURE 10 Scatter dot plots illustrating the effects of CdCl at different holding potentials. (a) At -10 mV, under control conditions (n=14 cells), bath application of 15 μM STO (n=8 cells), and co-application of STO with CdCl (n=6 cells). Unpaired t-test was applied to compare all experimental conditions. $p=0.0011$, 0.0008, and 0.3808; $t=3.7$, 4, 0.9; $df=20$, 18, 12, for control conditions, under STO and co-application of STO with CdCl, respectively. (b) At -50 mV, under control conditions (n=13 cells), bath application of 15 μM STO (n=6 cells), and co-application of STO with CdCl (n=7 cells). $p=0.04$, 0.008, and 0.19; $t=2.1$, 4, 1.3; and $df=17$, 18, 11, for control conditions, under STO and co-application of STO with CdCl, respectively. (c) At -90 mV, under control conditions (n=13 cells), bath application of 15 μM STO (n=7 cells), and co-application of STO with CdCl (n=6 cells). $p=0.004$, 0.04, and 0.11; $t=3.2$, 2.2, 1.7; $df=18$, 17, 11, under control conditions, STO, and co-application of STO with CdCl, respectively. * $p<0.05$.

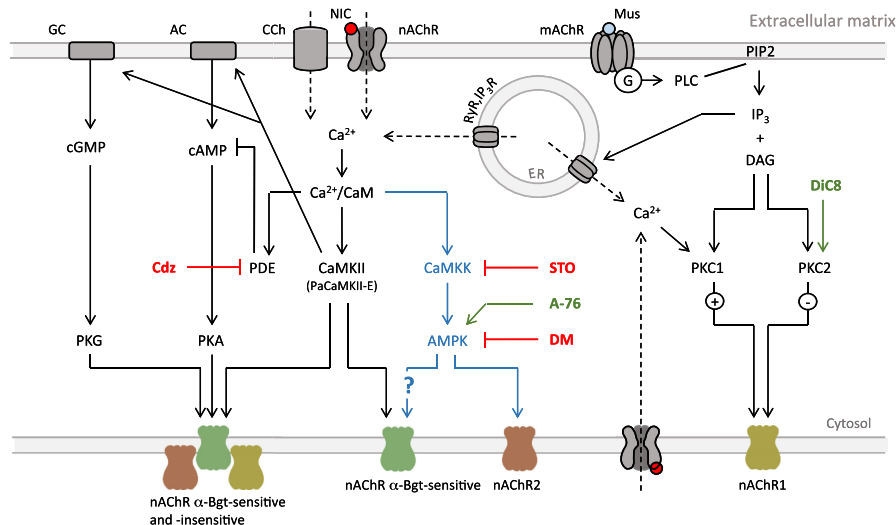


FIGURE 11 Diagram illustrating a partial overview of the intracellular calcium pathways involved in the regulation of α -Bgt-sensitive and -insensitive nAChRs expressed on cockroach DUM neurons. The diagram highlights complex pathways that have been studied and the potential pathways. Elevation in intracellular Ca^{2+} is caused by nAChRs, Ca^{2+} channels (CCh), or Ca^{2+} release from endoplasmic reticulum (ER). High Ca^{2+} activated Ca^{2+} -CaM complex that activates downstream enzymes such as PDE, CaMKK, and PaCaMKII-E. AC, adenylyl cyclase; AMPK, adenosine monophosphate-activated protein kinase; Ca^{2+} , calcium; A-76, A-769662; CaM, calmodulin; CaMKII, calcium-calmodulin-dependent protein kinase II; CaMKK, CaM kinase kinase; cAMP, cyclic adenosine monophosphate; CCh, calcium channel; cGMP, cyclic guanosine monophosphate; Cd²⁺, cadmium chloride; DAG, diacylglycerol; DM, dorsomorphin; DIC8, dioctanoylglycerol; GC, guanylyl cyclase; Mus, muscarine; NIC, nicotine; NO-GC, nitric oxide-sensitive guanylyl cyclase complex; IP₃, inositol triphosphate; PaCaMKII-E, *Periplaneta americana* CaMKII-E isoform; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; PIP₂, phosphatidylinositol-4,5-bisphosphate; mAChR, muscarinic ACh receptor; nAChR, nicotinic acetylcholine receptor; STO, 7-oxo-7H-benzimidazo[2,1-a] benz[de]isoquinoline-3-carboxylic acid; RE, endoplasmic receptor; RyR, ryanodine receptor.

calcium permeability have been associated with homomeric receptors such as the mammalian $\alpha 7$ receptors (Castro & Albuquerque, 1995; Seguela et al., 1993) which were sensitive to α -Bgt. Data from insect nAChRs suggest that α -Bgt could bind to homomeric and heteromeric nAChR subtypes (Thany et al., 2007), and as mentioned above, we have already observed that α -Bgt partially reduces nicotinic current amplitudes. Therefore, we suspected that a small part of the responses to nicotine could be mediated by α -Bgt-insensitive nAChR subtypes. This hypothesis is not surprising because (1) NIC activated both kinds of receptors, (2) Cd²⁺, a high-affinity CaM inhibitor, co-applied with α -Bgt did not reduce nicotine-evoked currents (Houchat et al., 2022), and (3) different nAChR subtypes are expressed in DUM neurons (Courjaret et al., 2003; Courjaret & Lapied, 2001; Sun et al., 2017; Thany et al., 2008). Thus, different populations of DUM neuron nAChRs could vary with respect to the magnitude of NIC-evoked current amplitudes, which was demonstrated with nornicotine (Calas-List et al., 2012). Nornicotine induced two distinct ionic currents, named types 1 and 2. In the presence of α -Bgt, type 1 currents were rapidly desensitized, whereas type 2 currents were completely blocked (Calas-List et al., 2012). Interestingly, pre-treatment of DUM neurons with STO prevented the increase in intracellular Ca^{2+} levels, which was not dependent on α -Bgt. This finding suggested a complex mechanism regulating the STO effect of nicotine-evoked currents and in intracellular Ca^{2+} , through activation of DUM neuron nAChRs. We checked whether this effect was associated with Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCCs). This hypothesis is based on the idea that

DUM neurons express a variety of VGCCs which could affect intracellular Ca^{2+} levels (Wicher et al., 1994, 2004; Wicher & Penzlin, 1994). Moreover, it was demonstrated that Ca^{2+} background currents were blocked by CdCl (Wicher et al., 1994; Wicher & Penzlin, 1994), which strongly reduced nicotine-induced currents through the nAChR2 subtype (Thany et al., 2008). In the present study, we found that CdCl did not induce an additional decrease in intracellular Ca^{2+} levels, when co-applied with STO, at hyperpolarizing potential known to activate CdCl-sensitive Ca^{2+} -resting channels (Heine & Wicher, 1998; Wicher et al., 1994). Thus, we argue that activation of nAChRs eliciting Ca^{2+} response through direct Ca^{2+} permeation via nAChRs did not activate voltage-dependent Ca^{2+} channels. Indeed, their activation should further augment intracellular Ca^{2+} signals generated by nAChRs, as found in other studies (Dajas-Bailador et al., 2002; Rathouz & Berg, 1994; Toulorge et al., 2011).

We then explored to what extent nAChR1 and nAChR2 subtypes were linked to the activation of CaMKK through intracellular Ca^{2+} . First, NIC-evoked currents were reduced by STO, and completely blocked when STO was co-applied with Mec, which is a selective inhibitor of the nAChR2 subtype (Courjaret & Lapied, 2001). A similar effect was found when we used LMA, which was known to activate the nAChR2 subtype (Mathe-Allainmat et al., 2012). STO partially reduced LMA-evoked currents, and its effects were reversed by A-76. LMA also induced an increase in intracellular Ca^{2+} , which was lower compared to the NIC effects, and Mec inhibited the increase in intracellular Ca^{2+} induced by LMA. These results confirmed a link



between nAChR2 and CaMKK activation, which is the most intriguing result of the present study. Thus, we propose that in the absence of STO, nicotine elicited significant increase in intracellular Ca^{2+} through the activation of α -Bgt-sensitive receptors. In this context, α -Bgt-sensitive nAChR-mediated Ca^{2+} responses are dependent on intracellular Ca^{2+} levels, leading to their inactivation under STO application. This hypothesis is compatible with patch-clamp recordings and the blocking of nicotine-induced intracellular Ca^{2+} increase by STO. In other words, α -Bgt-sensitive receptors could be involved in the rapid and strong entry of Ca^{2+} in the DUM neurons, whereas α -Bgt-insensitive nAChR2 subtype could maintain a low level or residual intracellular Ca^{2+} levels. Nevertheless, we cannot exclude that (1) nAChR1 accounted for the increase in intracellular Ca^{2+} . We demonstrated that when nAChR2 was blocked by Mec, a down-regulation of cyclic guanosine monophosphate (cGMP) pathways was found through the activation of nAChR1 (Mannai et al., 2016). (2) A link could exist between the Ca^{2+} -CaMKK-AMPK signaling pathway and ryanodine receptors, which could lead to a release of intracellular Ca^{2+} . For example, it was demonstrated that Ca^{2+} inflow through Ca^{2+} -permeable AMPA receptors triggers Ca^{2+} release from the internal store of fast-spiking interneurons in the rat prefrontal cortex (Camire & Topolnik, 2014; Wang & Gao, 2010). Nevertheless, it has to be kept in mind that in mammals, two CaMKK genes (α and β , respectively) have been cloned (Tokumitsu et al., 1995), and highly expressed in the brain tissues (Green et al., 2011; Lin et al., 2011). In the present study, we were unable to clearly specify how many CaMKKs were expressed in the DUM neurons, and are activated by the Ca^{2+} -CaM complex. Nevertheless, it was demonstrated that CaMKK β isoform is more sensitive to STO (Tokumitsu et al., 2003). We recently found that although CaMKII expresses five isoforms, only PaCaMKII-E is present in the DUM neurons, and accounts for the CaMKII activation (Taillebois et al., 2013). It will be interesting to explore the link between PaCaMKII-E isoform and CaMKK β in DUM neurons, through the activation of nAChRs.

5 | CONCLUSION

From an electrophysiological point of view, and taking previous studies into consideration, we conclude that at least three different pathways could be involved in the regulation of insect nAChRs in neurosecretory cells (Figure 11). The first pathway is CaM/PDE/cAMP, with its inhibition directly linked to a decrease in nicotine-evoked currents. The second is the CaM/PaCaMKII-E pathway, which involves both α -Bgt-insensitive nAChR1 and nAChR2 subtypes. In contrast, inhibition of the third, and novel CaMKK/AMPK pathway, involves both α -Bgt-sensitive and -insensitive nAChRs, without an activation of extracellular Ca^{2+} through voltage-gated calcium channels. This could be caused by STO activating other mechanisms, such as Ca^{2+} being released from intracellular stores. In terms of the intracellular regulation of Ca^{2+} pathways in insect neurosecretory cells, our main goal will be to investigate the functional coupling between the three calcium pathways, DUM neuron nAChRs, and the release

of internal stores. Indeed, in several studies, it was found that $\alpha 7$ nAChR-mediated Ca^{2+} signals arising from calcium-induced calcium released through the ryanodine receptors (Dickinson et al., 2007; Sharma & Vijayaraghavan, 2001; Ziviani et al., 2011).

AUTHOR CONTRIBUTIONS

Maria Taha: Data curation; formal analysis; investigation; writing – original draft; writing – review and editing. **Jean-Noël Houchat:** Data curation; formal analysis; investigation; writing – original draft; writing – review and editing. **Emiliane Taillebois:** Methodology; validation; writing – original draft; writing – review and editing. **Steeve Thany:** Conceptualization; funding acquisition; project administration; supervision; validation; writing – original draft; writing – review and editing.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter IV: The link between calcium release from ER through RyRs and IP₃Rs and flupyradifurone (FLU) mode of action

I. Presentation of the article

Context: Previous research has highlighted the importance of calcium influx through the cell membrane and calcium-binding proteins in regulating insect nAChRs. This regulation affects the insects' responses to many insecticides, including FLU. Notably, intracellular calcium stores are also known to modulate currents through insect nAChRs, as demonstrated by (Campusano *et al.*, 2007; Thany *et al.*, 2008). However, the specific types of receptors involved in this modulation had not been identified until now.

Objective: This study aimed to explore the specific involvement of RyRs and IP₃Rs in FLU' mode of action on nAChRs expressed in DUM neurons. Given that DUM neurons express two subtypes of nAChRs, nAChR1 and nAChR2 (Courjaret *et al.*, 2001), the initial objective was to identify which subtype is primarily involved in FLU mode of action. This was accomplished using nAChRs blockers, α -Bgt, d-TC, and Mec. Following this, the study investigated the effects of blocking calcium release through RyRs and IP₃Rs on FLU-induced currents and intracellular calcium increase to determine the contribution of each receptor type to these processes.

Results: we found that only d-TC and Mec were able to reduce FLU-evoked currents, suggesting that FLU can activate α -Bgt-insensitive nAChRs, including both nAChR1 and nAChR2 subtypes. FLU was able to induce an increase in intracellular calcium level which was almost completely inhibited by bath application of α -Bgt, d-TC, and Mec. IP₃R reduced FLU-induced currents but it did not affect the intracellular calcium increase after FLU pulse application demonstrating that IP₃ pathways and/or IP₃R were not associated to this process. In contrast, blocking RyRs significantly inhibited both the intracellular calcium rise and the FLU-induced currents

Conclusion: our study provides valuable insights into the mechanisms by which FLU modulates nAChRs in DUM neurons. We propose an interplay between FLU evoked nAChR activity and RyR, which is of critical interest since RyR have already been used as an insecticide target to control agricultural pests.

II. Synthèse de l'article

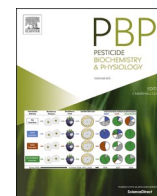
Contexte : Des études antérieures ont mis en évidence l'importance de l'afflux de calcium à travers la membrane cellulaire et les protéines de liaison au calcium dans la régulation des nAChRs des insectes. Cette régulation affecte les réponses des insectes à de nombreux insecticides, dont le FLU. Notamment, les réserves de calcium intracellulaires sont également connues pour moduler les courants nicotiques chez les insectes, comme l'ont démontré (Campusano *et al.*, 2007; Thany *et al.*, 2008). Cependant, les types spécifiques de récepteurs nicotiques impliqués dans cette modulation n'avaient pas été identifiés jusqu'à présent.

Objectif : Cette étude visait à explorer l'implication spécifique des RYR et des IP₃R dans le mode d'action du FLU sur les nAChR exprimés dans les neurones DUM. Etant donné que les neurones DUM expriment deux sous-types de nAChRs, nAChR1 et nAChR2 (Courjaret *et al.*, 2001), l'objectif initial était d'identifier quel sous-type est principalement impliqué dans le mode d'action du FLU. Pour ce faire, nous avons utilisé des bloqueurs de nAChRs, l' α -Bgt, la d-TC et la Mec. Ensuite, nous avons examiné les effets du blocage de la libération de calcium par les RYR et les IP₃R sur les courants induits par le FLU et l'augmentation du calcium intracellulaire afin de déterminer la contribution de chaque type de récepteur à ces processus.

Résultats : Nous avons constaté que seuls la d-TC et la Mec étaient capables de réduire les courants induits par le FLU, ce qui suggère que le FLU peut activer les nAChRs insensibles à l' α -Bgt, y compris les sous-types nAChR1 et nAChR2. Le FLU a pu induire une augmentation du niveau de calcium intracellulaire qui a été presque complètement inhibée par l'application de l' α -Bgt, la d-TC et la Mec. Le 2-APB, un inhibiteur des récepteur à l'IP₃, a réduit les courants induits par le FLU mais n'a pas affecté l'augmentation du calcium intracellulaire après l'application du FLU, ce qui démontre que les voies IP₃ et/ou l'IP₃R n'ont pas été associées à ce processus. En revanche, le blocage des RyRs a significativement inhibé à la fois l'augmentation du calcium intracellulaire et les courants induits par le FLU.

Conclusion : Notre étude apporte des informations précieuses sur les mécanismes par lesquels le FLU module les nAChRs dans les neurones du DUM. Nous proposons une interaction entre l'activité nAChR évoquée par le FLU et les RYR, ce qui est d'un intérêt crucial puisque les RYR ont déjà été utilisés comme cible d'insecticides pour contrôler les insectes nuisibles dans les cultures.

III. Publication n°2: Taha *et al.*, 2024



Flupyradifurone activates DUM neuron nicotinic acetylcholine receptors and stimulates an increase in intracellular calcium through the ryanodine receptors

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ABSTRACT

Insect neuronal nicotinic acetylcholine receptors (nAChRs) are transmembrane receptors that play a key role in the development and synaptic plasticity of both vertebrates and invertebrates, and are considered to be major targets of several insecticides. We used dorsal unpaired median (DUM) neurons, which are insect neurosecretory cells, to explore what type of nAChRs are involved in flupyradifurone's (FLU) mode of action, and to study the role of calcium release from intracellular stores in this process. Using whole-cell patch-clamp and fura-2-AM calcium imaging techniques, we found that inhibition of IP₃Rs through application of 2-APB reduced FLU inward currents, but did not affect the intracellular calcium release induced by FLU. In contrast, inhibition of RyRs using ryanodine, led to reduction of intracellular calcium increase following FLU pulse application. These results suggested that FLU inward currents are likely due to a combination of the direct effects of FLU on DUM neuron nAChRs and the subsequent calcium release from RyRs.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast synaptic transmission in the insect nervous system, and are major targets of various insecticides. Flupyradifurone (FLU) is a novel butenolide insecticide that selectively targets insect nervous systems. It acts reversibly as an agonist on insect nAChRs and has an effective action against a wide range of pest insects, including neonicotinoid-resistant populations (Jeschke et al., 2015; Nauen et al., 2015). Its unique insecticidal and ecotoxicological properties and safety profile have made it an important tool for pest management (Nauen et al., 2015). Dorsal unpaired median (DUM) neurons are pacemaker neurons that have the intrinsic ability to generate spontaneous rhythmic activity. They express two α -bungarotoxin (α -Bgt)-insensitive nAChR subtypes, nAChR1 and nAChR2, which are differently regulated by intracellular calcium pathways (Courjaret and Lapied, 2001; Courjaret et al., 2003). While extensive research has investigated the mode of action of insecticides, including their interaction with calcium signaling, no studies have examined the connection between FLU and intracellular calcium pathways. For example, previous studies showed that inhibition

of calcium influx through the plasma membrane can increase nAChR2 sensitivity to acetamiprid (ACE) and clothianidin (CLT), which are both well-known neonicotinoids (Bodereau-Dubois et al., 2012). The role of the calcium binding protein in regulating the mode of action of neonicotinoids has also been proven. Blocking the expression of the isoform E of calcium/calmodulin-dependent protein kinase II (CaMKII-E) in *Periplaneta americana* cockroach DUM neurons led to a decrease in both the currents and intracellular Ca²⁺ concentration induced by CLT (List et al., 2014). In our recent study, we also demonstrated the involvement of the calcium/calmodulin-dependent protein kinase kinase (CaMKK)/adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway in the regulation of DUM neurons nAChR2, by using the selective CaMKK inhibitor, STO-609 (Taha et al., 2024).

Furthermore, studies on *Drosophila* Kenyon cells showed that calcium-induced calcium release (CICR) from intracellular stores contributes to the nicotine response. Both nicotine (NIC)-induced amplitude and intracellular calcium increase were reduced in cultures treated with thapsigargin (Campusano et al., 2007). In addition, on *P. americana* DUM neurons, the application of 1 μ M thapsigargin produced a significant reduction of nAChR2 mediated inward current, concluding that

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CICR process can modulate nicotine responses following nAChR2 activation (Thany et al., 2008). However, recent electrophysiological recordings on insect neurons provided evidence that calcium release from intracellular stores can modulate insect nAChRs regulation, no studies indicate whether the specific types of receptors involved in this process are ryanodine receptors (RyRs) or inositol 1,4,5-triphosphate receptors (IP₃Rs) (Courjaret and Lapied, 2001). Therefore, our aim in this study is to further characterize the involvement of both RyRs and IP₃Rs in the calcium release induced by FLU through DUM neuron nAChRs. This was achieved by using dantrolene and ryanodine, selective inhibitors for RyRs (Zhao et al., 2001; Ostrovskaya et al., 2007; Inan and Wei, 2010; Chiantia et al., 2023), and 2-APB, a selective IP₃R inhibitor (Hagenston et al., 2009; Xiao et al., 2010). RyRs are non-voltage-gated calcium channels located on the endoplasmic reticulum (ER) of neurons (Sattelle et al., 2008). Their name originally comes from the natural insecticide, ryanodine, which is a plant metabolite from *Ryania speciosa*. Ryanodine has been found to affect calcium release by blocking channels in a partially opened state (Lahm et al., 2007). IP₃Rs are also intracellular Ca²⁺ channels, predominantly localized in the ER, and activated by the binding of IP₃. Signals initiated by IP₃Rs can evoke a wide variety of cellular events that can regulate many neuronal functions (Prole and Taylor, 2016; Schmitz et al., 2022). The involvement of IP₃Rs in neuronal nAChR signaling in mammals was demonstrated when IP₃R-selective antagonists reduced nAChR-induced calcium responses (Shen and Yakel, 2009). In this study we aim to further explore the role of IP₃Rs and RyRs in modulating insect nAChRs, and specifically how calcium release from ER through these receptors can affect FLU's mode of action on nAChRs.

2. Materials and methods

2.1. Animals

Experiments were performed using adult male cockroaches *P. americana* reared at 29 °C on a 12:12 h light/dark cycle.

2.2. Dissection of cockroach terminal abdominal ganglion

The cockroach terminal abdominal ganglion (TAG) was isolated by dissection along the longitudinal dorsal-median line. Forceps were used to remove the alimentary canal as well as the overlying muscle and trachea.

2.3. DUM neuron isolation

After being carefully dissected, the TAG was placed in the standard saline extracellular solution. Each compound in this solution was purchased from Sigma Aldrich, France. The TAG was incubated (35 min, 29 °C) in the saline supplemented with collagenase (type I, 1.5 mg/ml, GIBCO), rinsed twice and placed in the saline supplemented with foetal calf serum (5 % by volume), 50 UI/ml penicillin, and 50 µg/ml streptomycin (Sigma Aldrich, France). They were then mechanically dissociated by gentle trituration using fire-polished Pasteur pipettes. Before adding the cell culture medium, the isolated DUM neurons were left for 10 min to settle and adhere to Petri dishes (35/09 mm, NEST, Grosseron, France) coated with poly-D-lysine hydrobromide (MW, 70,000-150,000; Sigma Aldrich, France) for patch-clamp experiments, or to coverslips (22 × 40 × 0.17 mm, HIRSCHMANN, Verrerie Villeurbannaise, France) for calcium imaging. Cell cultures were incubated overnight at 29 °C. All preparations were carried out under semi sterile conditions i.e., all equipment was sterilized with ethanol and solutions were filtered.

2.4. Electrophysiological recordings

Signals were recorded using the patch-clamp technique in the whole-cell recording configuration in voltage-clamp mode using an Axopatch

200B (Patch-clamp amplifier, Axon Instruments, Foster City, CA). They were digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments) and Axoscope 10.2 software (Axon Instruments). Signals were low pass-filtered at 5 kHz. The Petri dishes containing isolated DUM neurons were placed onto an inverted microscope (AE 2000, Motic), and continuously bathed with the standard saline using a gravity perfusion system positioned within 100 µm from the cell body. In all experiments DUM neuron capacitance ranged from 200 to 250 pF to ensure reproducible current amplitudes. Patch pipettes (borosilicate glass capillary tubes: GC 150 T-10; Clark Electromedical Instruments, Harvard Apparatus) were filled with a standard internal solution (in mM: 160 K-D gluconic acid, 10 K-fluoride, 10 NaCl, 1 MgCl₂, 0.5 CaCl₂, 20 HEPES, 10 EGTA, 3 ATP; pH 7.4 adjusted with KOH). Their resistances ranged from 1.5 to 2 MΩ. FLU was applied via pneumatic pressure ejection (15 psig, 300 ms; Miniframe, Medical System Corporation, USA). The pressure ejection was made using a glass micropipette, with resistance ranging from 1.5 to 2 MΩ, positioned in a solution within 100 µm of the isolated cell body. All compounds were purchased from Sigma Aldrich, France.

2.5. Intracellular calcium recordings

Intracellular calcium levels were recorded using the ratiometric fluorescent dye fura-2-AM. Coverslips containing isolated cell bodies were rinsed twice and incubated in the dark (45 min, 37 °C) in the saline supplemented with sucrose (50 mM), Pluronic F-127 (0.1 %) and fura-2-AM (10 µM). All compounds were purchased from Sigma Aldrich, France. They were rinsed twice more and mounted on a perfusing chamber (PM-1, SA-OLY/2-AL, Warner Instruments) under an inverted microscope (IX73, Olympus) installed in a light-tight box. Neurones were bathed using a gravity perfusion system from the outside of the box, with tubes entering the box through 1 mm holes. This allowed us to control the levels of saline without exposing the samples to exterior light. Pressure ejection of FLU (15 psig, 300 ms; PLI 100, Medical System Corporation, USA) was carried out using the same glass micropipettes as those used for the patch-clamp. Control cells were subjected to a pulse application of 1 µM FLU in saline solution, whereas tested cells were pre-treated for 15–30 min with 2-APB, ryanodine, caffeine, d-tubocurarine (d-TC), mecamlamine (Mec), or α-Bgt before pulse application of FLU. For all recordings, the following parameters were applied: exposure time: 100–200 ms, gain: 0 dB, recording time: 2 min, and acquisition interval: 1.5 s. Calcium intracellular levels were measured using a brightfield/340/380 nm LED camera (CoolLED pE-340^{tura}) and the *cellSens Dimension* acquisition software (Olympus).

2.6. Drugs and solutions

For all experiments (patch-clamp and calcium imaging), FLU was diluted in saline solution, then applied using a pulse application of 300 ms (15 psig) at a concentration of 1 µM. α-Bgt was diluted in saline solution to a final concentration of 0.5 µM (Courjaret and Lapied, 2001; Thany et al., 2008). The compounds d-TC (nAChR1 antagonist), Mec (nAChR2 antagonist), dantrolene (RyR antagonist), ryanodine (RyR blocker), 2-Aminoethoxydiphenyl borate (2-APB, IP₃R inhibitor) were first dissolved in DMSO (stock solution), and then diluted in saline solution to make a final solution of 0.01 % DMSO (working solution) to be used in the experiment. α-Bgt, Mec, d-TC, 2-APB, ryanodine, and caffeine, were applied using a gravity bath perfusion system positioned within 100 µm from the cell body (VC-6 valve controller, Warner Instrument, USA). Only dantrolene was diluted in the intrapipette solution. All currents were recorded at −50 mV holding potential.

2.7. Statistical analyses

Ionic currents were normalized according to the mean amplitude in control conditions (I/I_{mean}) and intracellular levels of calcium were

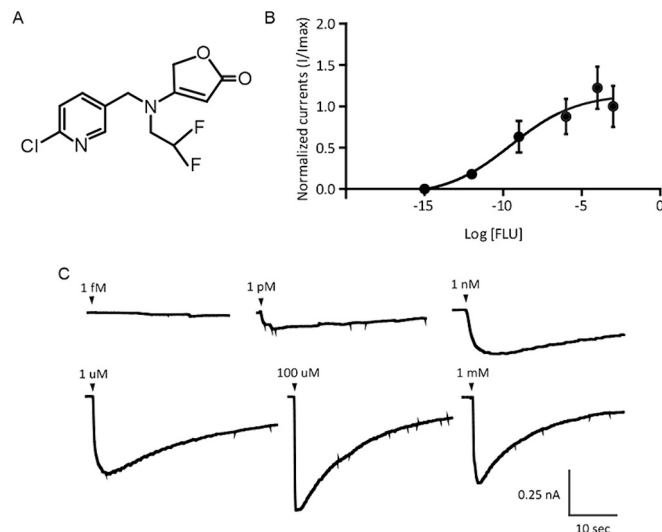


Fig. 1. (A) Chemical structure of flupyradifurone. (B) Flupyradifurone (FLU) dose-response curve. Responses are normalized to the mean current induced by 1 μ M FLU. Each point represents mean $\pm$ SEM, $n = 6$. (C) Examples of inward currents induced by different concentrations of flupyradifurone. Currents were recorded at -50 mV holding potential. Arrowheads represent pulse application (300 ms, 15 psig) of FLU.

expressed as R values (R being the 340 nm / 380 nm ratio value). R-studio 8.0 software and Prism 9 (GraphPad Software Inc) were used to compare mean current amplitudes and mean calcium peaks. Data were analysed using Kruskal-Wallis with Holm post-hoc test, or Student *t*-test, depending on the size of the group. Normal distribution (Gaussian distribution) of the data was tested using a Shapiro-Wilk test. All tests used were two-tailed. All data points were included in each assay. The difference was considered significant when the *p* value was <0.05 . For all scatter dot plot graphs, a dot represents one cell, error bars represent mean with SD. n = number of independent cells.

3. Results

3.1. Effect of flupyradifurone on nicotinic acetylcholine receptors expressed in the cockroach DUM neurons

FLU induced inward currents in a dose-dependent manner (Fig. 1). The maximum response was obtained at 1 μ M FLU, and the EC_{50} value was 0.3 ± 0.01 μ M. According to our previous data (Houchat et al., 2022; Taha et al., 2024), we aimed to identify whether α -Bgt and d-TC and Mec were able to inhibit currents induced by pulse application of 1 μ M FLU. One major question was regarding the involvement of both α -Bgt-sensitive and -insensitive nAChRs, nAChR1 and nAChR2 subtypes in FLU's mode of action. First, we bath applied DUM neurons with 0.5 μ M α -Bgt to study the involvement of α -Bgt-sensitive nAChRs. Bath application of 0.5 μ M α -Bgt did not significantly reduce FLU-evoked

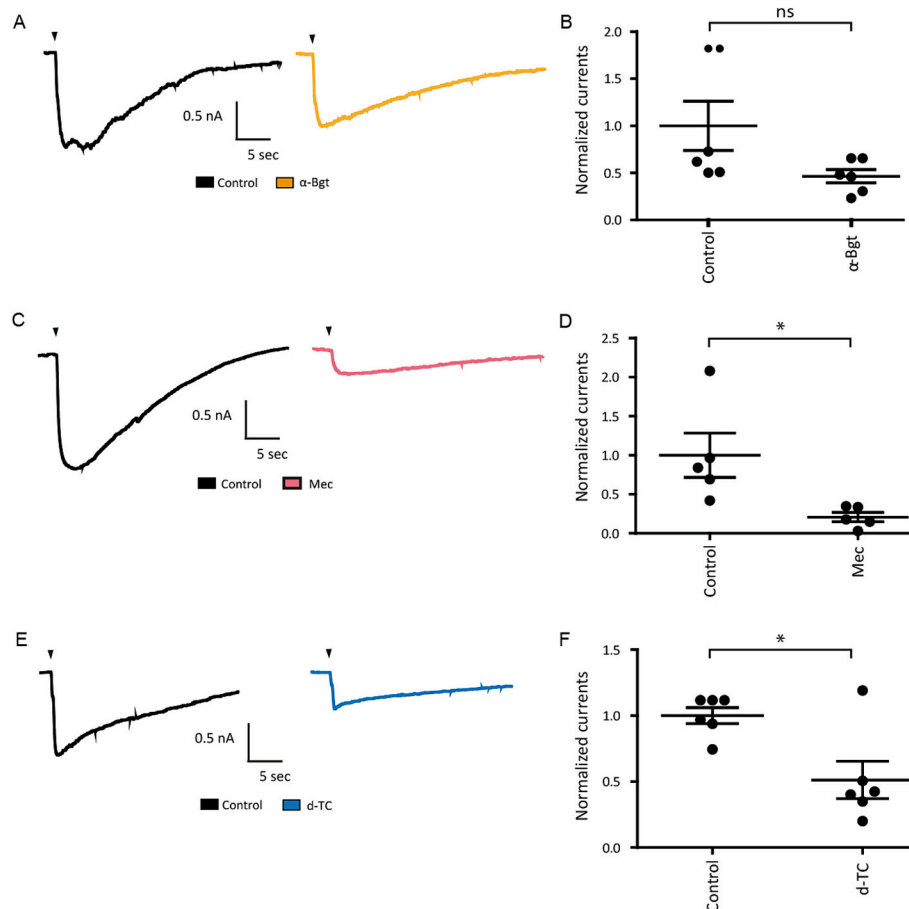


Fig. 2. Effects of α -Bgt, d-TC, and Mec bath application on FLU induced currents. In each case, data are paired results obtained with the same cells using 1 μ M FLU (300 ms, 15 psig) under control conditions and bath application of 0.5 μ M α -Bgt (A and B), 20 μ M d-TC (C and D), and 20 μ M Mec (E and F). Each histogram represent mean $\pm$ SEM of $n = 6$ cells. * $p < 0.05$, paired *t*-test, $p = 0.08$, $t = 2.2$, and $df = 5$ between control and α -Bgt; $p = 0.01$, $t = 3.6$, $df = 5$ between control and d-TC; $p = 0.03$, $t = 3.1$, and $df = 4$ between control and Mec. ns: no significant. In all cases, arrowheads indicate pulse application (300 ms, 15 psig) of 1 μ M FLU. Currents were recorded at -50 mV holding potential.

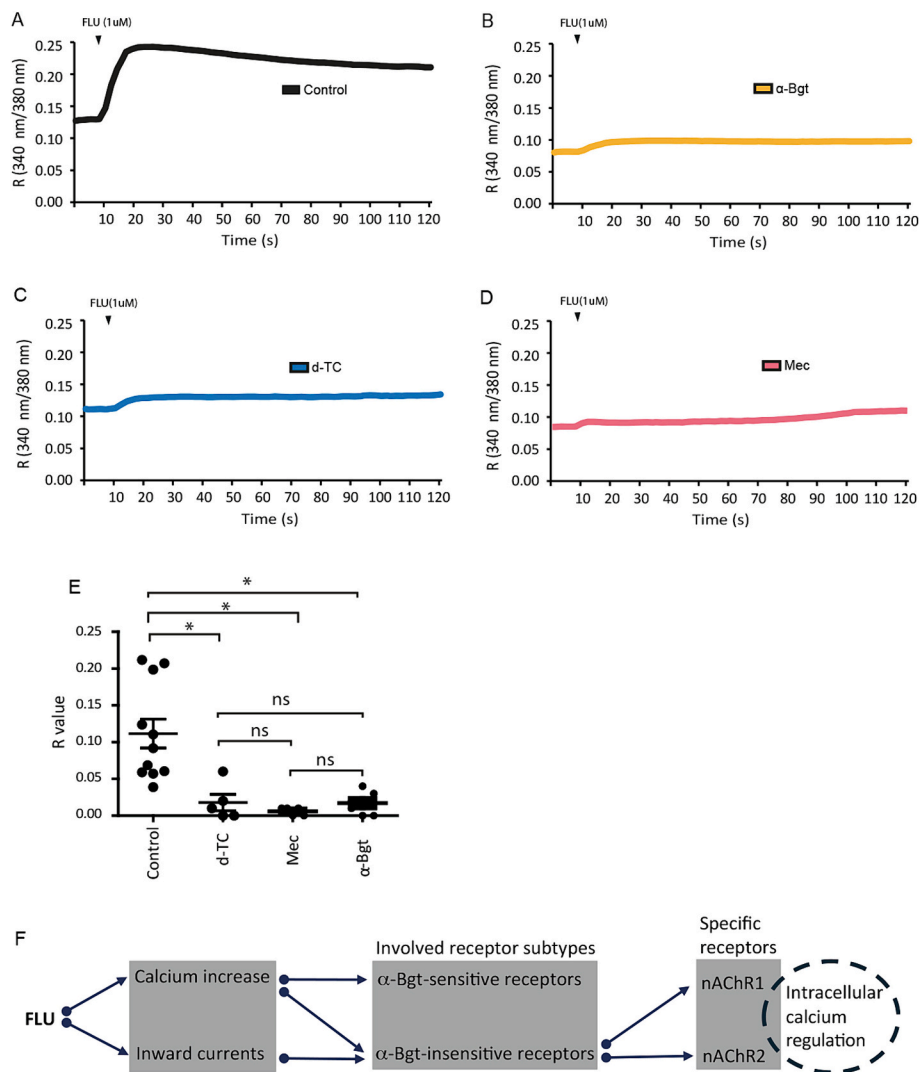


Fig. 3. Effects of α -Bgt, d-TC, and Mec bath application on FLU induced intracellular Ca^{2+} increase. (A) Intracellular calcium increase induced by 1 μM FLU (control conditions) was strongly blocked by bath application of 0.5 μM α -Bgt (B), 20 μM d-TC (C), and 20 μM Mec (D). (E) Scatter dot plots illustrating calcium peak (R value) induced by FLU under control conditions ($n = 11$ cells), and the inhibitory effect of α -Bgt ($n = 7$ cells), d-TC ($n = 5$ cells), and Mec ($n = 5$ cells). * $p < 0.05$, Kruskal-Wallis with Holm post-hoc test. Arrowheads indicate pulse application (300 ms, 15 psig) of 1 μM FLU. Note that, each histogram represents an independent recording. (F) Summary of results from Figs. 2 and 3. We suggest that FLU induces ionic currents through α -Bgt-insensitive receptors, probably through an activation of both nAChR1 and nAChR2 subtypes. The increase in intracellular calcium involved both α -Bgt-sensitive and -insensitive receptors, a mechanism which is associated to intracellular calcium regulation.

current amplitudes ($n = 6$ cells, Fig. 2A and B, $p > 0.05$), which suggested the involvement of α -Bgt-insensitive nAChR subtypes. To study this hypothesis, we used both d-TC and Mec, selective nAChR1 and nAChR2 blockers, respectively (Courjaret and Lapied, 2001; Courjaret et al., 2003). Application of 20 μM d-TC ($n = 6$ cells, $p < 0.05$, Fig. 2C and D) and 20 μM Mec (-0.4 ± 0.1 nA, $n = 6$ cells, $p < 0.05$, Fig. 2E and F), significantly reduced FLU-induced currents from -1.35 ± 0.08 and -2 ± 0.57 nA to -0.69 ± 0.2 and -0.42 ± 0.12 nA, respectively. We concluded from these studies that α -Bgt-insensitive nAChR1 and nAChR2 subtypes may be the main subtypes involved in FLU's mode of action. However, varying results were obtained when studying the involvement of the same inhibitors in intracellular calcium modulation after pulse application of 1 μM FLU (Fig. 3). First, pulse application of 1 μM FLU was able to induce an increase in intracellular calcium which was maintained after FLU application (Fig. 3A). But, contrary to what we observed with the current amplitudes, the three nicotinic inhibitors, including α -Bgt (Fig. 3B, C and D), strongly blocked the increase in intracellular calcium (Fig. 3E). However, this inhibition was never complete, as a residual calcium was observed during the recordings. We

proposed that mechanisms other than calcium entry via the DUM neuron nAChRs were involved and could be activated by FLU-induced calcium release through the activation of nAChR1 and nAChR2 subtypes, respectively. A summary of our hypothesis is illustrated in Fig. 3F. Our main idea was that the activation of DUM neuron nAChRs by FLU does not have the same consequences for current amplitudes and intracellular calcium activity (Fig. 3F).

3.2. The release of intracellular calcium involves ryanodine receptors more than IP_3 receptors

It is known that nAChR activation leads to the opening of the ion channels, an influx of cations, and in particular an increase in intracellular calcium (Seguela et al., 1993; Castro and Albuquerque, 1995). Electrophysiological recording using 1 μM FLU demonstrated that 15 min bath application of 70 μM 2-APB (2-Aminoethoxydiphenyl borate), a blocker of store-operated Ca^{2+} entry through IP_3R (Prakriya and Lewis, 2001; Ma et al., 2002; Peinelt et al., 2008; Littlechild et al., 2015), significantly decrease current amplitudes. Currents were significantly

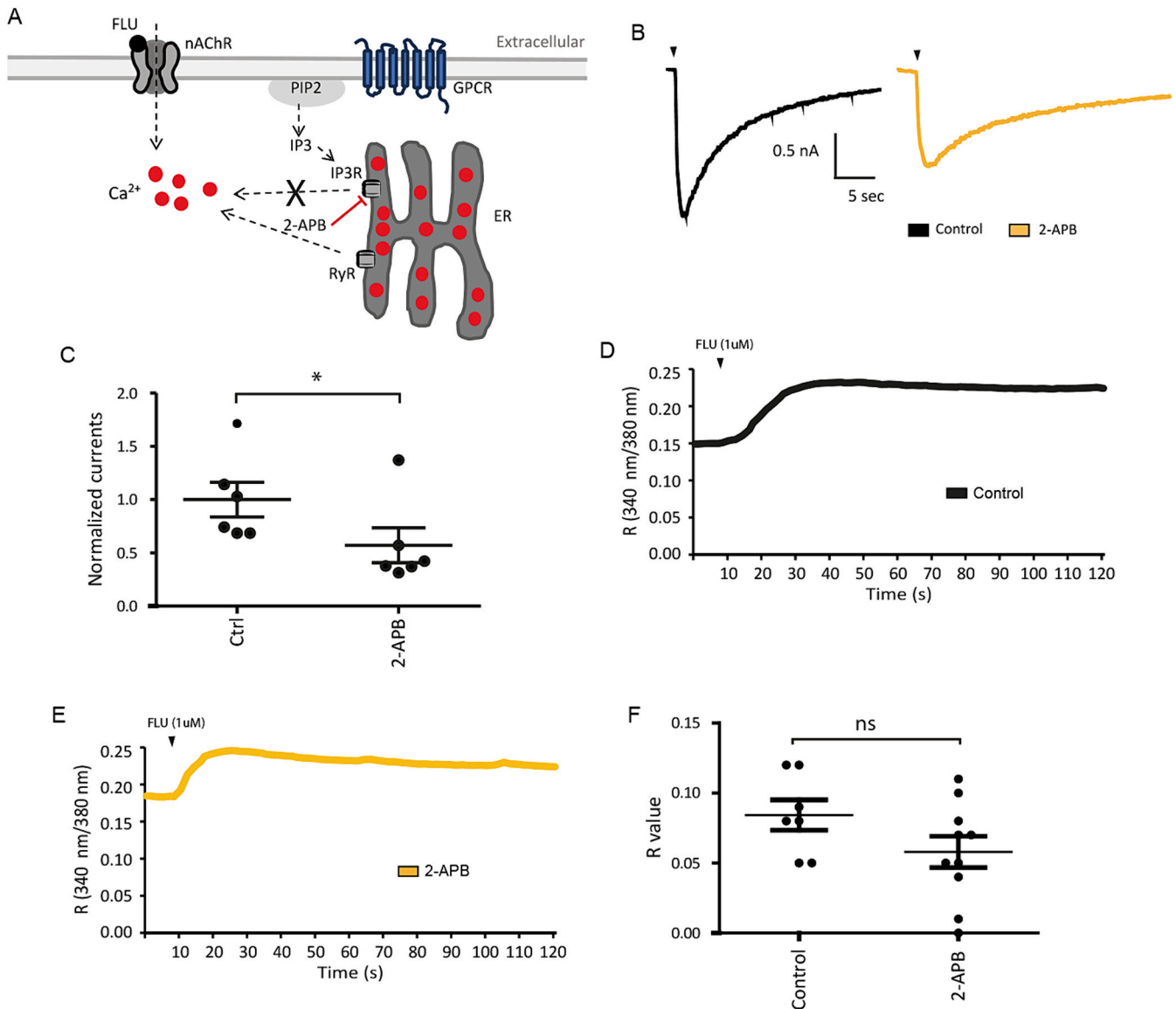


Fig. 4. Effects of 2-APB bath application on FLU-induced currents and intracellular calcium increase. (A) Ca^{2+} release from ER via IP_3Rs were blocked by 2-APB (The cross indicated that this pathway is blocked by 2-APB). (B) Examples of FLU-induced currents under control condition and bath application of 70 μM 2-APB. (C) Scatter dot plots representing the effect of 1 μM FLU under control conditions ($n = 6$ cells) and bath application of 70 μM 2-APB ($n = 6$ cells, paired t -test, $p = 0.0009$, $t = 7.033$, and $df = 5$). Currents were recorded at -50 mV holding potential. $*p < 0.05$. (D and E) Pulse application of 1 μM FLU (D) induced an increase in intracellular calcium levels which was not affected by the bath application 70 μM 2-APB (E). Cells were incubated for 15–30 min with 70 μM 2-APB. Arrowheads indicate pulse application of 1 μM FLU (300 ms pulse duration, 15 psig). (F) Scatter dot plots illustrating FLU-induced calcium peak (R value) in control conditions ($n = 7$ cells), and after bath application of 2-APB ($n = 10$ cells). Unpaired t -test. ns: no significant. In all cases, arrowheads indicate pulse application (300 ms, 15 psig) of 1 μM FLU.

reduced from -1.75 ± 0.3 nA under control conditions to -0.68 ± 0.17 nA under bath application of 70 μM 2-APB ($p < 0.05$, $n = 6$ cells, Fig. 4A, B and C). Similar experiments were carried out when intracellular calcium variations were recorded after 300 ms pulse application of 1 μM FLU. In this condition, no specific changes and/or variation in intracellular calcium were found ($p > 0.05$, $n = 6$ cells, Fig. 4D, E and F). To reevaluate the effect of 2-APB, we added 5 mM caffeine to the bath. Caffeine had previously been shown to act as an activator of the intracellular calcium release (Nagasaki and Kasai, 1984; Klein et al., 1990; Alonso et al., 1999). Fig. 5 clearly demonstrated that IP_3 pathways and/or IP_3R were not involved in the increase in intracellular calcium induced through the activation of nAChRs by FLU. Caffeine did not induce an increase in intracellular calcium compared to control ($p > 0.05$, $n = 6$, Fig. 5A and B). In a similar way, co-application of both 0.5 mM caffeine and 70 μM 2-APB did not modulate intracellular calcium

levels ($p > 0.05$, $n = 6$, Fig. 5C and D). The other hypothesis we wanted to explore concerned the involvement of RyR due to the results found with 2-APB. We therefore decided to use dantrolene, a selective RyR blocker (Ryglewski et al., 2007). Intrapipe application of 100 nM dantrolene had no significant effect on FLU-evoked currents. Currents were -1.35 ± 0.18 nA and -1.00 ± 0.12 nA ($p > 0.05$, $n = 6$, Fig. 6A and B), under control conditions and for patch pipette application of dantrolene, respectively. Similarly, caffeine had no significant effect on FLU-induced currents (-0.96 ± 0.11 nA, $p > 0.05$, $n = 6$, Fig. 6A and B). Interestingly, in the presence of both caffeine and dantrolene, FLU-induced currents were significantly reduced from -1.35 ± 0.18 nA to -0.67 ± 0.11 nA ($p < 0.05$, $n = 6$ cells, Fig. 6A and B). Since dantrolene is not a cell-permeable compound (Ryglewski et al., 2007), we evaluated the effect of bath applied inhibitory ryanodine to suppress RyR-mediated Ca^{2+} release (Arias-Cavieles et al., 2018). To do that, we

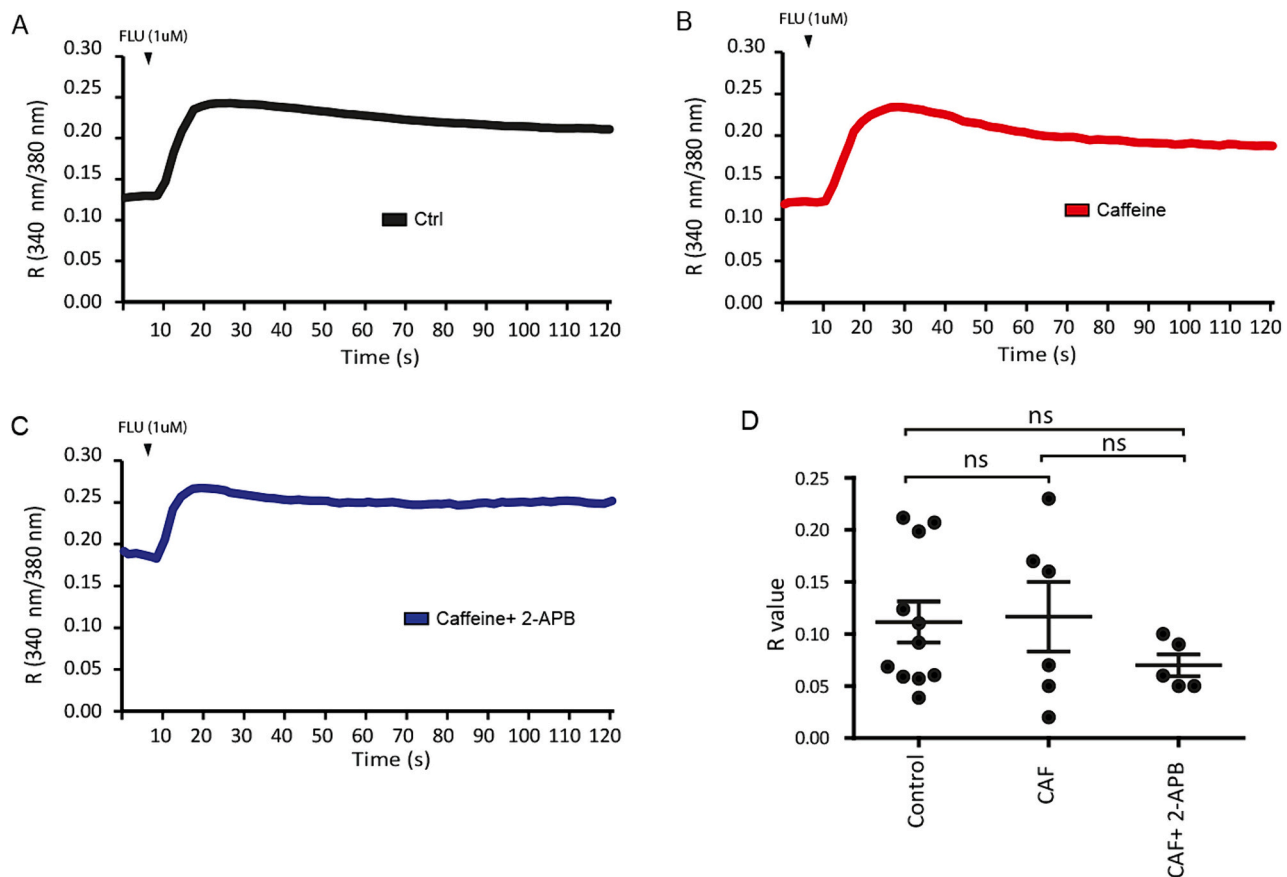


Fig. 5. Effects of bath application of caffeine and 2-APB on intracellular calcium increase induced by FLU. Pulse application of 1 μ M FLU (A) evoked an increase in intracellular calcium levels which was not affected by bath application of 5 mM caffeine (B), or by the co-application of caffeine and 70 μ M 2-APB (C). Cells were incubated for 15–30 min. Arrowheads indicate pulse application of 1 μ M FLU (300 ms pulse duration, 15 psig). (D) Scatter dot plots representing FLU-induced calcium peak in control conditions ($n = 11$ cells), under bath application of 5 mM caffeine (CAF) ($n = 6$ cells), and co-application of caffeine with 2-APB ($n = 5$ cells). * $p < 0.05$, Kruskal-Wallis with Holm post-hoc test. In all cases, arrowheads indicate pulse application (300 ms, 15 psig) of 1 μ M FLU.

bath applied the cells with 20 μ M ryanodine. As shown in Fig. 7, ryanodine had no significant effect of currents induced by 1 μ M FLU. Currents were -1.21 ± 0.45 nA under control conditions and -1.00 ± 0.25 nA after ryanodine bath application ($p > 0.05$, $n = 6$, Fig. 7). Data obtained from recordings of intracellular calcium changes demonstrated that ryanodine bath application almost completely prevented calcium increase induced by 300 ms pulse application of 1 μ M FLU ($p < 0.05$, $n = 6$ cells, Fig. 8).

4. Discussion

We have previously shown that calcium influx through cell membrane and calcium binding proteins have a critical role in regulating insects nAChRs, which in turn can affect their responses to many insecticides (Houchat et al., 2020; Houchat et al., 2022). However, even though the role of intracellular calcium stores in modulating currents through insects nAChRs has been demonstrated (Campusano et al., 2007; Thany et al., 2008), no studies to date have evaluated a possible crosstalk between the activation of insect nAChRs and the release of intracellular calcium induced through the ER. In this study, we aimed to further explore how calcium release from RyRs and/or IP₃R may be involved in FLU's mode of action on nAChRs expressed in DUM neurons. Since DUM neurons express two subtypes of nAChRs (nAChR1 and nAChR2) (Courjaret and Lapied, 2001), we started by identifying which type of nAChR is involved in FLU's mode of action. To do this, we used the nAChR inhibitors α -Bgt, d-TC, and Mec, to identify the involvement of α -Bgt-sensitive and -insensitive, nAChR1 and nAChR2 subtypes, respectively. Using patch clamp recordings, we found that only d-TC and

Mec were able to reduce FLU-evoked currents, suggesting that FLU activated α -Bgt-insensitive nAChRs, including both nAChR1 and nAChR2 subtypes, respectively. Moreover, data obtained with FLU shed further light on the pharmacology of nAChR1 and nAChR2 subtypes. Indeed, in previous studies, we considered that nAChR1 and nAChR2 had distinct pharmacological properties according to the current-voltage curve induced by nicotine and ACh, in which nAChR1 and 2 were blocked by d-TC and Mec, respectively (Courjaret and Lapied, 2001; Courjaret et al., 2003; Thany et al., 2008; Bodereau-Dubois et al., 2012). The present data suggest a more complex native pharmacology in which at -50 mV DUM neuron holding potential, both nAChR1 and nAChR2 were present and activated by FLU. This assumption is exemplified by the finding that FLU was able to induce an increase in intracellular calcium levels which was almost completely inhibited by bath application of α -Bgt, d-TC, and Mec. Here, patch clamp and calcium imaging data point to the same conclusion, helping us to clearly confirm that the increase induced by FLU partly comes from the DUM neuron nAChRs as previously found with nicotine and LMA10203 (Taha et al., 2024). It was also interesting to note that in our latter study, we found that current amplitudes and the increase in intracellular calcium evoked by LMA10203 were not significantly blocked by α -Bgt but were with Mec, confirming the involvement of nAChR2 (Taha et al., 2024). Thus, the present results lead us to consider the following observation: the inhibition of nicotine-evoked intracellular calcium increase in the presence of both calmidazolium and α -Bgt could be associated to the unique effect of α -Bgt (Houchat et al., 2022). FLU activates different mechanisms compared to nicotine. During this experiment, we observed that intracellular calcium increase induced by FLU still remains high

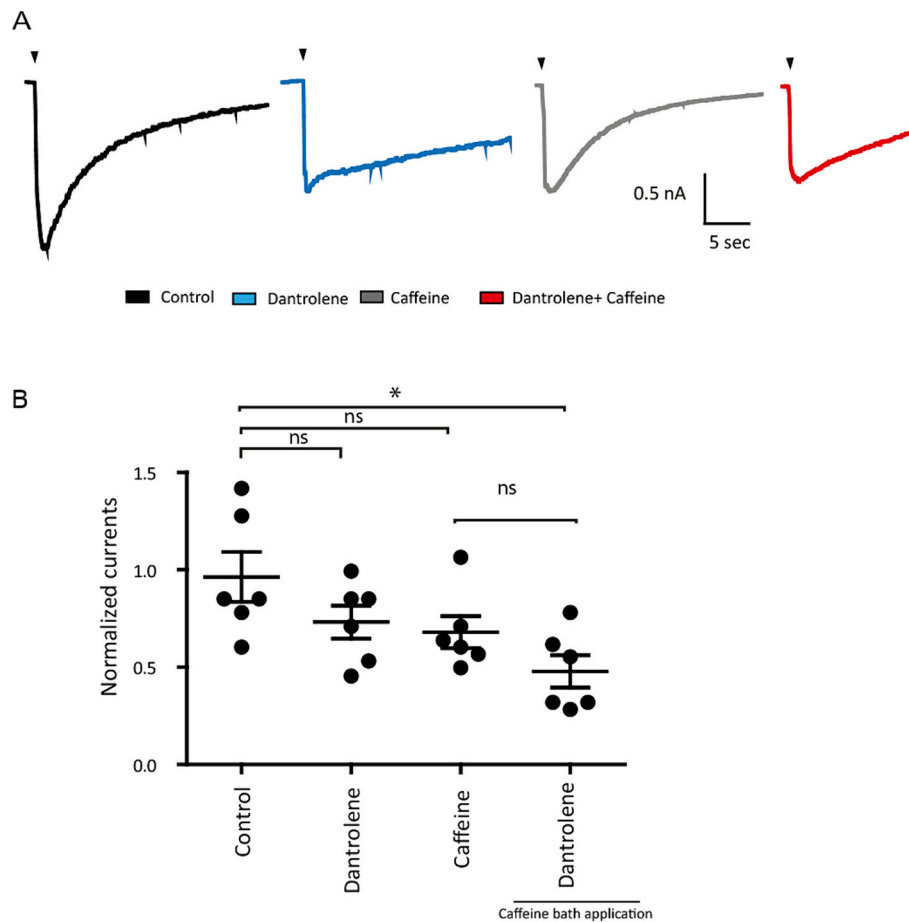


Fig. 6. Effects of intrapipette application of dantrolene and bath application of caffeine on FLU-induced currents. (A) Examples of 1 μ M FLU-induced currents (300 ms, 15 psig) in control conditions, under bath application of 5 mM caffeine, intrapipette application of 100 nM dantrolene, and co-application of caffeine and 100 nM dantrolene. (B) Scatter dot plots summarizing the effect of 1 μ M FLU under control conditions (n = 6 cells), intrapipette application of dantrolene (n = 6), bath application of 5 mM caffeine (n = 6 cells), and co-application of caffeine and 100 nM dantrolene (n = 6 cells). * p < 0.05, Kruskal-Wallis with Holm post-hoc test. ns: no significant. In all cases, arrowheads indicate pulse application (300 ms, 15 psig) of 1 μ M FLU. Currents were recorded at -50 mV holding potential.

after pulse application of FLU, which was not found with nicotine. Our results, clearly show that FLU activates nAChRs but the increase in intracellular calcium concentration is a second mechanisms which involves the ER. The ER is known to be crucial for maintaining intracellular calcium homeostasis in the cells (Berridge, 1998; Guzel et al., 2017). Importantly, we established that while the blocking of IP₃R with 2-APB reduced FLU-induced currents, it did not affect the intracellular calcium increase, indicating a minor role of IP₃R in this process. Indeed, several studies reported that calcium can be mobilized from the ER into the cytoplasm through the IP₃R (Bartok et al., 2019; Tiscione et al., 2021). Generally, IP₃R release calcium from intracellular stores in response to binding of IP₃ and calcium (Foskett et al., 2007; Taylor and Tovey, 2010), and there are three types of IP₃R isoforms which are important for cell survival in mammalian cells (Mendes et al., 2005; Bartok et al., 2019). They are encoded by three distinct genes (Sugawara et al., 1997; Mendes et al., 2005). Interestingly, it was found that IP₃R1, IP₃R2 and IP₃R3 were required for maintaining ER-mitochondrial contacts such as facilitating calcium signal transmission from the ER to the mitochondria (Bartok et al., 2019). However, their role seems to be independent to the calcium fluxes (Bartok et al., 2019). At the present, we don't know if these three IP₃R are expressed in the DUM neurons or how they bind to calcium and IP₃. We can only speculate that IP₃R in our experiment were not involved in intracellular calcium variation which could suggest that signaling pathways involving phospholipase C (PLC) and the regulation of IP₃R are not activated when FLU binds to DUM neuron nAChRs. This calcium pathway involving PLC has been

previously identified in DUM neurons and associated to muscarinic receptors activation (Courjaret et al., 2003). In contrast, blocking the RyRs significantly inhibited both the intracellular calcium rise and the FLU-induced currents. These findings suggest that calcium release from RyRs plays a crucial role in amplifying the FLU-evoked intracellular calcium increase and sustaining the inward currents, highlighting the intricate interplay between calcium signaling and nAChR activity in insect neurons. Nevertheless, we do not suggest that FLU directly activates the release of calcium present in the ER, although it does remain a possibility. Indeed, recent studies demonstrated that diamide insecticides could activate RyR (Ren et al., 2022; Du and Fu, 2023; Liu et al., 2024). It was found that when neurons from the third instar larvae of *Mythimna separata* were treated with evodiamine and chlorantraniliprole insecticides, the peaks in intracellular calcium were elevated (Liu et al., 2024), suggesting that they were able to induce the release of stored Ca^{2+} . In this study, they suspected that the compounds had the capacity to activate IP₃R or RyRs (Liu et al., 2024). In our study, because ryanodine inhibited the FLU-evoked increase in intracellular calcium, we suspected that RyRs promoted the release of free calcium from the ER, leading to the elevation in intracellular calcium. This assumption is also supported by the finding that dantrolene, a ryanodine receptor antagonist, reduced the current amplitudes induced by FLU. The second hypothesis was that FLU interacts with the nAChRs first, promoting enhanced calcium influx to the cell and leading to a secondary effect where the ryanodine receptors are activated through a CICR mechanism. Similar results were obtained in mammalian nAChRs

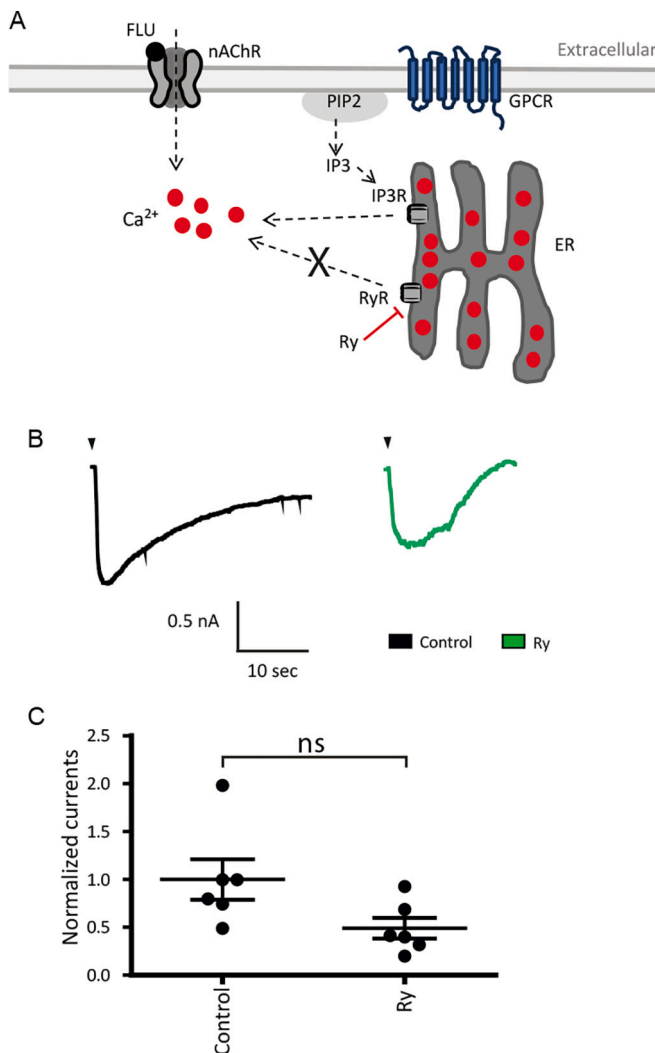


Fig. 7. Effects of ryanodine bath application on FLU-induced currents. (A) Inhibition of ryanodine receptors (RyRs) by ryanodine (Ry). The cross indicated that this pathway is blocked by ryanodine. (B) Examples of FLU-induced currents under control condition and bath application of 20 μ M ryanodine (Ry). (C) Scatter dot plots representing the effect of 1 μ M FLU under control conditions ($n = 6$ cells) and bath application of 20 μ M ryanodine (Ry, $n = 6$ cells, paired t -test, $p = 0.056$, $t = 2.2$, $df = 8$), paired t -test, ns: no significant. In all cases, arrowheads indicate pulse application (300 ms, 15 psig) of 1 μ M FLU. Currents were recorded at -50 mV holding potential.

when the allosteric modulator PNU120596 induced calcium release through RyRs by CICR activation (King et al., 2018; Sinclair and Kabani, 2023).

To conclude, our study provides valuable insights into the mechanisms by which FLU modulates nAChRs in DUM neurons. FLU activates different mechanisms compared to neonicotinoid insecticides. Previous studies demonstrated that clothianidin-evoked current amplitudes were reduced by α -Bgt (Calas-List et al., 2013), and imidacloprid activated α -Bgt-insensitive nAChR1 preferentially (Courjaret and Lapied, 2001). However, for all these compounds, activation of an ionic current through DUM neuron nAChRs is associated to an increase in intracellular calcium, which does not necessarily involve identical signaling pathways. In the present study, the interplay between FLU-evoked nAChR activity and the RyRs is of critical interest because RyRs have been used as an insecticide target to control agricultural pests (Ziviani et al., 2011; Ren et al., 2022). In the management strategy of arthropod vectors of animal and human diseases, including in the agricultural context, it will be interesting to evaluate the synergic effect between

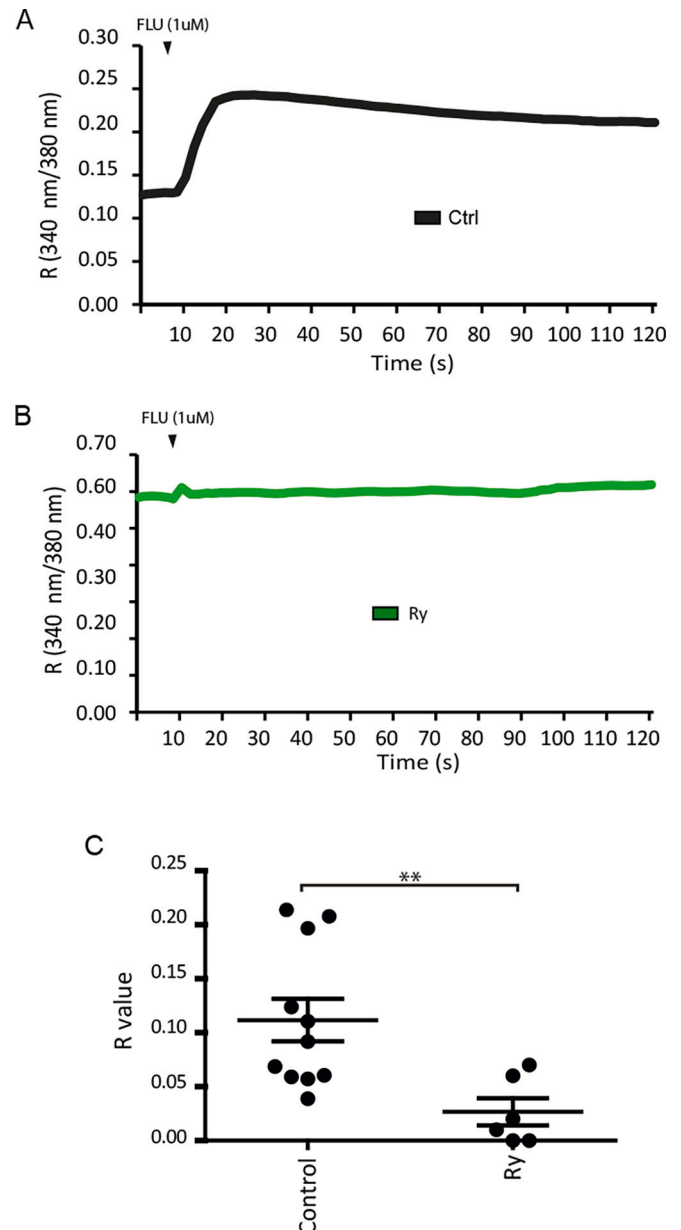


Fig. 8. Effects of ryanodine bath application on the increase in intracellular calcium levels induced by FLU. Pulse application of 1 μ M FLU (Control, Ctrl) evoked an increase in intracellular calcium levels (A). Bath application 20 μ M ryanodine (Ry) almost completely prevented this increase (B). Cells were incubated for 15–30 min with 20 μ M ryanodine. Arrowheads indicate pulse application of 1 μ M FLU (300 ms pulse duration, 15 psig). (C) Scatter dot plots illustrating FLU-induced calcium peak (R value) in control conditions ($n = 11$ cells), and after bath application of ryanodine (Ry, $n = 6$ cells, unpaired t -test, $p = 0.0094$, $t = 2.976$, $df = 15$). $*p < 0.05$.

compounds acting on nAChRs and RyRs. One approach would be to combine neonicotinoids and similar families with diamide insecticides with the aim of reducing the concentrations used and thereby resistance phenomena (Taillebois and Thany, 2022). This strategy would also have the advantage of identifying new alternatives to the insecticides currently on the market, which have demonstrated their effectiveness despite arthropod resistance mechanisms.

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Data statement

Data will be available upon request.

CRedit authorship contribution statement

Maria Taha: Investigation, Formal analysis. **Alison Cartereau:** Validation, Formal analysis. **Emiliane Taillebois:** Writing – original draft, Validation, Formal analysis. **Steeve H. Thany:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

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Conclusion and perspectives

Studying insect nAChRs becomes a matter of high importance since they are the main targets of neonicotinoids, a widely used class of insecticides. Nowadays, neonicotinoids are facing significant challenges due to the increase of insect resistance, in addition to their harmful effects on non-target organisms. Understanding the structure and regulation of insects nAChRs is essential to address these issues and develop more effective and environmentally friendly pest control strategies.

In term of resistance, the first mechanisms identified correspond to metabolic resistance, for example overexpression of CYP450 can cause an over-detoxification of neonicotinoids converting them to less effective compounds (Bass *et al.*, 2015; Nikookar *et al.*, 2019; Puinean *et al.*, 2010; Wang *et al.*, 2022). Resistance by modification of the molecular target is also described in literature. . Indeed, several insects' strains started to developed mutations in specific nAChRs subunits, affecting the overall receptor sensitivity to neonicotinoids (Crossthwaite *et al.*, 2014). Also, modification of subunit expression pattern could lead to variation in nAChR molecular composition and/or stoichiometry, that could imply pharmacological properties variations of the receptors (Chen *et al.*, 2017; Zhang *et al.*, 2015). In addition, changes in the intracellular calcium pathways regulating nAChRs, was linked to insects' resistance by up- or down- regulating nAChRs, modifying their expression levels, and as a result reducing the insecticides efficacy (Bantz *et al.*, 2022; Benzidane *et al.*, 2017) .

In American cockroaches DUM neurons, two types of α -Bgt-insensitive have been identified. nAChR1 and nAChR2. Recently, and using RNA interference (RNAi) techniques, it was found that the Pam α 3, Pam α 8, and Pam β 1 subunits are involved in the composition of nAChR1, whereas the Pam α 1, Pam α 2, and Pam β 1 subunits are part of nAChR2 (Sun *et al.*, 2017). These two receptors exhibit different voltage dependences and pharmacological properties, for example, nAChR1 is sensitive to IMI, while nAChR2 is insensitive. These differences were first attributed to the different subunit's composition of the two receptors (Courjaret *et al.*, 2003; Courjaret *et al.*, 2001). Indeed, chronic exposure to sublethal doses of IMI was found to reduce the participation of nAChR1 while optimizing the functional properties of nAChR2. This occurred alongside increased expression of the α 1 subunit and decreased expression of the α 2 subunit, both of which contribute to nAChR2 formation. One hypothesis suggests that changes in subunit composition might result in conformational states of nAChR2 that make them more resistant to IMI (Bantz *et al.*, 2022; Benzidane *et al.*, 2017). Interestingly, the subunit composition of nAChR1 remained unchanged, yet its sensitivity to IMI was reduced following chronic exposure. Based on previous studies, calcium ions are believed to play a role. One

hypothesis is that chronic exposure to sublethal doses of IMI elevates resting calcium levels, activating Ca^{2+} -dependent pathways, such as the cAMP/AMPK pathway, which has been shown to downregulate IMI-sensitive nAChRs, nAChR1 (Benzidane *et al.*, 2017).

These results were similar to result found in mammals in nicotine addiction. Indeed, studies showed that chronic exposure of nicotine can upregulate nAChRs. However, this upregulation was subtypes sensitive, it means only $\alpha 4\beta 2$ nAChRs, which have high affinity to nicotine, were upregulated by nicotine chronic exposure, while $\alpha 7$ nAChRs, which have low affinity to nicotine, were resistance to nicotine-induced upregulation (Govind *et al.*, 2009; Walsh *et al.*, 2008). Interestingly, studies showed that these two subtypes can be differently regulate by intracellular calcium pathways. Indeed, cAMP analog enhanced nicotine-induced $\alpha 4\beta 2$ nAChRs upregulation and PKC inhibitors prevented this upregulation (Gopalakrishnan *et al.*, 1997). However, no studies about the involvement of these pathways in $\alpha 7$ nAChRs regulation. Concluding that these two nAChRs subtypes are differently regulated by calcium signaling pathways which may explain their distinct responses to prolonged nicotine exposure. Consequently, and with the recent surge in neonicotinoid resistance, researchers have increasingly turned their focus to investigating the specific role of calcium-dependent pathways in regulating insects nAChRs.

Indeed, in insect nAChRs, specifically in DUM neurons of the cockroach *Periplaneta americana*, recent studies have highlighted the complex intracellular pathways involved in the modulation of nAChRs. Previous studies had identified pathways involving protein kinase A (PKA), two distinct protein kinase C (PKC), protein kinase G (PKG), and calcium/calmodulin-dependent protein kinase (CaMK) as critical regulators of nAChRs (Courjaret *et al.*, 2003; Courjaret *et al.*, 2001; Thany *et al.*, 2007). However, the specific mechanisms by which these intracellular regulatory pathways affect nAChRs remain poorly understood, despite their critical role in the pharmacological properties of nAChR, as demonstrated by Courjaret and Lapied, using IMI (Courjaret *et al.*, 2001). In this thesis, we aimed to further investigate the regulation of insect nAChRs by Ca^{2+} dependent intracellular pathways.

In the first part of our work, we identified a new CaM-dependent regulatory pathway involved in the modulation of insect nAChRs: the CaMKK/AMPK pathway.

CaMKK is an extremely abundant protein in mammals' brain and plays a fundamental role in synaptic plasticity and memory formation. Two CaMKK isoforms have been identified, CaMKK1(α) and CaMKK2(β), that are encoded by two separate genes. In mammals, CaMKK2

dysregulation contributes to Alzheimer's disease progression, caused by calcium signaling attribution. CaMKK acts also as a key mediator that translates the Ca^{2+} signals after $\alpha 7$ nAChRs activation into protective cellular responses, helping in reducing the toxicity in the neurodegenerative, amyotrophic lateral sclerosis (ALS) (Axtman, 2021; Ghosh *et al.*, 2015; Ito *et al.*, 2020). AMPK activation through CaMKK2 has already been proven in mammals, particularly in mammals' neurons and T cells. Importantly, AMPK phosphorylation *via* CaMKK2 also play a significant role in tumor suppression lung and cervical cancers (Anderson *et al.*, 2008; Mihaylova *et al.*, 2011; Tamás *et al.*, 2006). Furthermore, in mice macrophages, STO-609 inhibited the activation of AMPK, which implies that AMPK is downstream of CaMKK. Interestingly, CaMKK activation in macrophages was induced by a nicotine binding to nAChRs (Cheng *et al.*, 2007). All these studies in mammals support the hypothesis that CaMKK/AMPK could also have a significant role in insect's nervous system.

In our study we measured nicotinic currents and Ca^{2+} influxes induced by both nicotine and LMA10203, a selective nAChR2 agonist, in the presence of the CaMKK inhibitor, STO-609, and the AMPK inhibitor and activator, DM and A-76, respectively.

Electrophysiological recordings indicate that STO-609 significantly reduces nicotinic currents in the presence of α -Bgt. Furthermore, the co-application of Mec and α -Bgt completely blocks the currents, whereas the co-application of d-TC and α -Bgt has no effect. These results demonstrate that CaMKK is involved in the regulation of nicotinic currents through nAChR2 subtype. We also found that the intrapipette application of A-76, maintained the control currents induced by both nicotine and LMA10203, despite the presence of DM and STO-609. This observation suggests that AMPK is a downstream molecule in the nAChR2 regulatory pathway involving CaMKK.

Since nAChRs have a relatively high calcium permeability, we also used calcium imaging techniques to study the effect of CaMKK inhibition on intracellular calcium increase induced by nicotine and LMA10203. Calcium imaging recordings revealed that CaMKK inhibition by STO prevents the increase in intracellular Ca^{2+} levels induced by NIC, and it was independent on α -Bgt. This finding suggested a complex mechanism regulating the STO effect of nicotine-evoked currents and in intracellular Ca^{2+} , through activation of DUM neuron nAChRs. One obstacle in this study was testing the effects of CaMKK inhibition simultaneously with AMPK activation on calcium influxes, as A-76 is applied intracellularly. Unlike the patch-clamp, this is technically unachievable with the laboratory's imaging device. In addition, a link between

CaMKK/AMPK pathway and other mechanisms like Ca^{2+} release from intracellular stores still need to be investigated.

Our results showed that insect nAChRs in neurosecretory cells can be regulated by at least three different pathways, including CaM/PDE/cAMP, CaM/PaCaMKII-E, and the novel CaMKK/AMPK pathway which involves both α -Bgt-sensitive and -insensitive nAChRs.

Since to date no studies have evaluated a possible crosstalk between the activation of insect nAChRs and calcium release from intracellular stores in insecticides mode of action, in the second part of our experiments, we aimed to investigate for the first time how calcium release from ryanodine (RyRs) and/or inositol trisphosphate receptors (IP₃Rs) can be involved in flupyradifurone (FLU) mode of action on nAChRs expressed in DUM neurons.

Electrophysiological recordings using the patch clamp technique showed that only d-TC and Mec were able to reduce FLU-evoked currents, suggesting that FLU activated α -Bgt-insensitive nAChRs, including both nAChR1 and nAChR2 subtypes, respectively. While calcium imaging recordings indicated FLU was able to induce an increase in intracellular calcium level which was almost completely inhibited by bath application of α -Bgt, d-TC, and Mec, confirming that a part of the increase induced by FLU comes from DUM neuron nAChRs. These results align with our first part of the experiments using nicotine and LMA10203.

Since intracellular Ca^{2+} is known to affect the currents induced by NIC on DUM neurons of cockroach (Campusano *et al.*, 2007; Thany *et al.*, 2008), we investigated the role of calcium release through RyRs and IP₃Rs on FLU induced nAChR activation. IP₃R inhibition reduced FLU-induced currents without affecting intracellular calcium increase, indicating a minor role of IP₃R in this process. In contrast, blocking RyRs significantly inhibited both the intracellular calcium rise and the FLU-induced currents. These findings suggest that calcium release from RyRs plays a crucial role in amplifying the FLU-evoked intracellular calcium increase and sustaining the inward currents, highlighting the intricate interplay between calcium signaling and nAChR activity in insect neurons.

This supports the hypothesis that Ca^{2+} release from ER via RyRs can amplify FLU-evoked Ca^{2+} increase. These results get along with a previous study demonstrates that intracellular Ca^{2+} is an important modulator of the nAChR-mediated inward currents, where NIC-induced currents through nAChR2 were reduced when intracellular Ca^{2+} was chelated by BAPTA (Thany *et al.*, 2008). In addition, a previous study on *Drosophila* Kenyon cells, also showed a reduction on both NIC-induced currents and intracellular calcium increase when cells were incubated with

5 μ M thapsigargin, a concentration used to deplete internal calcium reservoirs in a number of systems including insects (Campusano *et al.*, 2007).

In mammals' brain, a complex relationship between RyRs and nAChRs has been demonstrated. Nicotine-mediated activation of nAChRs leads to RyRs upregulation and enhanced calcium signaling, which in turn influences synaptic plasticity and neuronal function. Furthermore, activation of $\alpha 4\beta 2$ nAChRs by nicotine or ACh results in a prolonged enhancement of glutamatergic input to neurons in the dorsal raphe nucleus. Calcium-induced calcium release (CICR) from presynaptic stores in glutamatergic terminals through RyRs is essential for the nicotine-evoked release of glutamate (Garduño *et al.*, 2012; Ziviani *et al.*, 2011).

However, in our case, more studies are needed to investigate if there is a direct link between FLU and RyRs activation and other calcium ion channels present in the ER. Even though, recent studies demonstrated that diamide insecticides could activate RyR directly (Du *et al.*, 2023; Liu *et al.*, 2024; Ren *et al.*, 2022). In our study, because ryanodine inhibition abolished FLU-evoked increase in intracellular calcium, we suspected that RyR promoted the release of free calcium from the ER, leading to the elevation in intracellular calcium. This assumption is also supported by the finding that dantrolene, a ryanodine receptor antagonist, reduced the current amplitudes induced by FLU.

In summary, our findings revealed the complexity of nAChR regulation in insect neurosecretory cells, that involves multiple intracellular pathways and receptor subtypes. The identification of the CaMKK/AMPK pathway as a key regulatory mechanism opens new avenues for understanding nAChR function and its implications for insecticide resistance.

Our future research should focus on the interactions between CaMKK, AMPK, and other intracellular pathways along with calcium release from ER in regulating nAChRs and exploring the potential roles of other ligand-gated ion channels in modulating nAChR responses.

To conclude, by studying the link between nAChRs regulation and intracellular calcium pathways, new attractive molecular targets for insecticides can be developed, primarily by disrupting the Ca^{2+} homeostasis in insect neurons, and potentially revealing novel strategies to counteract resistance in pest populations. Indeed, Insects rely heavily on a tuned Ca^{2+} homeostasis for most of their physiological processes. Developing new compounds that target $\text{IP}_3\text{Rs/RyRs}$ along with nAChR for example, could overcome resistance issues by attacking multiple critical points simultaneously in the insect nervous system. This shift in research

direction highlights the growing recognition of calcium ions not only as key modulators of nAChRs function but also as pivotal players in the development of resistance to neonicotinoids.

Conclusion en français

Les neurones DUM de la blatte *Periplaneta americana* possèdent deux populations de récepteurs sensibles et insensibles à l' α -bungarotoxine. Dans la population des récepteurs insensibles, les sous-types nAChR1 et nAChR2 présentent des propriétés pharmacologiques et des mécanismes de régulation particuliers. Le sous-type nAChR2 a été décrit comme insensible aux insecticides néonicotinoïdes comme l'imidaclopride. Nous avons postulé que des mécanismes de régulation intracellulaire dépendante du calcium pouvait expliquer l'insensibilité de cette population de récepteurs vis-à-vis des néonicotinoïdes. Au cours de cette thèse nous avons mis en évidence l'existence d'une autre voie de régulation intracellulaire dépendante du calcium qui permet de réguler les récepteurs nicotiniques neuronaux des insectes. Cette voie implique la CaMKK et l'AMPK et une libération du calcium au travers le réticulum endoplasmique. La mise en évidence de cette voie et son implication dans la modulation de l'effet du flupyradifurone montre la complexité des mécanismes intracellulaires dans la modulation de l'effet des insecticides néonicotinoïdes et de leurs dérivés, les buténolides et les sulfoximines.

Cependant, nos travaux sur l'effet du flupyradifurone sont partiels car nous n'avons pas pu tester l'effet d'autres insecticides sur la libération du calcium intracellulaire au niveau du réticulum endoplasmique. Il serait intéressant de voir si les insecticides néonicotinoïdes active de la même manière la libération du calcium intracellulaire comme le flupyradifurone. La connaissance de ces mécanismes de régulation et comment ils contrôlent les propriétés pharmacologiques des récepteurs nicotiniques neuronaux des insectes nous aider à mieux comprendre le mode d'action des insecticides.

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My contribution to the following article entitled: Isolation and electrophysiological recording of *Ixodes ricinus* synganglion neurons

In the current study, our team aimed to investigate the functional and pharmacological properties of nicotinic and muscarinic receptors expressed in tick neuronal cells.

This study serves as a proof of concept for the mechanical isolation of tick synganglion neurons followed by their electrophysiological and calcium imaging recording.

Similarly to my studies on cockroach DUM neurons, we aimed to investigate the involvement of intracellular calcium pathways in the modulation of ticks nAChR and mAChR subtypes, where it was my main contribution.

To achieve this, nAChRs and mAChRs agonists were applied on isolated neurons from hard ticks (*Ixodidae*), and intracellular calcium levels were recorded using the ratiometric fluorescent dye fura-2-AM.

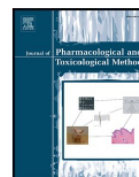
While nicotine induced a reversible increase in intracellular calcium concentrations in *P. americana* DUM neurons (Houchat et al., 2022; Taha et al., 2024a), interestingly, in tick neurons it induced a strong and irreversible increase. In contrast, both muscarine and ACh induced a reversible increase in intracellular calcium levels after pulse application. Thus, both mAChRs and nAChRs could be involved in intracellular calcium modulation and the functioning of the tick synganglion neurons.

This study offers valuable insights into the pharmacological properties of tick neurons, provides a step towards developing effective tick control measures, and improving the necessary tools for the identification of drug-targeted sites in ticks neurons.



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Isolation and electrophysiological recording of *Ixodes ricinus* synganglion neurons

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ABSTRACT

The central nervous system of hard ticks (*Ixodidae*) consists of a concentrated merged nerve mass known as the synganglion. Although knowledge of tick neurobiology has dramatically improved over the last two decades, this is the first time that isolation and electrophysiological recordings have been carried out on tick neurons from the synganglion. **Method:** We developed a simple protocol for synganglion neuron isolation and used a whole-cell patch clamp to measure ionic currents induced by acetylcholine, nicotine and muscarine. Relatively large neurons (~25 µm and ~35 µm) were isolated and 1 mM acetylcholine was used to induce strong inward currents of -0.38 ± 0.1 nA and -1.04 ± 0.1 nA, respectively, with the corresponding cell capacitances being at around 142 pF and 188 pF. In addition, successive application of 1 mM acetylcholine through ~25 µm and ~35 µm cells for increasing amounts of time resulted in a rapid reduction in current amplitudes. We also found that acetylcholine-evoked currents were associated with a reversible increase in intracellular calcium levels for each neuronal type. In contrast, 1 mM muscarine and nicotine induced a strong and non-reversible increase in intracellular calcium levels. This study serves as a proof of concept for the mechanical isolation of tick synganglion neurons followed by their electrophysiological recording. This approach will aid investigations into the pharmacological properties of tick neurons and provides the tools needed for the identification of drug-targeted sites and effective tick control measures.

1. Introduction

Ticks are ectoparasites of major medical importance for human and animal health. The development of effective methods for controlling ticks and tick-borne diseases is now an urgent issue due to the rapid expansion of tick vectors into new zoogeographic areas, caused in part by the current climatic changes (Hvidsten et al., 2015; Jaenson, Jaenson, Eisen, Petersson, & Lindgren, 2012; Leger, Vourc'h, Vial, Chevillon, & McCoy, 2013), and host movement essential to tick dispersal (Tardy et al., 2023). In Europe the tick population is increasing, and there is every reason to believe that ticks, along with the pathogens they carry will become an escalating problem for years to come (Voyiatzaki et al., 2022). Therefore, it remains fundamental to find adequate solutions to limit their spread. In Europe, the tick *Ixodes ricinus*, which is the major vector of borrelia spirochetes and the tick-borne encephalitis virus, is expanding its ranges towards higher latitudes and altitudes (Cunze, Glock, Kochmann, & Klimpel, 2022). Recently the European continent

experienced an invasion of new, mostly North African tick species, which carry new pathogens capable of establishing a tick-host-tick cycle (Jameson, Morgan, Medlock, Watola, & Vaux, 2012; Pascucci et al., 2019). Ticks distinguish themselves from other blood feeding arthropods due to their distinctive parasitic lifestyle, which is driven by unique physiological and behavioural features regulated primarily by the activities of their central nervous system, the synganglion (Lees & Bowman, 2007; Šimo, Daniel, Park, & Žitňan, 2014).

Multiple chemical agents were found to directly or indirectly activate tick salivary gland secretions through synganglion neurons (Kaufman, 1978; Kim, Šimo, & Park, 2014; Sauer, Essenberg, & Bowman, 2000; Sauer, McSwain, Bowman, & Essenberg, 1995). Earlier studies significantly contributed to the understanding of the general anatomy and ultrastructure of the tick synganglion (Obenchain, 1974; Obenchain & Oliver, 1975; Roma et al., 2012). More recent investigations into the tick neuropeptidergic system have allowed in-depth knowledge about the distribution of specific neuronal cells across the synganglion and their

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connections with visceral tissues (Šimo & Park, 2014; Šimo, Slovak, Park, & Zitnan, 2009), and in particular, the connections of the three types of spherical acini (type I, II and III), in the salivary glands (SG) (Mateos-Hernandez et al., 2020; Šimo, Zitnan, & Park, 2009, 2012). In our previous study, we used microtransplanted membranes from the synganglion to demonstrate that it expresses acetylcholine receptors (AChRs) which are sensitive to nicotine and muscarine (Le Mauff et al., 2020). Furthermore, *I. ricinus* synganglion transcriptome analyses identified an exhaustive catalogue of the Cys-loop ligand-gated ion channels, including neuronal nicotinic acetylcholine receptors (nAChRs) (Rispe et al., 2022), which could be targets for new active compounds used as acaricides. In this context, functional and pharmacological identification of native tick receptors through their expression in distinct cells is of critical interest in order to develop new chemical compounds targeting this crucial aspect of their physiology. This will help to promote the development of alternative methods or strategies for tick control.

Here, we present the first implementation and validation of electrophysiological recordings of tick neurons isolated from the synganglion. Acetylcholine (ACh)-, nicotine- and muscarine-evoked current amplitudes were recorded through two different neuronal types. In order to complete our analysis, Fura-2 dye was used to measure calcium increases in these neurons. The proposed assay can be implemented easily in other laboratories to study the pharmacological properties of specific tick neuronal cells.

2. Materials and methods

2.1. Ticks

Experiments were performed using unfed adult female ticks *I. ricinus* which were obtained from the *Ixodes* rearing facility of UMR-BIPAR, Maisons-Alfort, France. We used unfed ticks (5–6 weeks) because the expression of cys-loop gated ion channel genes could be lower in fed ticks (Rispe et al., 2022), and to compare our data with previous study using unfed ticks (Le Mauff et al., 2020). Ticks were maintained in plastic vials containing sterile wood shavings in an incubator (PHCbi, Japan) at >90% relative humidity at 22 °C with a 12 h/12 h light-dark cycle. Before the experiment, ticks were transferred to a glass desiccator containing 4 M MgSO₄ at room temperature.

2.2. Tick dissection and extraction of the synganglion

Unfed *I. ricinus* females were fixed to a Petri dish using double-sided tape, and then observed under a stereomicroscope. An incision on the side of the tick was made with a scalpel followed by a cut using small scissors to remove the entire dorsal cuticle. To avoid tissue desiccation, ticks were covered with a filtered phosphatase buffered-saline (PBS), containing: 137 mM NaCl, 1.45 mM NaH₂PO₄·H₂O, 20.5 mM Na₂HPO₄, pH = 7.2 (328 mOsm). SG, intestines and trachea were removed with forceps to help extract the synganglion, which was incubated for 30 min at 29 °C in a PBS supplemented with collagenase (Type I, 1.5 mg/ml, GIBCO, France). Incubation was done in 2 ml microtubes. For each experiment, we dissected three ticks in order to extract the cells from the corresponding synganglia.

2.3. Neuron isolation

Neurons were isolated from the synganglia using the mechanical dissociation previously described with cockroach dorsal unpaired median (DUM) neurons (Lapied, Malecot, & Pelhate, 1989) and currently used in the laboratory (Houchat, Cartreau, Taillebois, & Thany, 2022; Houchat, Taillebois, & Thany, 2020). The following modifications were added in order to adapt the protocol to tick neurons. The synganglion was rinsed twice in a solution containing: PBS, 50 mM sucrose, 50 U/ml penicillin and 50 µg/ml streptomycin (SIGMA Aldrich, France).

Synganglia were then transferred to the same solution supplemented with fetal calf serum (5% volume, SIGMA Aldrich, France) before being mechanically dissociated by a delicate trituration using fire-polished Pasteur pipettes. Isolated neurons were deposited onto Petri dishes (35/09 mm, NEST, GROSSEON, France) containing a layer of poly-D-lysine hydrobromide (MW, 70,000–150,000, SIGMA Aldrich, France), and incubated overnight at 29 °C to allow them to adhere. For these experiments, all equipment was sterilized with ethanol and all solutions were filtered.

2.4. Electrophysiological recordings

Currents were measured using the patch-clamp technique in the whole-cell configuration with the Axopatch 200B amplifier (Axon Instruments, Forest City, CA). Signals were acquired and digitized using the analog-digital converter MiniDigidata 1440 (Axon Instruments, Forest City, CA) and Axoscope 10.2 software (Axon Instruments, Forest City, CA). Recordings were filtered at 5 kHz using a low-pass filter. The Petri dishes with isolated cells were placed on an inverted microscope (AE 2000, Motic), and the cells were constantly perfused with standard extracellular solution (200 mM NaCl, 3.1 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES, pH = 7.4 adjusted with NaOH) using a gravity perfusion system (403 mOsm). Ejection and patch pipettes were made from borosilicate glass capillary tubes internal diameter 1.15 mm / length 100 mm (HIRSCHMANN, Eberstadt, Germany) using a P-97 puller (Sutter Instrument Company, USA). Patch pipettes were filled with a standard internal solution (160 mM K-D gluconic acid, 10 mM K-fluoride, 10 mM NaCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 20 mM HEPES, 10 mM EGTA, 3 mM ATP, pH = 7.4 adjusted with KOH). The electrode resistance was between 2 and 3 MΩ, and 420 mOsm. The glass micropipette was placed 100 µm from the cell body. Neurons were voltage-clamped at a steady-state holding potential of −50 mV.

2.5. Intracellular calcium recordings

Intracellular calcium levels were recorded using the ratiometric fluorescent dye fura-2-AM (Abcam, Paris, France). The Petri dish containing isolated cells was incubated in the dark (45 min, 37 °C) in the saline supplemented with sucrose (50 mM), Pluronic F-127 (0.1%), and fura-2-AM (10 µM). The Petri dish was mounted on an inverted microscope (IX73, Olympus) installed in a light-tight tissue- and wood-made box. Cells were bathed with standard extracellular solution (See above), using a gravity perfusion system. Pressure ejection of the agonists (15 psig, 300 ms, Medical System Corporation, USA) was carried out using the same glass micropipettes as those used for the patch-clamp. Intracellular calcium levels were measured using a brightfield/340/380 nm LED camera (CoolLED pE-340^{full}) and the *cellSens Dimension* acquisition software (Olympus, Rungis, France).

2.6. Compounds

All compounds were purchased from Sigma Aldrich, France. ACh, nicotine and muscarine were applied with a pneumatic pressure ejection system (Miniframe, Medical System Corporation, USA), at 15 psig.

2.7. Data analyses and statistics

Currents were normalized according to the maximum currents recorded under the same experimental condition. Currents were compared using Student's *t*-test and Prism 9 software (GraphPad Software, San Diego, CA).

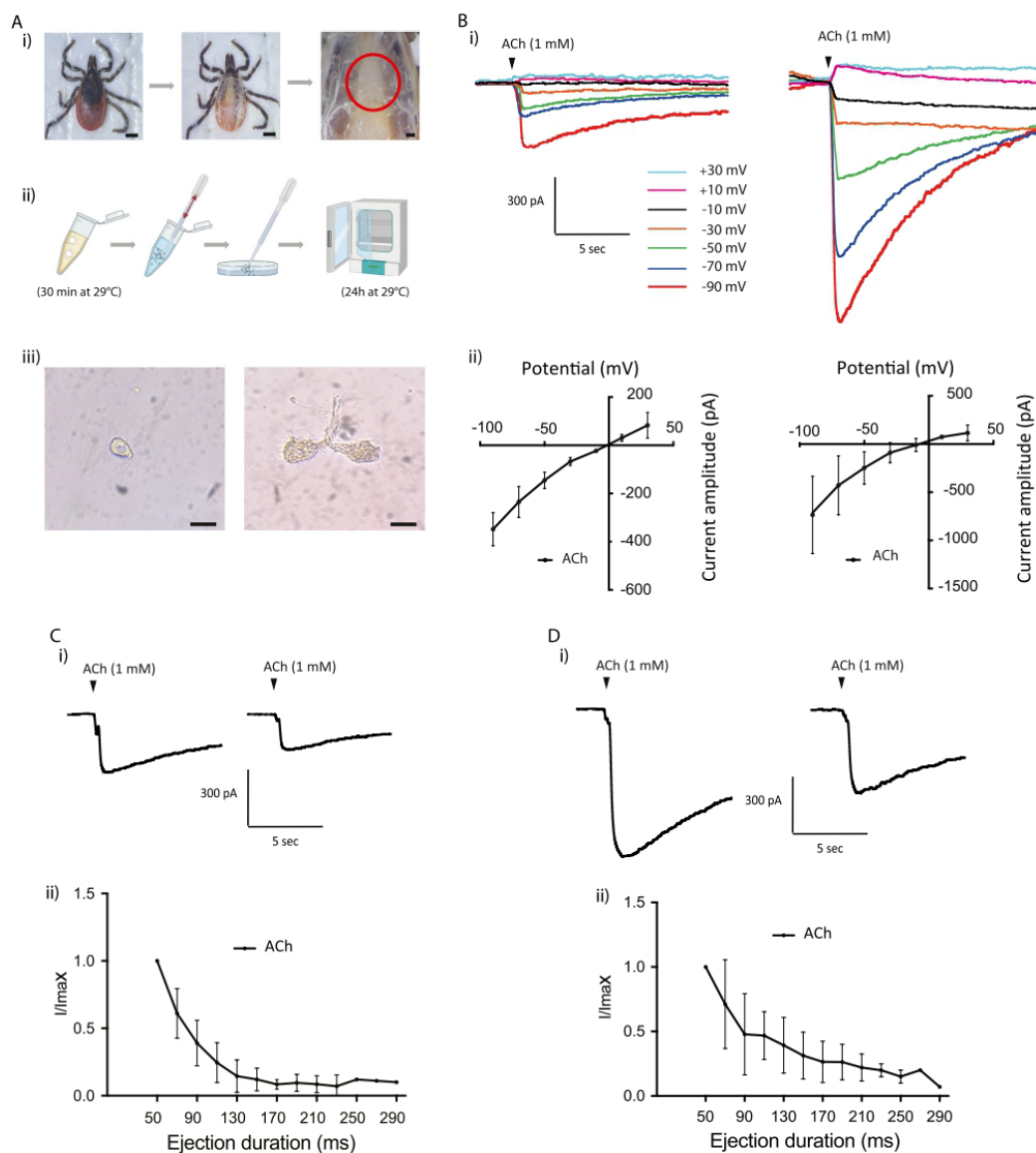


Fig. 1. Different steps for isolating tick neurons from the synganglion, and electrophysiological recordings. (A) Tick dissection and synganglion extraction. (i) The red circle shows the location of the synganglion. (ii) Enzymatic treatment with collagenase. From left to right: 30 min incubation at 29 °C followed by mechanical dissociation using Pasteur pipettes. Neurons were deposited in a Petri dish containing poly-D-lysine for 24 h, incubation at 29 °C. (iii) Isolation of ~25 μm (left panel) and ~35 μm (right panel) cells identified in the Petri dish after mechanical dissociation. The scales indicate 500 μm (i) and 24 μm (iii). (B) Electrophysiological recordings. (i) Typical example of ACh-evoked currents recorded at different potentials, through ~25 μm (left panel) and ~35 μm (right panel) cells. (ii) Current-voltage curve recorded through ~25 μm (left panel) and ~35 μm (right panel) cells. (C and D) Successive applications of 1 mM ACh on ~25 μm and ~35 μm cells. (C) Applications of 1 mM ACh recorded at 5 min intervals (i), successive pulse applications of increasing time ejection (ii) of 1 mM ACh through ~25 μm. (D) Same recordings as in (C) for ~35 μm cells. All currents were recorded at -50 mV holding potential and 300 ms pulse applications (15 psig). $n = 5$. Arrowheads indicate pulse applications of the agonists.

3. Results

3.1. Extraction of tick neurons from the synganglion

Three female ticks were dissected to extract the corresponding synganglia in the PBS (Fig. 1A, i and ii). Based on the different sizes of the neurons, three common cell types were constantly identified in the Petri dish, and had spherical diameters of $\sim 9\ \mu\text{m}$, $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$, respectively. The most abundant cell type obtained were those measuring $\sim 9\ \mu\text{m}$. Due to their size and low number in the synganglion, we focused our attention on the characterization of the $\sim 25\ \mu\text{m}$ (ranging between 20 and $25\ \mu\text{m}$) and $\sim 35\ \mu\text{m}$ (ranging between 30 and $35\ \mu\text{m}$) neurons (Fig. 1A, iii). For each synganglion, only up to six $\sim 25\ \mu\text{m}$ and two $\sim 35\ \mu\text{m}$ neuronal cells were routinely observed in the Petri dish. In the dissociation process the isolated neurons often had a spherical round shape, although occasionally an axonal projection exiting the cell somata was also observed. This particular case was more obvious with the $\sim 35\ \mu\text{m}$ cells (Fig. 1A, iii). It should be noted that we found the neurons to be highly sensitive to the mechanical dissociation, which often led to their loss during the process.

3.2. Electrophysiological recordings of tick neurons

The isolated neurons were recorded using the patch clamp method, 24 h after the mechanical dissociation. We first aimed to identify the presence of acetylcholine (ACh)-evoked current amplitudes in $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neurons. For the $\sim 25\ \mu\text{m}$ neurons, 1 mM pulse applications (300 ms, 15 psig) of ACh induced a mean inward current of $-0.38 \pm 0.1\ \text{nA}$ at $-50\ \text{mV}$ ($n = 7$). Their cell capacitance and membrane resistance values were $141.6 \pm 10\ \text{pF}$ and $97.4 \pm 28\ \text{M}\Omega$. For the $\sim 35\ \mu\text{m}$ neurons we found a cell capacitance and membrane resistance of $187.7 \pm 24.1\ \text{pF}$ ($n = 6$) and $104.6 \pm 44\ \text{M}\Omega$ ($n = 6$), respectively. They were activated by 1 mM ACh leading to inward currents composed of a fast inward current followed by a depolarizing phase (Fig. 1B, i). The ACh induced inward current amplitude was $-1.04 \pm 0.1\ \text{nA}$ at $-50\ \text{mV}$ ($n = 4$). The current-voltage curve recorded from $-90\ \text{mV}$ to $+30\ \text{mV}$ showed the voltage dependence of the ACh currents through $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neurons (Fig. 1B, ii). Interestingly, successive applications of 1 mM ACh induced a decreased response 5 min after the first recording (Fig. 1C and D). In this condition, successive pulse application of 1 mM ACh for increasing amounts of time induced a strong reduction in ACh-evoked currents. This was true for both $\sim 25\ \mu\text{m}$ (Fig. 1C) and $\sim 35\ \mu\text{m}$ neuronal cells (Fig. 1D). ACh-induced currents recorded through $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neurons were found to be significantly different ($p < 0.05$, $n = 6$, Fig. 2A). Interestingly, for $\sim 25\ \mu\text{m}$ neurons, 1 mM nicotine and muscarine induced inward currents of $-0.06 \pm 0.01\ \text{nA}$ and $-0.11 \pm 0.01\ \text{nA}$, respectively ($p > 0.05$, $n = 6$, Fig. 2B). However, currents obtained through $\sim 35\ \mu\text{m}$ neurons were $-0.67 \pm 0.3\ \text{nA}$ and $-0.78 \pm 0.34\ \text{nA}$, respectively ($p > 0.05$, $n = 6$, Fig. 2C). Successive pulse application of nicotine and muscarine did not significantly reduce currents (Fig. 2). These results demonstrated that both $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neuronal cells expressed nAChR and mAChR subtypes. To examine this possibility, we measured the inhibition of ACh and nicotine currents in the presence of methyllycaconitine (MLA), a nAChR antagonist, and atropine, a mAChR antagonist. Current induced through $\sim 35\ \mu\text{m}$ neurons demonstrated that $0.5\ \mu\text{M}$ MLA induced 25% ($p < 0.05$, $n = 4$, Fig. 3A) reduction of ACh currents, and completely inhibited nicotine current amplitudes ($p < 0.05$, $n = 4$, Fig. 3B). $0.5\ \mu\text{M}$ atropine, a mAChR antagonist, reduced muscarine evoked currents to 50% ($p < 0.05$, $n = 4$, Fig. 3C). Cells were incubated with MLA and atropine during 10 min prior to testing the effect of ACh, nicotine and muscarine through $\sim 35\ \mu\text{m}$ neurons.

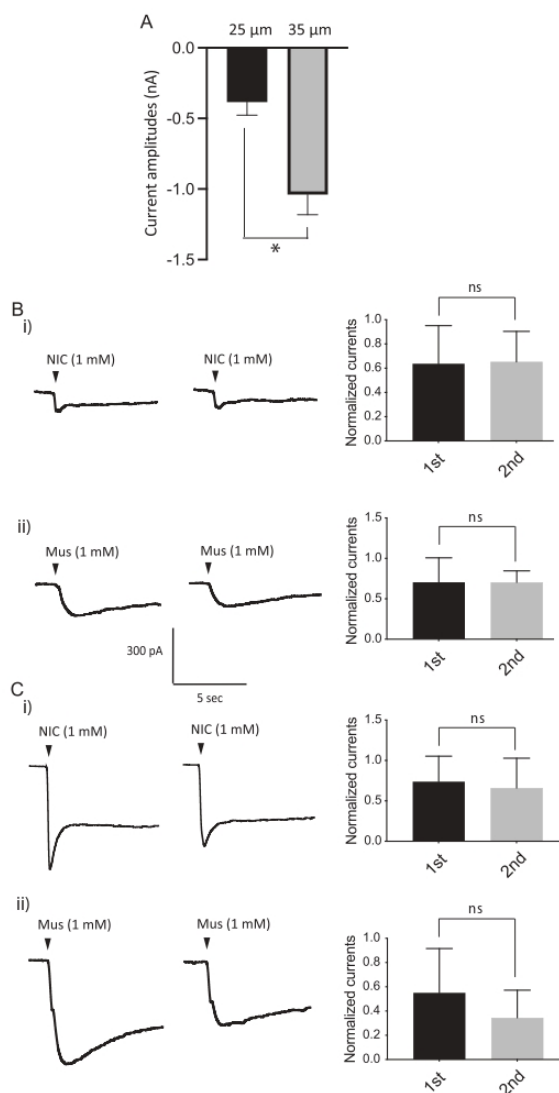


Fig. 2. Currents recorded through $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neurons. (A) Histogram illustrating the current amplitudes recorded through $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ neurons. Each histogram represents $n = 6$ cells, data are mean $\pm$ SEM. * $p < 0.05$. (B, i) Successive applications (1st and 2nd) of 1 mM nicotine and (ii) muscarine, recorded at 5 min interval through $\sim 25\ \mu\text{m}$. (C) Similar recordings with 1 mM nicotine (i) and muscarine (ii) through $\sim 35\ \mu\text{m}$ cells. All currents were recorded at $-50\ \text{mV}$ holding potential and 300 ms pulse applications (15 psig). Arrowheads indicate pulse applications of the agonists. Each histogram represents mean $\pm$ SEM of the current amplitudes. ns: no significant.

3.3. Calcium imaging of tick neurons after pulse application of acetylcholine

Once the electrophysiological recordings were done, we recorded the intracellular calcium variation of the isolated neurons. We aimed to identify whether activation of AChRs through $\sim 25\ \mu\text{m}$ and $\sim 35\ \mu\text{m}$ cells induced changes in intracellular calcium after 300 ms pulse application of 1 mM ACh. Indeed, it had previously been demonstrated that activation of insect AChRs by nicotine or neonicotinoids is associated to an increase in intracellular Ca^{2+} concentration (Houchat et al., 2022; List

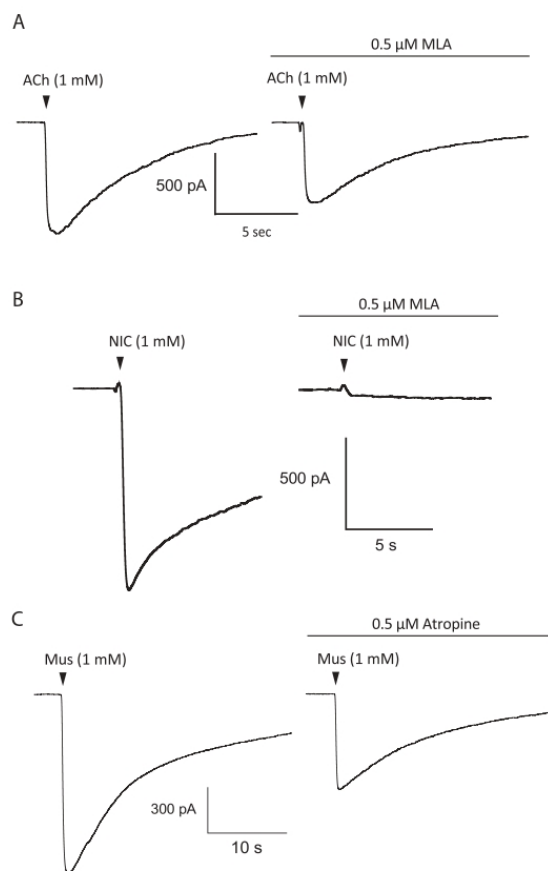


Fig. 3. Effects of acetylcholine receptor antagonists through $\sim 35 \mu\text{m}$ neurons. (A) Effect of $0.5 \mu\text{M}$ methyllycaconitine (MLA) on ACh- and (B) nicotine-induced currents. (C) Effect of $0.5 \mu\text{M}$ atropine on muscarinic currents. MLA and atropine were bath applied for 10 min. Arrowheads indicate pulse applications of 300 ms (15 psig).

et al., 2014). In control conditions, these neurons did not display a spontaneous increase in intracellular calcium levels without the application of an agonist (Fig. 4A). The $\sim 25 \mu\text{m}$ neuronal cells showed an increase in intracellular calcium concentration after pulse applications of 1 mM ACh, with a large peak shape, whereas the $\sim 35 \mu\text{m}$ neuronal cells presented a rapid and strong increase which declined rapidly (Fig. 4B), suggesting that in the addition to their size, the time course in intracellular calcium recordings could be used to differentiate each cell type. Interestingly, application of 1 mM muscarine and nicotine induced a strong and non-reversible increase in intracellular calcium levels through both $\sim 25 \mu\text{m}$ and $\sim 35 \mu\text{m}$ neuronal cells (Fig. 4C and D).

4. Discussion

The synganglion is thought to play a key role in the tick physiology and behavior during both on- and off- host periods (Lees & Bowman, 2007; Mateos-Hernandez et al., 2020; Šimo et al., 2014). Despite these facts, the functional and pharmacological properties of specific tick neurons remains undetermined because as the majority of current investigations focus on visceral tissues which are innervated by axons exiting the synganglion (Lees & Bowman, 2007; Mateos-Hernandez

et al., 2020; Šimo et al., 2014; Šimo & Park, 2014; Šimo, Slovak, et al., 2009; Šimo, Zitnan, & Park, 2009; Vancova et al., 2019). In this study, we optimized the technique previously used for cockroach neurosecretory cells (Houchat et al., 2020; Houchat et al., 2022) to isolate single cell from the synganglion of females *I. ricinus*. We found that both major neuronal types are easily identifiable and can be isolated according to their sizes. Electrophysiological recordings of neurons were performed 24 h after cell dissociation, demonstrating that they can survive and have stable electrophysiological properties, similarly to other arthropod neurons such as cockroach dorsal unpaired median (DUM) neurons (Houchat et al., 2020; Houchat et al., 2022) and mosquitoes *Anopheles gambiae* neurons (Lavialle-Defaix et al., 2011).

All neurons in tick synganglion were believed to be unipolar (Obenchain & Oliver, 1975), and this phenomenon was also observed in our experiments. In ticks, the neurons can be categorized into three types (I, II, and III.) based on their sizes (Šimo et al., 2014). Type I neurons are around ~ 6 to $\sim 9 \mu\text{m}$ in size and possess a pyriform shape. Type I neurons are believed to act as motor or motor/association neurons (Coons, Roshdy, & Axtel, 1974). Type II cells are the largest, measuring from ~ 25 up to $\sim 40 \mu\text{m}$, and found in obvious clusters in the synganglion cortex. These cells are believed to produce neuropeptides, which are released via neurosecretion or axonal transfer to the target tissues. In addition, the cytoplasmic-nuclear ratio of type II neurons is considerably large. Type III tick neurons are relatively small, around $\sim 6 \mu\text{m}$, and are clustered primarily in paired globuli and first pedal ganglion, but their function is currently unknown (Šimo et al., 2014). Although the morphology and size of particular cells could vary during our single neuron extraction process, the neurons in our experiments were considered as type II and thus to be peptidergic neurons. To confirm this assumption, either a single cell transcriptomic analysis, or immunocytochemistry staining using anti-neuropeptide antibodies, will be done in future studies. Furthermore, we speculate that the large $\sim 35 \mu\text{m}$ neurons could be the previously identified single pair of protocerebral peptidergic neurons (PcSG), which innervates tick SG (Mateos-Hernandez et al., 2020; Šimo et al., 2012). The PcSG is a giant pair of cells located on the dorsal protocerebrum just above the spot where the esophagus exits the synganglion, and their axons form a “double cherry” shape (Šimo, Zitnan, & Park, 2009). Interestingly, in some of our neuron dissociation attempts we were able to clearly observe up to two large cells per synganglion with this distinctive shape. Thus, we hypothesized that these cells may be the PcSG neurons identified in previous studies (Šimo, Zitnan, & Park, 2009). Moreover, our electrophysiological recordings showed that the ~ 25 and $\sim 35 \mu\text{m}$ cells did not present the same capacitance values and membrane resistances. The current amplitudes reinforce the finding that they are differently sensitive to ACh. Interestingly, successive pulse applications of the same concentration of ACh induced a decrease in current amplitudes. Similar results were found for muscarine and nicotine, confirming the presence of muscarinic and nicotinic receptors on the same cell, as found in our previous study (Le Mauff et al., 2020). Moreover, increasing the ejection time of ACh did not induce a semilogarithmic dose-response curve, suggesting the presence of desensitizing currents. Indeed, there is a linear relationship between the pressure ejection time and the molar concentration of the compound applied by the pressure ejection system (Bodereau-Dubois et al., 2012). For cockroach DUM neurons, we found that compounds applied in these conditions induced a semilogarithmic dose-response curve (Bodereau-Dubois et al., 2012; Calas-List, List, & Thany, 2012). These results suggested that *I. ricinus* AChRs expressed in the ~ 25 and $\sim 35 \mu\text{m}$ cells were strongly desensitized under pulse application of ACh. The electrophysiological properties of these desensitized receptors remain to be studied, as demonstrated using cockroach thoracic neurons (Salgado, 2016; Salgado & Saar, 2004). Moreover, muscarine-induced inward currents which were reduced by atropine, demonstrating the presence of mAChRs. The kinetics we observed were similar to mAChR currents previously recorded using tick membranes from the synganglion (Le Mauff et al., 2020). The contribution of muscarinic inward

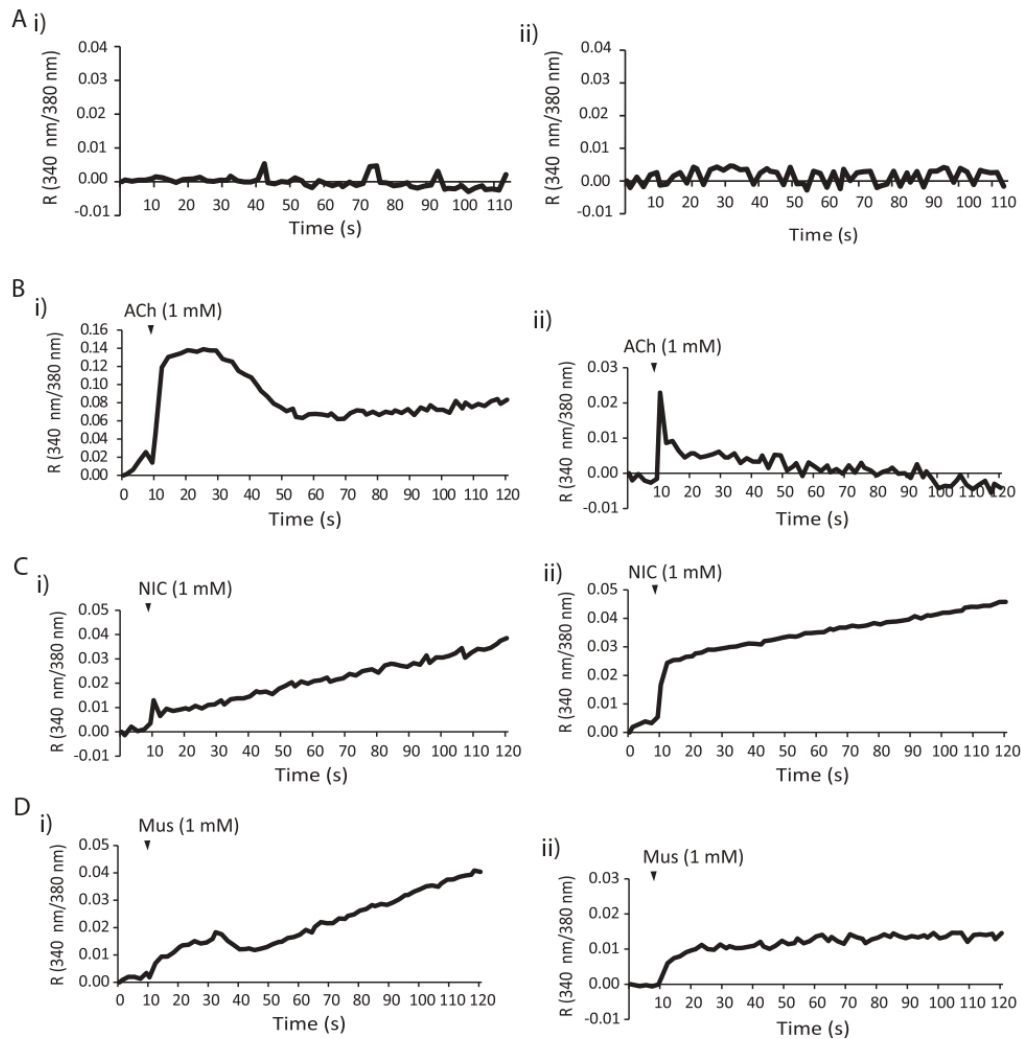


Fig. 4. Effects of pulse applications of 1 mM ACh, nicotine (NIC) and muscarine (Mus) on intracellular calcium levels. (A) control conditions. Cells with Fura2 dye. No spontaneous increase in intracellular calcium levels. (A,i) $\sim 25 \mu\text{m}$ cell and (A,ii) $\sim 35 \mu\text{m}$ cell. (B) 1 mM ACh induced a strong increase in intracellular calcium levels through both $\sim 25 \mu\text{m}$ (i) and $\sim 35 \mu\text{m}$ (ii) cells. The increase was reversible through $\sim 35 \mu\text{m}$ cells (ii). (C and D) 1 mM nicotine (C) and muscarine (D) led to a strong increase which was not reversed after pulse application of the agonists through both $\sim 25 \mu\text{m}$ (i) and $\sim 35 \mu\text{m}$ (ii) cells. Arrowheads indicate pulse application (300 ms, 15 psig) of each compound.

currents to the electrophysiological properties of $\sim 25 \mu\text{m}$ and $\sim 35 \mu\text{m}$ cells suggest the activation of ion channels, as found in several studies (Bronson & Kalluri, 2023; Li et al., 2022; Zhang et al., 2007). We hypothesize that tick mAChRs could contribute to the modulatory function of ~ 25 and $\sim 35 \mu\text{m}$ neurons which could influence the synaptic events in the tick SG.

Intracellular calcium recordings also suggested that the activation of tick AChRs induces a specific increase in calcium, which differs from observations made on insects. Indeed, pulse application of nicotine on cockroach DUM neurons induced a reversible increase in intracellular calcium concentrations (Houchat et al., 2022; List et al., 2014), whereas it induced a strong and irreversible increase in tick neurons. A similar result was found for muscarine, although ACh induced an increase in intracellular calcium which was reversed after pulse application. We

speculate that intracellular calcium pathways may be involved in the modulation of ticks nAChR and mAChR subtypes, as found with the mosquito *Anopheles gambiae* (Perrier et al., 2021) and cockroach *Periplaneta americana* (Houchat et al., 2020; Houchat et al., 2022; List et al., 2014; Mannai, Bitri, & Thany, 2016). In the cockroach, intracellular calcium plays a fundamental role in DUM neurons. In particular, it maintains pacemaker activity and hormone secretion (Heine & Wicher, 1998). We speculate that the $\sim 35 \mu\text{m}$ neurons could be the previously identified single pair of protocerebral peptidergic neurons, which innervates tick SG (Mateos-Hernandez et al., 2020). Thus, mAChRs and nAChRs could be involved in intracellular calcium modulation and the functioning of the tick SG.

5. Conclusion

The current study investigates the functional and pharmacological properties of nicotinic and muscarinic receptors expressed in tick neuronal cells. This pioneer approach is expected to be a stepping stone for helping to identify the distinct roles of different tick neurons, as well as contributing all together to general understanding of synganglion functioning. Subsequently, the in-dept knowledge regarding the tick central nervous system should help to uncover vulnerable spots in their physiology, in order to generate new and effective control of these medically important arthropods.

Credit authorship contribution statement

Khalid Boussaine: Conceptualization, investigation, writing - original draft. **Maria Taha:** Conceptualization, investigation, writing - original draft. **Cáinà Ning:** Methodology, Ressources, Writing - original draft. **Sabine Rakotobe:** Methodology, Ressources. **Lourdes Mateos-Hernandez:** Methodology, Ressources. **Emiliane Taillebois:** Methodology, Supervision, Validation, Writing - original draft. **Ladislav Simo:** Funding acquisition, Methodology, Writing - original draft. **Steeve H. Thany:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Maria TAHA

Rôle des voies de régulation intracellulaire calcium- dépendante dans la modulation des récepteurs cholinergiques de type nicotinique chez les insectes

Ces dernières années, l'utilisation des pesticides a évolué rapidement. Ils sont aujourd'hui considérés comme l'une des clés de voûte de l'agriculture moderne. Leur importance vient de leur rôle dans la protection des cultures contre une variété de ravageurs et de maladies d'origine fongique, végétale ou animale. Cependant, de nombreuses controverses concernant l'utilisation de certains pesticides, notamment les néonicotinoïdes, sont apparues ces dernières années en raison de leurs effets nocifs potentiels sur les pollinisateurs, en particulier les abeilles mellifères. Par exemple, l'utilisation des néonicotinoïdes a été liée au déclin des populations d'abeilles mellifères, ce qui a suscité de nombreuses inquiétudes quant à leur impact sur l'environnement. En réponse à ces préoccupations, l'Union européenne a interdit en 2018 l'utilisation de trois néonicotinoïdes : la clothianidine, l'imidaclopride et le thiaméthoxame. Cependant, cette mesure a affecté de nombreux agriculteurs qui comptent sur ces produits pour protéger leurs cultures contre les ravageurs et les maladies. En l'absence d'alternative efficace, l'interdiction des néonicotinoïdes pourrait entraîner une baisse des rendements agricoles et des pertes économiques. Par conséquent, la société agricole est désormais confrontée à un défi de taille : trouver de nouveaux insecticides efficaces qui n'ont aucun effet sur les organismes non ciblés. Cela permettra d'assurer un équilibre entre la productivité agricole et la conservation de la biodiversité. La principale source de ces défis provient de notre mauvaise compréhension du mode d'action des néonicotinoïdes et d'autres produits utilisés. Pour surmonter ces difficultés, les chercheurs étudient désormais les mécanismes moléculaires qui sous-tendent les effets des insecticides sur les espèces cibles et non cibles. Par exemple, l'étude des récepteurs nicotiniques de l'acétylcholine (nAChRs), cibles principales des néonicotinoïdes, est cruciale pour comprendre à la fois le mode d'action de ces composés et les mécanismes de résistance des insectes à ces derniers. Dans cette thèse, notre objectif était d'étudier comment les voies calciques intracellulaires régulant les nAChRs peuvent modifier les effets de divers agonistes sur ces récepteurs. En particulier, nous avons étudié le lien entre les voies calciques et le mode d'action de la nicotine et d'autres insecticides sur les nAChRs sensibles et insensibles à l' α -Bgt (type 1 et 2) dans les neurones de la blatte américaine. Mots clés : Récepteurs nicotiniques, néonicotinoïdes, électrophysiologie, insectes, signalisation cellulaire, Imagerie calcique.

The role of intracellular calcium regulatory pathways in the modulation of insects' nAChRs

In recent years, the use of pesticides has evolved rapidly. They are considered now a main keystone in modern agriculture. Their importance comes from their roles in crops protection from a variety of pests and diseases of fungal, plant, or animal origins. However, many controversies surrounding the use of certain pesticides, including neonicotinoids, have emerged in the last few years due to their potentially harmful effects on pollinators, particularly honeybees. For example, the use of neonicotinoids has been linked to declines in honeybees' populations, raising many concerns about their impact on the environment. As a response to these concerns, in 2018, the European Union banned the use of three neonicotinoids: clothianidin, imidacloprid, and thiamethoxam. However, this action affected many farmers who rely on these products to protect their crops from pests and diseases. Without any effective alternative, neonicotinoids ban could lead to reduced crop yields and economic losses. As a result, the agriculture society is now facing a serious challenge of finding new effective insecticides without any effect on the non-target organisms. This will ensure a balance between agricultural productivity and biodiversity conservation. The main source of these challenges comes from our poor understanding of the mode of action of neonicotinoids and other used products. Thus, to overcome these challenges, researchers are now studying the molecular mechanisms underlying the effects of insecticides on both target and non-target species. For example, studying nicotinic acetylcholine receptors (nAChRs), the main targets of neonicotinoids is crucial to understand both the mode of action of these compounds and insects' resistance mechanisms to them. In this thesis, our goal was to investigate how intracellular calcium pathways regulating nAChRs can modify the effects of various agonists on these receptors. In particular, we studied the link between calcium pathways and the mode of action of nicotine and other insecticides on α -Bgt-sensitive and -insensitive nAChRs (type1 and 2) in American cockroaches' neurons. Keywords: Insects, nicotinic receptors, intracellular regulation, neonicotinoids, patch clamp, calcium imaging.

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