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Different efficiency of auxiliary/chaperone proteins to promote the functional reconstitution of honeybee glutamate and acetylcholine receptors in *Xenopus laevis* oocytes

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Abstract

Heterologous expression systems (e.g., *Xenopus laevis* oocytes) are useful to study the biophysical properties and pharmacology of ionotropic receptors such as ionotropic glutamate (iGLuRs) and nicotinic acetylcholine (nAChRs) receptors. However, insect receptors often require the co-expression of chaperone proteins to be functional. Only few iGluRs and nAChRs have been successfully expressed in such systems. Here, we compared the efficiency of chaperone proteins to promote the functional expression of one *Apis mellifera* iGluR and several nAChR subunit combinations (α1α8β1, α7, α2α8β1 and α2α7α8β1) in Xenopus oocytes. To this end, we cloned a new iGluR (GluR-1) and potential chaperone proteins (e.g., SOL-1, Neto, NACHO) and tested more than 40 combinations of human, nematode and honeybee proteins. We obtained robust expression of GluR-1 and α1α8β1 when co-expressed with honeybee chaperone proteins and found that nAChR expression critically depended on the α1 subunit N-terminal sequence. We recorded small ACh-gated currents in few oocytes when the α7 subunit was co-expressed with *Caenorhabditis elegans* RIC-3, but none of the chaperone proteins allowed efficient expression of α2α8β1 or α2α7α8β1. Our results show that only some protein combinations can reconstitute functional receptors in Xenopus oocytes and that protein combination efficient in one species is not always efficient in another species.

KEYWORDS
chaperone proteins, honeybee, ionotropic glutamate receptors, nicotinic acetylcholine receptors, *Xenopus laevis* oocytes

INTRODUCTION

Glutamate (Glu) and acetylcholine (ACh) are two major neurotransmitters in mammals and insects that can bind to ligand-gated ion channels known as ionotropic Glu receptors (iGluRs) and nicotinic ACh receptors (nAChRs), respectively. Mammalian genomes encode 18 iGluR subunits and 17 nAChR subunits that assemble as homo- or hetero-tetramers and -pentamers, respectively. Contrary to their mammalian counterparts (Hansen et al., 2021), little is known about the precise stoichiometry of native insect receptors. Indeed, functional reconstitution of insect receptors in heterologous system is often problematic and requires the co-expression of auxiliary/chaperone proteins with the target receptor, for instance, STG-1, SOL-1 and Neto for iGluRs (Han et al., 2015; Walker et al., 2006,

2006), and RIC-3, UNC50 and UNC74 for nAChRs (Ihara et al., 2020). Most of these proteins were discovered by functional or genetic screening in *Caenorhabditis elegans* as required for proper maturation of membrane receptors. The *C. elegans* GLR-1 is closely related to the mammalian AMPA-type iGluR receptors (Brockie & Maricq, 2003) and does not elicit Glu-gated currents when expressed alone in *X. laevis* oocytes. However, the co-expression of *C. elegans* STG-1 and SOL-1 facilitates the reconstitution of functional GLR-1 receptors (Walker et al., 2006). Interestingly, *C. elegans* STG-1 and SOL-1 can be replaced by *Drosophila melanogaster* orthologues (Walker et al., 2006; Walker et al., 2006). On the other hand, *D. melanogaster* GluR-1, that is closely related to *C. elegans* GLR-1, does not elicit Glu-gated currents in *X. laevis* oocytes when expressed alone and also when expressed with *C. elegans* STG-1 and SOL-1 but only when co-expressed with *D. melanogaster* STG-1 (Walker et al., 2006). Moreover, mammalian but not *C. elegans* Neto greatly increases rat GluK2 currents (Wang et al., 2012) and *D. melanogaster* Neto hugely increases the Glu-gated currents recorded in *X. laevis* oocytes that express the *D. melanogaster* GluRII subunits from the neuromuscular junction glutamate receptors (Han et al., 2015). However, it is not known whether honeybee iGluR subunits can reconstitute functional Glu-gated receptors in *X. laevis* oocytes on their own or upon co-expression of chaperone proteins.

As for iGluRs, only some nAChR subunit combinations, such as mammalian α4β2 and α3β4, produce robust expression in *X. laevis* oocytes, while others such as α7 and α6β2, or *C. elegans* DEG3/DES2 and ACR-16, yield low expression when expressed alone (see, for examples, Bennett et al., 2012, and Halevi et al., 2003). Both human and nematode RIC-3 increase ACh-gated currents in *X. laevis* oocytes injected with mammalian α7 or with *C. elegans* DEG3/DES2 or ACR-16 (Castillo et al., 2005; Halevi et al., 2002, 2003; Williams et al., 2005). Except in very few examples (Cartereau et al., 2020), insect nAChR subunits do not produce functional receptors when expressed alone in *X. laevis* oocytes and RIC-3 improves the expression of *D. melanogaster* α7, α5α6, α5α7 and α5α6α7 but not α1β1 (Ihara et al., 2020; Lansdell et al., 2012; Watson et al., 2010), nor cockroach α7 (Cartereau et al., 2020). Besides RIC-3, a levamisole-sensitive AChR from *C. elegans* requires two additional proteins to yield robust expression in *X. laevis* oocyte: UNC50 and UNC74 (Boulin et al., 2008). *D. melanogaster* UNC74 (also called TMX-3) is sufficient to promote functional expression of the *D. melanogaster* α1β1, although higher expression is obtained by combining UNC74 with UNC50 and RIC-3 (Ihara et al., 2020). These three proteins from *A. mellifera* or *Bombus terrestris* improve also expression of α1α8β1 from the same species but not the expression of α1β1 (Ihara et al., 2020). Finally, human or *D. melanogaster* NACHO promote functional expression of mammalian α7 and α4β2 in HEK cells, and human NACHO can synergize with RIC-3 for α7 expression (Gu et al., 2016). However, little is known about the effects of NACHO on insect nAChR expression in *X. laevis* oocytes.

Heterologous expression failure deprives us of a valuable mean for developing biochemical or pharmacological tools aimed at elucidating the role played by the various iGluR and nAChR subunits in insect physiology. In the present study, we therefore tried to express honeybee iGluRs and nAChRs with different combinations of human, nematode and honeybee chaperone proteins in *X. laevis* oocytes. We obtained robust expression of the honeybee GluR-1 receptor when it was co-expressed with chaperone proteins from the same species. We found that the expression of the α1α8β1 nAChR subunits with chaperone proteins critically depended on a specific sequence in the α1 *N* terminus. We did not obtain efficient expression for honeybee α2α8β1 or α2α7α8β1 with any tested chaperone proteins, although these subunits are known to be expressed in antennal lobe neurons (Dupuis et al., 2011). Finally, we recorded small ACh-gated currents when we co-expressed the *A. mellifera* α7 subunit with *C. elegans* RIC-3, but not with honeybee chaperone proteins or with human chaperone proteins NACHO and RIC-3. Our results therefore highlight that the role of chaperone proteins in the functional reconstitution of iGluRs and nAChRs is species-specific and suggest that a genome-wide screening will probably be needed to uncover chaperone proteins suitable for functional reconstitution of specific insect receptors.

RESULTS AND DISCUSSION

Molecular cloning of *A. mellifera* GluR-1 and its chaperone proteins

In the honeybee genome, we identified the *sol-1* and *Neto* genes that encoded proteins harbouring the same functional domains as their homologues in *C. elegans* (Figure 1). Honeybee SOL-1 contained four CUB domains involved in protein–protein interactions and a single transmembrane segment and shared 28% and 25% sequence identity with *D. melanogaster* and *C. elegans* SOL-1, respectively. Honeybee Neto harboured one LDLa and two CUB domains and a single transmembrane segment and shared 17% and 28% sequence identity with *C. elegans* and *D. melanogaster* Neto (β isoform), respectively. We cloned *A. mellifera GluR-1*, the single gene in the honeybee genome with a domain typical of AMPA-type receptors. This cDNA encoded a protein of 910 amino acids with 34% and 59% of identity with its *C. elegans* and *D. melanogaster* homologues, respectively.

Molecular cloning of *A. mellifera* nAChR chaperone proteins

We cloned several *A. mellifera* RIC-3 variants (Figure 2): *ric-3A*, *3B* and *3D* that produced proteins of 455, 337 and 466 amino acids, respectively, which were similar to predicted sequences in GenBank® (XP_026301952, XP_026301954 and XP_026301953, respectively); and *RIC-3c* and *3E* that produced proteins of 248 and 210 amino acids, respectively, which lacked most of the C-terminus compared with the three previous isoforms. Multiple transcripts have been found also in fruit fly and human (Lansdell et al., 2008; Seredenina et al., 2008). *A. mellifera* RIC-3A shared 13% amino acids identity with *C. elegans* RIC-3 and 20% with the *D. melanogaster* variant RIC-3[6,7,9].

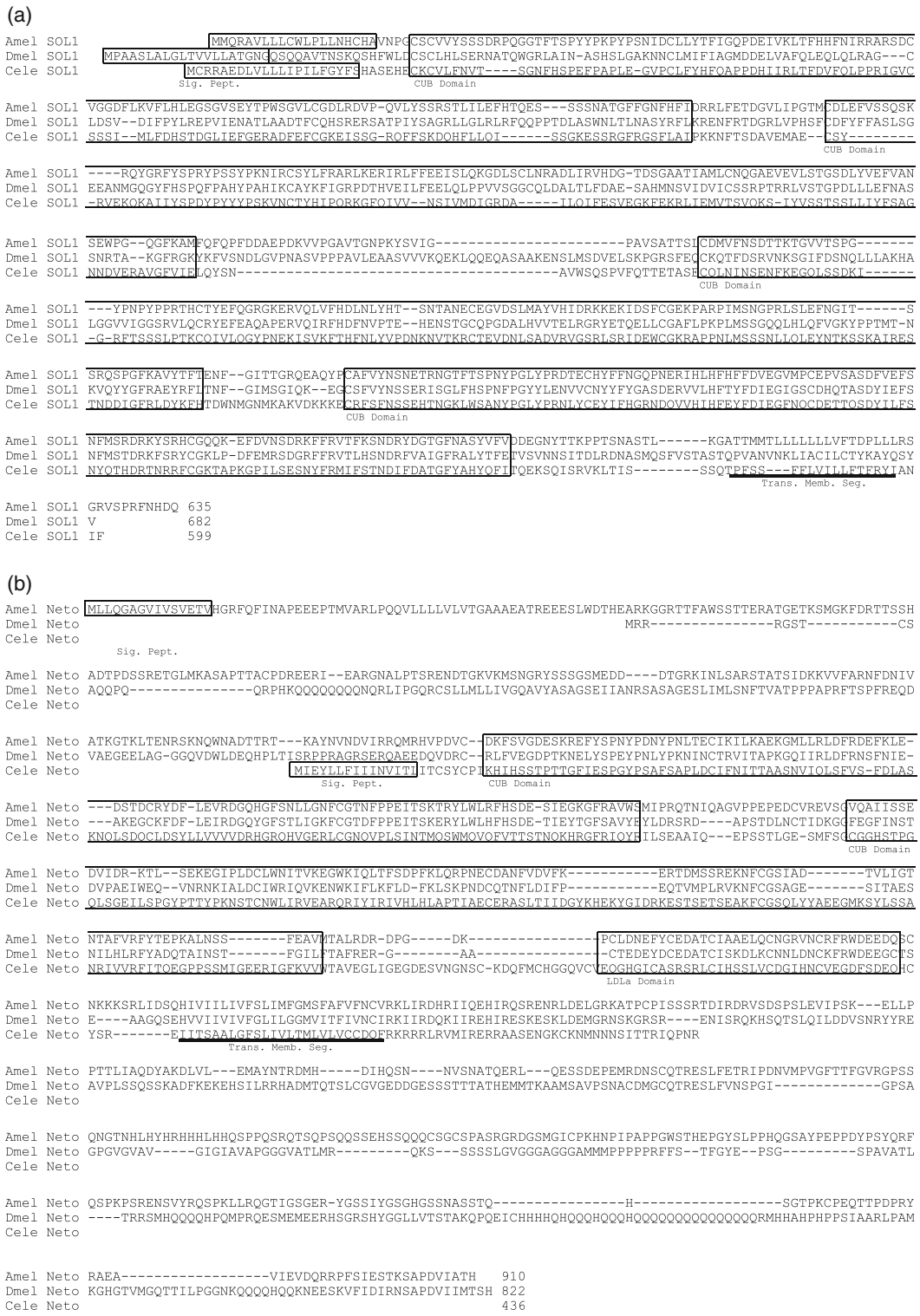

FIGURE 1 Amino acid sequences of *A. mellifera* SOL-1 (a), Neto (b), and GluR-1 (c). (a) *A. mellifera* (MZ198226), *D. melanogaster* (AAY81927), and *C. elegans* (MW021443) SOL-1 sequence alignment. (b) *A. mellifera* (MW021438), *D. melanogaster* (NP_001285211), and *C. elegans* (MW021439) Neto sequence alignment. (c) *A. mellifera* GluR-1 (MW021431), *D melanogaster* GluR-1 (NP_476855) and *C. elegans* GLR-1 (NP_498887) sequence alignment. The domains identified in the sequences are boxed. *CUB domain*: PF00431/IPR000859 (complement C1r/C1s, Uegf, Bmp1 domain); *LDLa domain*: PF00057/IPR002172 (Low-density lipoprotein receptor class A repeat domain); *PBP1-iGlu-AMPA*: cd06387 (N-terminal leucine–isoleucine–valine binding protein [LIVBP]-like domain of the GluR3 subunit of the AMPA receptor); and *PBP2-iGlu-AMPA*: cd13715 (ligand-binding domain of the AMPA subtypes of ionotropic glutamate receptors). *Sig. Pept.*: Signal peptide, *Trans. Memb. Seg.*: transmembrane segment.

FIGURE 1 (Continued)

The five *A. mellifera* variants contained a "*RIC-3 Domain*" and two hydrophobic segments. Some *D. melanogaster* RIC-3 variants included a predicted coiled-coil domain (Lansdell et al., 2008) and sequence analysis predicted a coiled-coil domain in the *A. mellifera* RIC-3C and D isoforms. *A. mellifera unc50* and *unc74* cDNAs encoded proteins of 268 and 432 amino acids, respectively. They included the same functional domains and shared 44% and 55%, and 30% and 49% sequence identity with their *C. elegans* and *D. melanogaster* orthologues, respectively. Finally, we identified in the honeybee genome a sequence close to human NACHO that harboured similar domains and shared 34% sequence identity.

Functional reconstitution of *A. mellifera* GluR-1 in *X. laevis* oocytes

As expected, the co-expression of the three *C. elegans* chaperone proteins STG-1, SOL-1 and Neto allowed recording of Glu-gated currents in most oocytes injected with GLR-1 cRNA (Figure 3a and Table 1). Surprisingly, we could record small Glu-induced current in 22% of the tested oocytes after injection of *A. mellifera* GluR-1 cRNA alone, and the mean current amplitude significantly increased, but remained small, when we co-expressed the three *C. elegans* chaperone proteins STG-1, SOL-1 and Neto. However, when GluR-1 was co-expressed with the three chaperone proteins from honeybee, we could measure Glu-induced currents in almost 100% of the tested oocytes, and the mean current amplitude was strikingly increased. Conversely, the three *A. mellifera* chaperone proteins were less efficient when co-expressed with GLR-1 because only 32% of the tested oocytes displayed Glu-induced currents.

We next wanted to determine the role of each individual honeybee chaperone protein (Figure 3b). We did not obtain any functional receptor when GluR-1 was co-expressed with Neto alone or with both Neto + SOL-1, and we recorded small currents in few oocytes when GluR-1 was co-expressed with SOL-1. However, the current amplitudes measured with GluR-1 alone and with GluR-1 + SOL-1

FIGURE 2 Amino acid sequences of *A. mellifera* RIC-3A (a), UNC50 (b), UNC74 (c) and NACHO (d). (a) Top, schematic representation of the alternative splice *A. mellifera* RIC-3 variants. Bottom, *A. mellifera* RIC-3A (MW021471), *D. melanogaster* RIC-3[6,7,9] (CAP16647) and *C. elegans* RIC-3 (MW021435) sequence alignment. (b) *A. mellifera* (KJ939605), *D. melanogaster* (NP_649813) and *C. elegans* (MW021436) UNC50 sequence alignment. (c) *A. mellifera* (KJ939606), *D. melanogaster* (NP_648847) and *C. elegans* (MW021437) UNC74 sequence alignment. (d) *A. mellifera* (MW021432) and human NACHO (MW021434) sequence alignment. The domains identified in the sequences are boxed. *RIC-3 domain*: PF15361/IPR032763 (resistance to inhibitors of cholinesterase homologue 3 domain); *UNC 50 domain*: PF05216/IPR007881; *Thioredoxin domain*: PF00085/IPR013766; and *Thioredoxin 6 domain*: PF13848; *DxoX-2 domain*: PF13564/IPR032808. *ER Sig.*: endoplasmic reticulum retention signal (KXD/E and KXKXX in invertebrates and vertebrates, respectively), *Sig. Pept.*: signal peptide, *Trans. Memb. Seg.*: transmembrane segment.

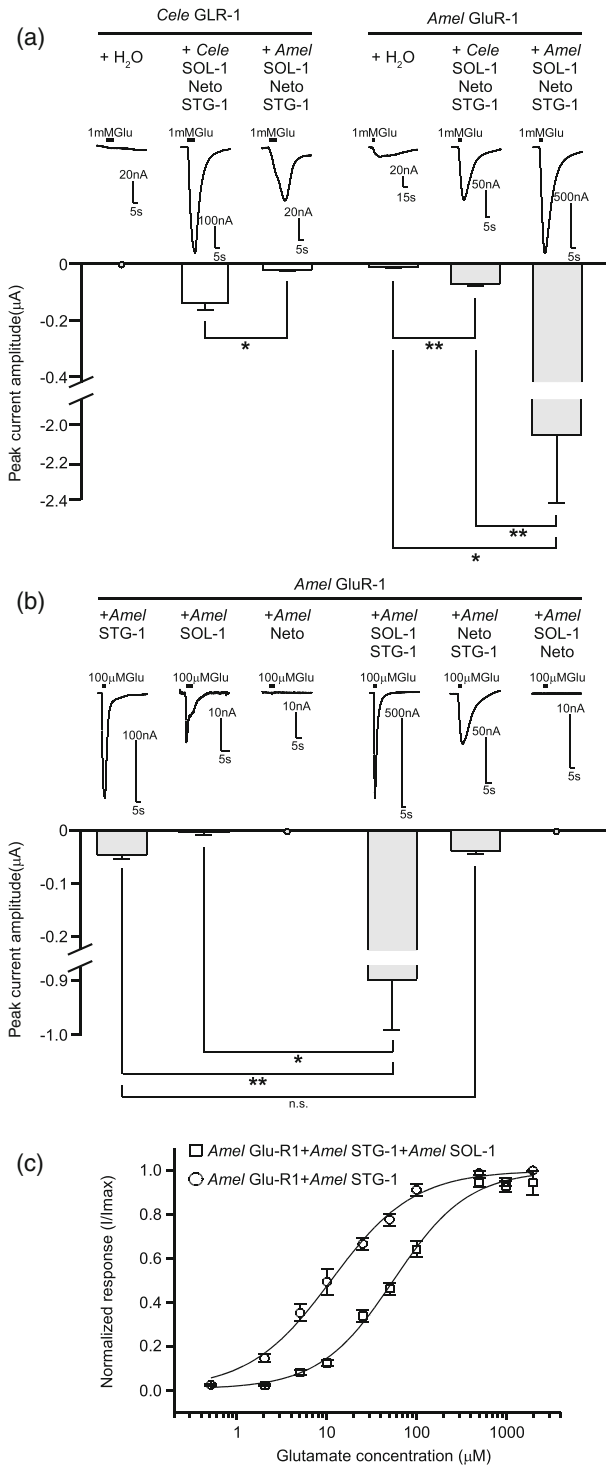

FIGURE 3 Functional reconstitution of *A. mellifera* GluR-1 in *X. laevis* oocytes. (a) and (b) top, representative current traces recorded in *X. laevis* oocytes that express *C. elegans* GLR-1 or *A. mellifera* GluR-1 without ($+H_2O$) or with the indicated chaperone proteins. Bottom, mean current amplitude for the different protein combinations. Bars represent the mean ± SEM of $n = 6–70$ oocytes. Values are given in Table 1. * $p < 0.01$, ** $p < 0.001$. The line above each trace illustrates the duration of agonist incubation.
(c) Concentration–response plots for glutamate-induced currents for *Amel* GluR-1 + *Amel* STG-1 with or without *Amel* SOL-1 fitted with the Hill equation. The data are the means ± SEM of $n = 15–18$ ovocytes. Glutamate $EC_{50} = 14 \pm 3$ and 61 ± 7 μM and nHill = 1.0 ± 0.1 and 1.1 ± 0.1 without and with *Amel* SOL-1, respectively

were similar, suggesting that SOL-1 did not improve the functional reconstitution of the receptor (Table 1). When GluR-1 was co-expressed with STG-1, 90% of injected oocytes produced functional receptors, in agreement with previous results obtained with the *D. melanogaster* GluR-1 homologue (Walker et al., 2006). Moreover, larger currents were detected when GluR-1 was co-expressed with STG-1 + SOL-1, but not with STG-1 + Neto, suggesting a lack of effect of Neto. Dose–response curves (Figure 3c) revealed that the EC50 is higher for GluR-1 + STG-1 + SOL-1 (61 ± 7 μM, $n = 18$) than for GluR-1 + STG-1 (14 ± 3 μM, $n = 15$). This difference in the EC50 values cannot explain the larger currents recorded in the presence of SOL-1. Moreover, receptor activation by other agonists, all applied at 100 μM, was also affected by the co-expression of SOL-1 (Figure 4). Responses to kainate were larger than those to glutamate for GluR-1 + STG-1 and conversely for GluR-1 + STG-1 + SOL-1. We did not obtain receptor activation with NMDA with or without SOL-1, and weak activation by AMPA or Quisqualate only without SOL-1. Altogether, our results suggest that both STG-1 and SOL-1 are necessary for proper functional expression of GluR-1. Invertebrate chaperone proteins like SOL-1 or STG-1 do not facilitate receptor trafficking, but rather promote iGluR functional expression by modification of receptor gating (activation time, deactivation and desensitization rates). For example, SOL-1 can slow the desensitization of GLR-1 (Walker et al., 2006). However, deciphering the precise role played by honeybee chaperone proteins requires additional experiments. At this level, their effects on GluR-1 trafficking and/or gating cannot be excluded. Our results demonstrate that reliable expression of *A. mellifera* iGluRs can be obtained with specific chaperone proteins, which opens the door to biophysical and pharmacological studies on these important receptors. We next utilized a similar approach for *A. mellifera* nACh receptors.

Functional reconstitution of nAChRs with RIC-3, UNC50 and UNC74 in *X. laevis* oocytes

We first tried to express α1α8β1 together with RIC-3A/UNC50/UNC74 all from *A. mellifera* (the combination similar to that used by Ihara and colleagues (Ihara et al., 2020)), but surprisingly, we could not record any ACh-evoked current (Figure 5a). Although all the other proteins were similar, the α1 variant used by Ihara and colleagues (here noted as α1b) had an additional N-terminal 26aa-long sequence. When expressed together with α8, β1, and the honeybee chaperone proteins, *A. mellifera* nAChRα1b led to the expression of functional receptors in 92% of injected *X. laevis* oocytes (Table 2). Bumblebee α1α8β1 subunits elicit also robust ACh-gated currents when expressed with chaperone proteins (Ihara et al., 2020). In GenBank®, there is a single transcript encoding the bumblebee nAChRα1 in which the *N* terminus is very similar to that of *A. mellifera* nAChRα1b, whereas they are three transcripts encoding three *D. melanogaster* nAChRα1 variants that all have the same short *N* terminus (Figure 5b). Since all these "long or short" α1 subunits do have a signal peptide, successful expression with honeybee α1b relies on an additional

TABLE 1 Expression of iGluR subunit combinations in *X. laevis* oocytes

iGluR subunit	Co-injected chaperone protein(s)	Mean current amplitude (nA)	Expressing oocytes/tested oocytes (from *N* frogs)
Caenorhabditis elegans GLR-1	-	-	0/40 ($N = 2$)
	C. elegans SOL-1/Neto/STG-1	-138 ± 23 ($n = 70$)	70/81 ($N = 3$)
	Apis mellifera SOL-1/Neto/STG-1	-20 ± 4 ($n = 23$)	23/72 ($N = 3$)
A. mellifera GluR-1	-	-11 ± 1 ($n = 13$)	13/58 ($N = 2$)
	C. elegans SOL-1/Neto/STG-1	-70 ± 8 ($n = 40$)	40/42 ($N = 2$)
	A. mellifera SOL-1/Neto/STG-1	-2059 ± 359 ($n = 34$)	34/35 ($N = 2$)
	A. mellifera STG-1	-46 ± 6 ($n = 64$)	64/71 ($N = 2$)
	A. mellifera SOL-1	-4 ± 1 ($n = 6$)	6/72 ($N = 2$)
	A. mellifera Neto	-	0/44 ($N = 1$)
	A. mellifera SOL-1/STG-1	-893 ± 94 ($n = 64$)	64/70 ($N = 2$)
	A. mellifera Neto/STG-1	-38 ± 5 ($n = 34$)	34/41 ($N = 1$)
	A. mellifera Neto/SOL-1		0/23 ($N = 3$)

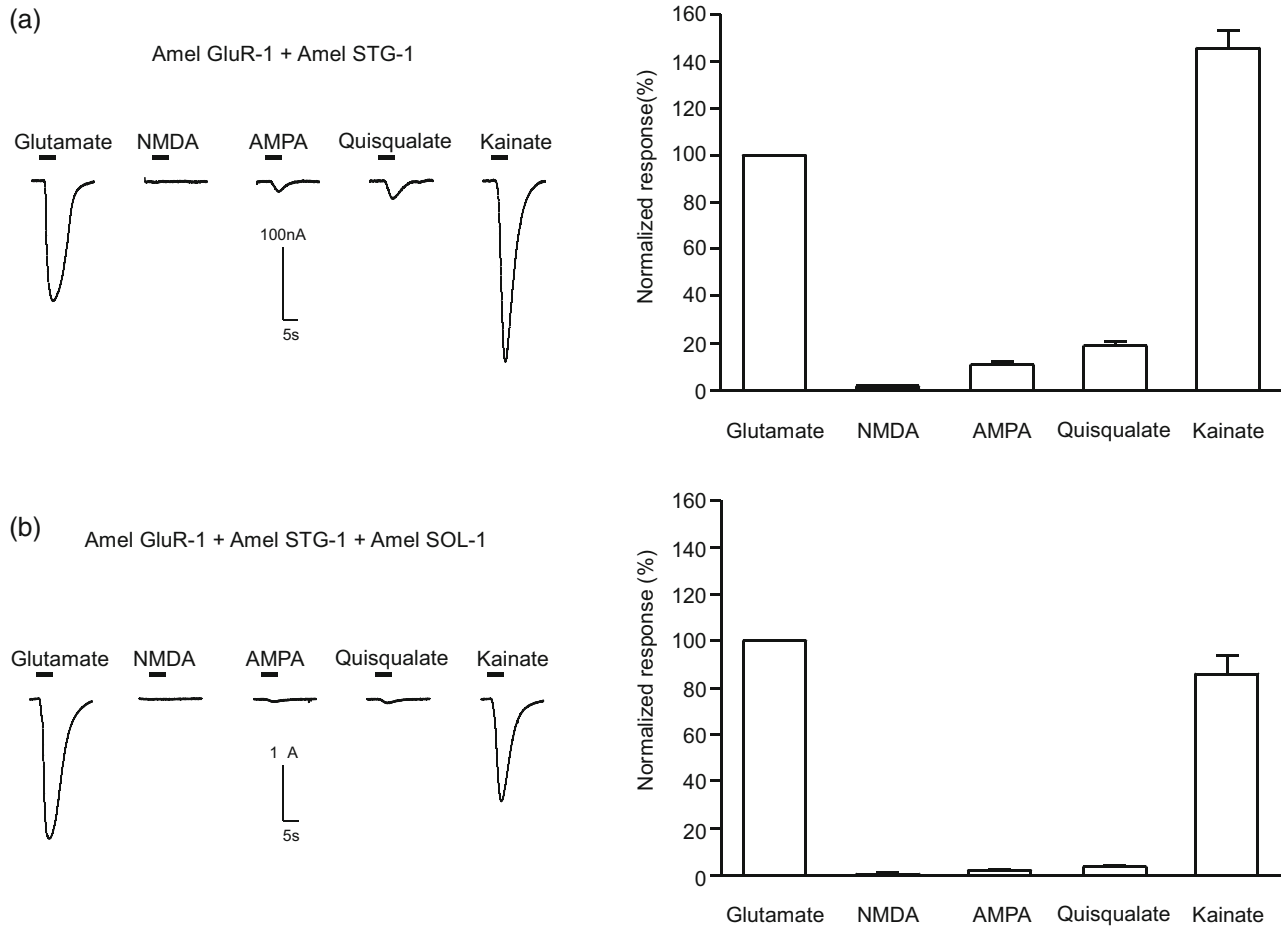

FIGURE 4 Responses of *Amel* GluR-1 to different ligands. (a) Right, responses to different ligands, all at 100 μM, from an oocyte injected with *Amel* GluR-1 + *Amel* STG-1. Left, peak current responses for the different agonists normalized to the glutamate response ($0.8 \pm 0.3\%$, $10.8 \pm 9\%$, $18.9 \pm 1.2\%$ and $145.7 \pm 6.8\%$ for NMDA, AMPA, quisqualate and kainate, respectively). (b) Same as in (a) for *Amel* GluR-1 + *Amel* STG-1 + *Amel* SOL-1 ($0.4 \pm 0.1\%$, $1.9 \pm 0.3\%$, $3.5 \pm 0.5\%$ and $85.8 \pm 7.8\%$ for NMDA, AMPA, quisqualate and kainate, respectively). The line above each trace illustrates the duration of agonist incubation. The data are the means ± SEM of $n = 5$–6 oocytes

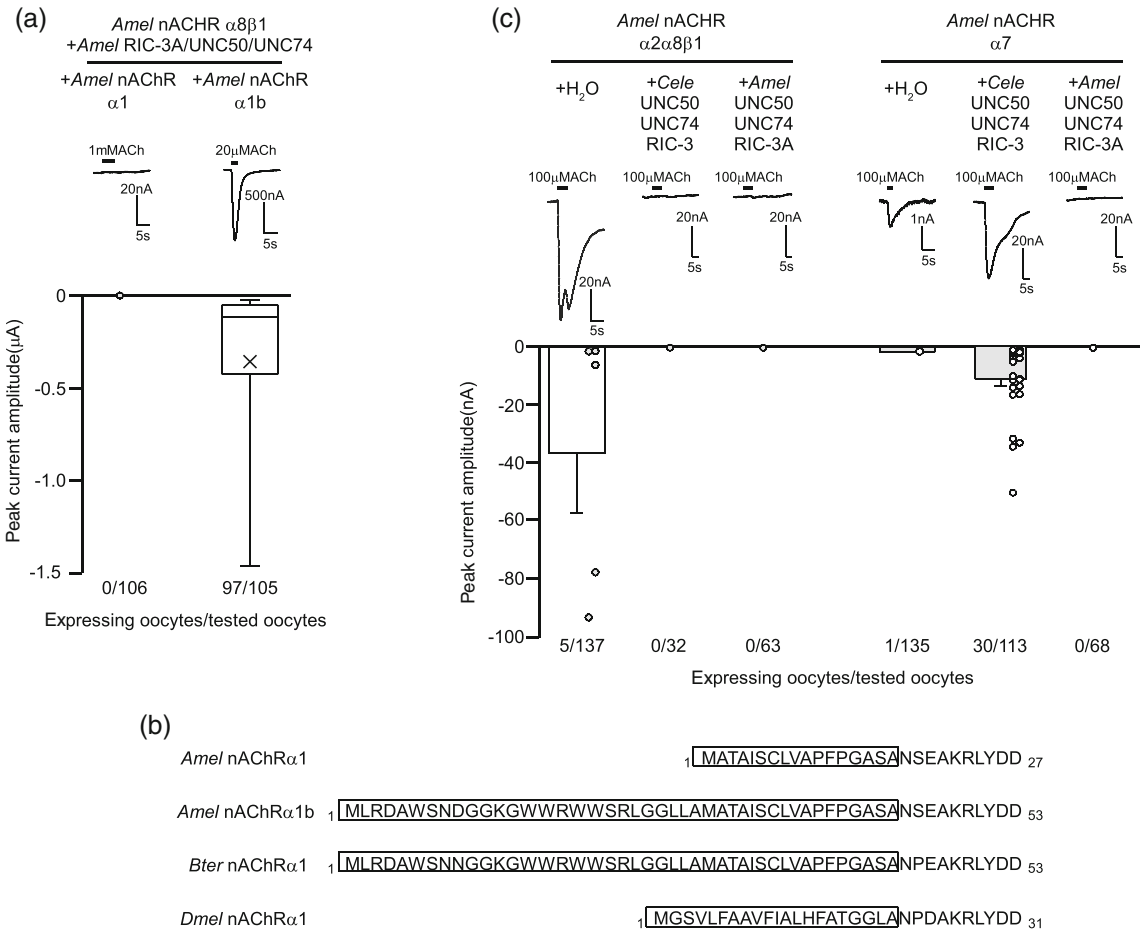

FIGURE 5 Reconstitution of honeybee nAChRs containing the α1 and α1b variants. (a) Top, representative current traces recorded in *X. laevis* oocytes that express either nAChRα1 or α1b with the indicated subunits and chaperone proteins. Bottom, box plots of current amplitudes for the two protein combinations. The cross symbol indicates the mean value of $n = 97$ oocytes from $N = 4$ frogs. (b) Amino acid sequences of the *N* terminus of *A. mellifera* nAChR α1 (AJE70259) and α1b (XP_026298411), *B. terrestris* α1 (XP_03397561) and *D. melanogaster* α1 (NP_524481). The identified signal peptides are boxed. (c) Top, representative current traces recorded in *X. laevis* oocytes that express the α2α8β1 or α7 honeybee subunits without ($+H_2O$) or with the indicated *C. elegans* or *A. mellifera* chaperone proteins. Bottom, mean current amplitude for the different protein combinations. Bars represent the means ± SEM of $n = 5–30$ oocytes from $N \geq 3$ frogs. The line above each trace illustrates the duration of agonist incubation

N terminus sequence that is unnecessary in fruit fly. The requirement of the α8 subunit also is species-specific. Indeed, the *D. melanogaster* α1β1 subunits co-expressed with chaperone proteins produce functional receptors but not *A. mellifera* α1bβ1 (Ihara et al., 2020). As *D. melanogaster* α1β1β2 (β2 being equivalent to honeybee α8) can also be functionally expressed (Ihara et al., 2020), an inhibitory role of the *A. mellifera* α8 subunit on the expression of nAChR with a short α1 isoform can be excluded, and this does not explain why we did not obtain functional receptors when we co-expressed honeybee α1 with α8 and β1. Additional experiments are needed to understand why the role played by the α1 *N* terminus and by the α8 subunit differs between species.

There is evidence for the existence of fruit fly receptors that include α1 and β1 nAChR subunits (Ihara et al., 2020), but to our knowledge, such combination of subunits in honeybee has never been demonstrated. In this species, single-cell PCR analysis in Kenyon cells and antennal lobe neurons indicated rather co-expression of α2, α8 and β1, and α2, α7, α8 and β1, respectively (Dupuis et al., 2011). Therefore, we decided to focus our efforts on the α2α8β1, α7 and α2α7α8β1 combinations of honeybee nAChR subunits. In *X. laevis* oocytes injected with cRNAs encoding the honeybee α2α8β1 subunits alone (without any chaperone proteins), we could sometimes record ACh-gated currents (Figure 5c). However, the expression rate was very low (5 of 137 tested oocytes from 5 frogs) and only two oocytes expressed a current with an amplitude higher than 50 nA with 100 μM ACh, which is not satisfactory for functional studies. We detected ACh-gated currents only in 1 of the 135 oocytes injected with *A. mellifera* nAChRα7 cRNA (Figure 5c). Co-expression of *A. mellifera* α2α8β1 and α7 subunits together also did not improve functional reconstitution of ACh-gated receptors (Table 2). GenBank® includes one α2 (NP_001011625) and four α7 (NP_001011621, XP_026300655, XP_026300656 and XP_026300658) *A. mellifera* variants. The α7 variant used in our study (similar to NP_001011621) harbours a signal peptide, and we did not identify in the different

TABLE 2 Expression of honeybee nAChR subunit combinations in *X. laevis* oocytes

nAChR subunit(s)	Co-injected chaperone protein(s)	Mean current amplitude (nA)	Expressing oocytes/tested oocytes (from *N* frogs)
Apis. mellifera nAChR α1α8β1	-	-	0/10 ($N = 1$)
	A. mellifera RIC-3A	-	0/23 ($N = 1$)
	A. mellifera UNC50	-	0/26 ($N = 1$)
	A. mellifera UNC74	-	0/30 ($N = 1$)
	A. mellifera RIC-3A/UNC50/UNC74	-	0/106 ($N = 3$)
A. mellifera nAChR α1bα8β1	*A. mellifera* RIC-3A/UNC50/UNC74	-352 ± 52 ($n = 97$)	97/105 ($N = 4$)
A. mellifera nAChR α2α8β1	-	-36.6 ± 20.6 ($n = 5$)	5/137 ($N = 5$)
	A. mellifera RIC-3A/UNC50/UNC74	-	0/63 ($N = 2$)
	Caenorhabditis elegans RIC-3/UNC50/UNC74	-	0/32 ($N = 2$)
	A. mellifera NACHO	-	0/63 ($N = 2$)
	Homo sapiens NACHO	-	0/23 ($N = 1$)
	H. sapiens RIC-3c/NACHO	-5.3 ± 0.2 ($n = 2$)	2/30 ($N = 1$)
	A. mellifera SOL-1	-	0/27 ($N = 1$)
	A. mellifera Neto	-	0/31 ($N = 1$)
	A. mellifera STG-1	-	0/30 ($N = 1$)
	A. mellifera SOL-1/Neto/STG-1	-	0/30 ($N = 1$)
	C. elegans SOL-1/Neto/STG-1	-	0/31 ($N = 1$)
	Rattus norvegicus nAChRβ2	-4.8 ± 0.5 ($n = 29$)	29/79 ($N = 2$)
A. mellifera nAChR α7	-	-1.6 ($n = 1$)	1/135 ($N = 6$)
	A. mellifera RIC-3A/UNC50/UNC74	-	0/68 ($N = 2$)
	C. elegans RIC-3/UNC50/UNC74	-10.3 ± 2.3 ($n = 30$)	30/113 ($N = 3$)
	A. mellifera NACHO	-	0/58 ($N = 2$)
	H. sapiens NACHO	-2.6 ($n = 1$)	1/26 ($N = 1$)
	H. sapiens RIC-3c	-3.0 ± 1.5 ($n = 3$)	3/45 ($N = 1$)
	H. sapiens RIC-3c/NACHO	-4.4 ± 1.0 ($n = 2$)	2/26 ($N = 1$)
	A. mellifera SOL-1	-	0/31 ($N = 1$)
	A. mellifera Neto	-	0/31 ($N = 1$)
	A. mellifera STG-1	-	0/30 ($N = 1$)
	A. mellifera SOL-1/Neto/STG-1	-	0/30 ($N = 1$)
	C. elegans SOL-1/Neto/STG-1	-	0/31 ($N = 1$)
	R. norvegicus nAChRβ2	-	0/36 ($N = 3$)
A. mellifera nAChR α2α7α8β1	-	-	0/35 ($N = 1$)
	A. mellifera RIC-3A/UNC50/UNC74	-	0/61 ($N = 2$)
	C. elegans RIC-3/UNC50/UNC74		0/26 ($N = 1$)
	A. mellifera NACHO	-18.6 ($n = 1$)	1/82 ($N = 3$)
	A. mellifera SOL-1/Neto/STG-1	-	0/22 ($N = 1$)
	C. elegans SOL-1/Neto/STG-1	-	0/22 ($N = 1$)

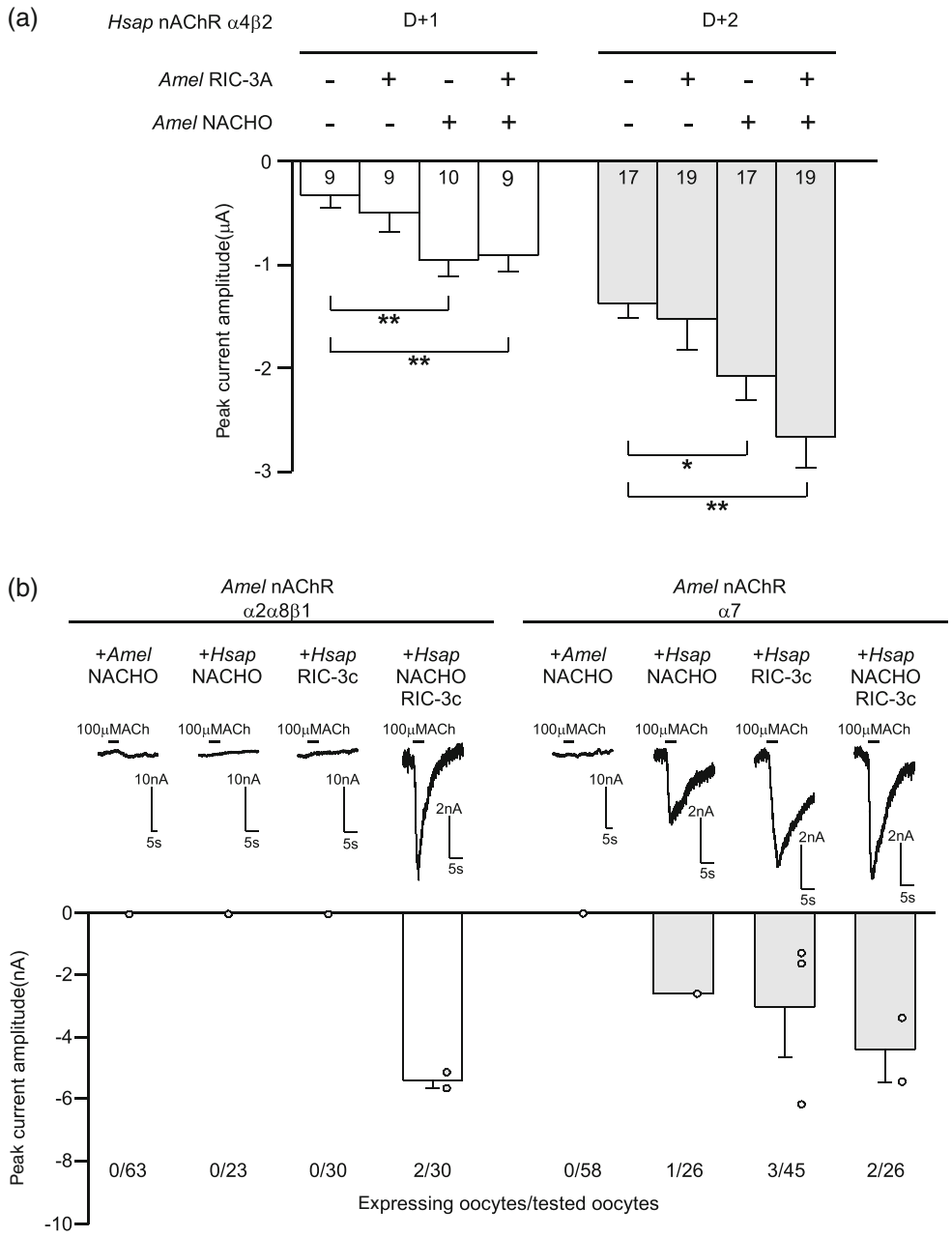

FIGURE 6 Functional reconstitution of honeybee nAChRs with NACHO. (a) Mean current amplitudes recorded at day (D) D + 1 or D + 2 after injection in *X. laevis* oocytes that express the different protein combinations. Bars represent the means ± SEM of the indicated number of oocytes from the same batch. The values at D + 1 and D + 2 were as follows: −330 ± 112 nA and −1375 ± 143 nA (α4β2 without chaperone proteins); −502 ± 190 nA and −1521 ± 298 nA (α4β2 with *A. mellifera* RIC-3A); −955 ± 166 nA and −2075 ± 229 nA (α4β2 with *A. mellifera* NACHO); and −909 ± 161 nA and −2665 ± 288 nA (α4β2 with *A. mellifera* RIC-3A and NACHO), respectively. * $p < 0.01$, ** $p < 0.001$. (b) Top, representative current traces recorded in oocytes that express the indicated protein combinations. Bottom, mean current amplitudes recorded from $n = 2–3$ oocytes that express the indicated protein combinations. The mean values are reported in Table 2. The line above each trace illustrates the duration of agonist incubation.

variants a sequence similar to that found in α1b susceptible to facilitate nAChR functional reconstitution. We therefore tried to identify chaperone proteins that might play this role.

Co-expression of honeybee α2α8β1 subunits with the *C. elegans* UNC50/UNC74/RIC-3 chaperone proteins did not allow functional expression of nAChRs (Figure 5c and Table 2). Interestingly, *A. mellifera* nAChRα7 displayed an expression rate of ∼26% when co-expressed with *C. elegans* chaperone proteins, but the current amplitudes did not exceed 50 nA. We obtained similar results with only the *C. elegans* RIC-3 chaperone protein (no UNC50 and UNC74, not shown). This is in line with the results obtained previously with *D. melanogaster* nAChrα7 (better expression with *C. elegans* RIC-3 than with *D. melanogaster* RIC-3^{7a,9} [Lansdell et al., 2012]). It has been suggested that the RIC-3 coiled-coil domain is required for proper

maturation of nAChRs in *X. Laevis* oocytes (Ben-David et al., 2016), but its deletion in nematode or human RIC-3 does not modify the regulation of mammalian α7 and of *C. elegans* ACR16 or DEG3/DES2 (Ben-Ami et al., 2005; Biala et al., 2009; Castillo et al., 2005). The *D. melanogaster* RIC-3 variants with or without coiled-coil domain were as efficient in increasing fruit fly α7 expression in tsA201 or S2 cells (Lansdell et al., 2008). Moreover, Ihara and colleagues used three very different RIC-3 variants in their study (Ihara et al., 2020). In *D. melanogaster* RIC-3^{6,7,9}, the coding sequence between the two hydrophobic segments is short, and this variant harbours the coiled-coil domain. This is exactly the opposite for *A. mellifera* RIC-3A used here (Figure 2a) and in the study by Ihara and colleagues. Moreover, the *B. terrestris* RIC-3 *N* terminus is very similar to that of *A. mellifera* RIC-3A, but the bumblebee variant (BCD56239) lacks the second hydrophobic segment, the coiled domain and the entire C-terminus. Rather than assigning a precise role to RIC-3, we decided to express combinations of *A. mellifera* nAChR subunits with their own UNC50/UNC74/RIC-3A chaperone proteins. However, even in this case, we did not obtain any ACh-gated current in any of the tested oocytes demonstrating that the chaperone proteins promote the functional reconstitution of only specific subunit combinations. In agreement with those obtained by Ihara et al, our results show that chaperone proteins UNC50/UNC74/RIC-3A allow the functional reconstitution of nAChRs with α1bα8β1, and our results demonstrate a lack of effect with α1α8β1, α2α8β1, α7 and α2α7α8β1 subunit combinations. Since our goal was to identify chaperone proteins allowing the functional expression with the set of nAChR subunits expressed together in honeybee neurons (α2, α7, α8 and β1), we decided to test other potential candidates.

Hybrid receptors and assessment of other potential chaperone proteins

Several studies described the successful expression of hybrid insect/vertebrate receptors when insect nAChR subunits (including honeybee nAChrα1b) are co-expressed with the rat or chicken nAChRβ2 subunit in *X. laevis* oocytes (see, for examples, Chen et al., 2019, and Shigetou et al., 2020). We failed to obtain such hybrid receptors by co-injecting the cRNAs encoding rat nAChRβ2 and *A. mellifera* nAChRα2 (not shown) or *A. mellifera* nAChRα7 (Table 2) in *X. laevis* oocytes, in agreement with a previous study by Chen and colleagues (Chen et al., 2019). Conversely, the expression rate increased from 4% to 36% upon co-expression of the honeybee α2α8β1 nAChR subunits with the rat nAChRβ2 subunit, but the current amplitude remained small (≤15 nA). This might result from the sole α8 subunit, as suggested by previous study (Chen et al., 2019), but we did not explore this possibility further.

Since the SOL-1/Neto/STG-1 allowed the functional reconstitution of iGluRs, we wonder whether they might also help for nAChRs. Indeed, SOL-1 and Neto share structural features with LEV-10; they all exhibit a single transmembrane segment with an intracellular *N* terminus and several extracellular CUB domains (Wang et al., 2012). LEV-10 is required for AChRs clustering at the neuromuscular junction in *C. elegans* (Gally et al., 2004). Moreover, STG-1 belongs to a protein family that can influence the expression of the AMPA-type glutamate receptor and also of the voltage-gated Ca^{2+} channels (Sandoval et al., 2007). We therefore decided to assess their potential effects on *A. mellifera* nAChRs. Unfortunately, co-expression of *A. mellifera* STG-1, SOL-1 or Neto, alone or all together, with the *A. mellifera* α2α8β1 or α7 subunits, did not facilitate the expression of nAChRs in *X. laevis* oocytes (not shown, Table 2). Similarly, the simultaneous expression of the three *C. elegans* chaperone proteins did not promote α7, α2α8β1 or α2α7α8β1 functional expression (not shown, Table 2).

We next decided to test the efficiency of NACHO. In the first set of experiments, we co-expressed *A. mellifera* NACHO and/or RIC-3A with human α4β2 nAChR subunits to test their potential effects on human receptor expression. At Day (D) +1 and D + 2 after oocytes injection, we observed a significant increase of the mean current amplitudes in the oocytes injected with *A. mellifera* NACHO alone or with NACHO + RIC-3A (Figure 6a). Conversely, we did not observe any effect of *A. mellifera* RIC-3A alone on α4β2 expression. Moreover, *A. mellifera* RIC-3A did not synergize with *A. mellifera* NACHO for α4β2 expression. Our results are more consistent with a lack of effect of insect RIC-3 on vertebrate α4β2 subunits as opposed to mammalian and nematode RIC-3 that decrease α4β2 currents when expressed in *X. laevis* oocytes (Ben-David et al., 2016; Castillo et al., 2005; Halevi et al., 2003). On the other hand, we show that *A. mellifera* NACHO increases human α4β2 currents in *X. laevis* oocytes and behaves as human NACHO (Gu et al., 2016). When co-expressed with *A. mellifera* α2α8β1, α7 or α2α7α8β1 subunits, however, neither honeybee NACHO nor human NACHO alone or with human RIC-3c led to functional reconstitution of nAChRs (Figure 6b, Table 2).

CONCLUSIONS

In this work, we tried to reconstitute honeybee iGlu and nACh receptors in a heterologous expression system. Although we obtained robust expression of an iGluR receptor when it was co-expressed with chaperone proteins from the same species, we failed to reconstitute functional nAChRs with any of the tested combination of chaperone proteins/nAChR subunits. Obviously, it seems easier to reconstitute homo-multimer receptors, such as GluR-1, than nAChR pentamers that may be assembled with any of the 11 α and β subunits in honeybee. Like in mammals, preferential subunit associations might also exist in honeybee nAChRs. Therefore, we tried to obtain functional nAChRs with subunits known to be expressed together in honeybee neuronal cells; however, we did not identify an efficient chaperone protein even for this limited set of subunits. Moreover, our results with nAChR α1 and α1b subunits demonstrate that specific variants could indeed play a critical role for assembly of functional receptor. In fact, the issue is not restricted to insect nAChRs but has been described also for the mammalian subunits. For example, the α7 subunit forms functional homo-pentamers in *X. laevis* oocytes but not in

many cell lines (including HEK). Only a screening of more than 17,000 cDNAs allowed identifying NACHO as an essential chaperone protein that promotes α7 folding, maturation and expression at the cell surface (Gu et al., 2016; Rex et al., 2017). The same methodology led to the identification of chaperone proteins specific for α6- and α9-containing AChRs that also do not form functional receptors in heterologous expression systems (Gu et al., 2019, 2020; Knowland et al., 2020). We may thus wonder whether genome-wide screening might be useful to identify chaperone proteins specific to given insect subunit combinations. Undoubtedly future studies will focus on testing the mammalian chaperone proteins identified in genome-wide screenings with insect receptors, identifying their potential homologues in insect species, and ultimately developing a similar screening with insect cDNAs.

EXPERIMENTAL PROCEDURES

Molecular biology

Honeybee total RNA was isolated and first-strand cDNA was obtained as previously described (Cens et al., 2015). Human brain total RNA was purchased from Clontech Laboratories Inc (catalogue n°636,530) and first-strand cDNA was obtained as previously described (Cens et al., 2015). *C. elegans* cDNA was a gift from Aymeric BAILLY (CRBM, Montpellier). *C. elegans glr-1* cDNA was purchased from Horizon Discovery Ltd (item number OCE1182). The α4 and β2 human nAChR subunit cDNAs were obtained from the ORFeome library (clone ID 55854 and 71,588). *C. elegans stg-1* (GenBank® accession number MW021444), *sol-1* (MW021443), *Neto* (MW021439), *ric-3* (MW021435), *unc50* (MW021436) and *unc74* (MW021437); *A. mellifera stg-1* (MW021441), *sol-1* (MZ198226), *Neto* (MW021438), *GluR1* (MW021431), *ric-3A* to *E* (MW021470 to MW021474), *unc50* (KJ939605), *unc74* (KJ939606), nAChR subunits α1 (KJ939588), α2 (KJ939589), α7 (KJ939594), α8 (KJ939595) and β1 (KJ939597) and *nacho* (MW021432); *Homo sapiens RIC-3c* (MW0214333) and *nacho* (MW021434) cDNAs were amplified by PCR using the Herculase II Polymerase (Agilent Technologies, Inc). Primers were designed based on the sequences previously published or deposited in WormBase, BeeBase and GenBank® (Figure S1). All cDNAs were first cloned in the pBluescript II cloning vector (Agilent Technologies, Inc.) and fully sequenced (Eurofins Genomics). Sequence analysis and domain identification were performed with InterProScan (Jones et al., 2014), the Conserved Domain Database (Lu et al., 2020) and SignalP (Nielsen et al., 2019). Sequences were managed and aligned with the Geneious Prime® software (Biomatters Ltd.). For the nAChRα1b honeybee subunit, a synthetic DNA fragment was purchased from Eurofins Genomics to extend the 5′ end of the α1 cDNA and cloned in frame with the coding sequence. Full-length cDNAs covering the entire ORF were then amplified with specific primers and cloned in the pcDNA3.1(+) vector, with the Alfalfa Mosaic Virus (AMV) sequence immediately before the start codon and the 3'-UTR sequence of the *X. laevis* β-globin gene immediately after the stop codon. For *X. laevis* oocyte injection, cRNAs were obtained from linearized plasmids using the Mmessage Mmachine Transcription Kit (Thermo Fisher), following the manufacturer's instructions. The cRNAs were pre-mixed at 1:1 ratio and diluted at a final concentration of 500 ng/μL.

X. laevis oocytes preparation and injection

Preparation and injection of *X. laevis* oocytes were previously described (Cens et al., 1996). Each oocyte was injected with 30 nL of cRNA solutions and cells were then maintained at 19°C in NDS (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, 2.5 mM Na-Pyruvate, 0.05 mM gentamycin, pH 7.2 with NaOH) renewed daily until recordings.

Electrophysiology and data analysis

Expressed currents were recorded at room temperature using the two-electrode voltage clamp method. Electrodes were pulled from borosilicate glass and filled with 3 M KCl. Oocytes were clamped at −60 mV, and ligand-activated currents were recorded at −60 mV with a Geneclamp 500 amplifier (Molecular Devices) and digitized with a Digidata 1200 converter (Molecular Devices) using the Clampex software (Molecular Devices). The external solution (NDherg; 96 mM NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4 with NaOH) was continuously perfused in the recording chamber at the rate of 1–5 ml/min. Ligands (stock solutions of 10 mM or 100 mM in H_2O) were diluted in NDherg solution. Functional expression in *X. laevis* oocytes was tested from day (D) D + 1 to D + 3 after injection and current amplitudes were measured at D + 2 except as otherwise noted. Glu concentration–response curves were generated by challenging oocytes with increasing concentrations of Glu. Peak current amplitudes were plotted against Glu concentrations, normalized to the maximal current recorded in individual oocyte and fitted with the Hill equation. Batch of oocytes in which non-injected oocytes displayed responses to ACh, revealing endogenous ACh receptors, were excluded from the analysis. Oocytes injected with only chaperone proteins did not display Glu- or ACh-gated currents. Data were analysed using Clampfit (Molecular Devices) and are presented as the mean ± SEM of *n* individual oocytes. The statistical significance of the difference between data was determined using the non-paired Student's *t*-test.

AUTHOR CONTRIBUTIONS

Thierry Cens designed the study. Thierry Cens, Lorène Brunello and Claudine Ménard performed the molecular biology, the electrophysiological experiments and analysed the results. Thierry Cens wrote the paper. Pierre Charnet, Matthieu Rousset and Michel Vignes provided guidance and support, and critically reviewed the manuscript.

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations and relevant guidelines of our institution. Surgery was performed under anaesthesia, and efforts were made to minimize suffering. The care and use of Xenopus conformed to institutional policies and guidelines. The experimental protocols were approved by the "Direction Départementale des Services Vétérinaires" (authorization N° C34.16).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Oligonucleotides used to amplify the specified cDNAs from Caenorhabditis elegans (Cele), Apis mellifera (Amel) and Homo sapiens (Hsap).

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