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Expression analysis of putative regulators of nicotinic acetylcholine receptors in Caenorhabditis elegans

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Title (English)

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Title (Swedish)

Abstract

Tobacco use is the major cause of death worldwide, largely due to the highly addictive nature of nicotine. Nicotine causes compulsive use of the drug by binding and activating nicotinic acetylcholine receptors (nAChRs) in the brain. Caenorhabditis elegans is a well suited system to identify molecules which modify nAChR function, abundance, or subcellular localization. We have recently preformed an RNAi screen of the entire *C. elegans* chromosome I, and identified 47 candidate genes which might effect nAChRs. In this study we report the expression patterns of some of the candidate genes by using the reporter GFP. This will be of great value understand the function of the genes and their roles in nicotine resistance.

Keywords

Nicotinic acetylcholine receptors, RNAi, nicotine addiction, nicotine resistance, cholinergic signaling, C. elegans

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Populärvetenskaplig sammanfattning

Tobak är den ledande dödsorsaken i världen, till stor del beroende på det beroendeframkallande nikotinet. Nikotin verkar genom att aktivera nikotinacetylkolinreceptorer i hjärnan, vilket leder till aktivering av belöningssystemen i hjärnan. Kroppen anpassar sig till nikotinanvändning genom att minska antalet nikotinreceptorer, och minska effekten av nikotinet. Det leder till att man intar ännu mer nikotin. Då man slutar bruka nikotin minskar aktiveringen av receptorerna och man får starka negativa upplevelser, såsom koncentrationssvårigheter och ångest. På så sätt uppstår ett beroende.

I vår forskningsgrupp undersöker vi hur nikotinreceptorerna regleras, i förhoppning att förstå nikotinberoende och kunna hitta botemedel. Vi använder en väletablerad modellorganism, en mask kallad *C. elegans*. Masken erbjuder ett antal mycket användbara verktyg för molekylära studier. Vi hittat ett antal kandidatgener som vi tror är inblandade i regleringen av nikotinacetylkolinreceptorer, och nu arbetar vi med att kategorisera dessa geners funktion. En viktig fråga är om kandidatgenen uttrycks i samma celler som nikotinreceptorerna. I denna studie har vi använt ett fluorescerande protein för att visualisera i vilka celler kandidatgenen uttrycks och utvärdera om genen ska undersökas närmare.

Examensarbete 20p
Civilingenjörsprogrammet Molekylär Bioteknik
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CONTENTS

1. INTRODUCTION

1.1 Nicotinic acetylcholine receptors

Function
Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand gated ion channels, which mediate fast synaptic transmission at the neuromuscular junctions between motor neurons and muscles. In response to binding of the neurotransmitter acetylcholine (ACh), the channels open, inducing fast depolarization of the cell. In the CNS, nicotinic signaling has been shown to be involved in arousal, attention, learning, memory and cognition.

Pharmacology
Mammals and birds have 17 different receptor subunits, which can be combined as homo- or heteropentamers, forming a large number of nAChRs with different properties such as gating kinetics, conductance and ion permeability. The nAChR subunits have been divided in five subfamilies, α, β, δ, ε and γ, based on sequence similarity and gene structure. The five subunits in the receptor are symmetrically arranged forming a central pore, and a binding site is formed by the alpha subunits. When ACh binds the two binding sites, a conformational change takes place, opening the gate and allowing inflow of ions [1] (Fig. 1).

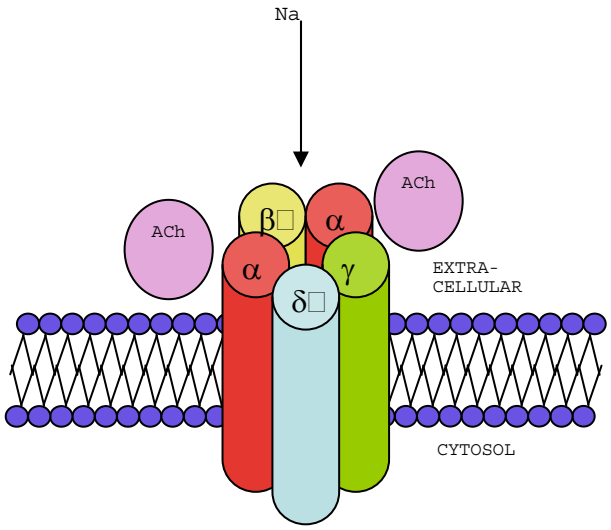

Figure 1. Scheme of muscle nAChRs. When ACh binds to both binding sites, a conformational change occurs allowing inflow of ions.

nAChRs in addiction and disease
nAChRs are implicated in several important pathological processes. Nicotine, the primary psychoactive component in tobacco, has a highly addictive nature and causes compulsive use of the drug by binding and activating brain nAChRs. Exposure of nAChRs to nicotine

results in increased activation of cholinergic pathways throughout the brain, increasing attention and memory, and stimulating reward pathways. The negative effects of withdrawal of tobacco use in smokers contributes to chronic tobacco use, and secondary diseases such as lung cancer and heart disease. Even though it is known that chronic exposure to nicotine causes long-lasting changes in abundance and activity of receptors in the brain, the molecular mechanisms underlying these effects remain unknown [2].

Neuronal nAChRs have also been linked to Alzheimer's, Parkinson's disease and schizophrenia. In both Parkinson's and Alzheimer's, cholinergic pathways are known to be affected, and the involvement of nAChRs in schizophrenia has been suggested by the high proportion of smokers in schizophrenic patients (90% compared to 33% in the general population) [3]. The identification of modulators of nAChRs may bring understanding of the molecular mechanisms of addiction and neurological diseases.

The regulation of the number and activity of nAChRs on the cell surface determines the response of the cell to extracellular stimulation by agonists such as ACh and nicotine. Even though nAChRs have a central role in the nervous system, and have been extensively studied, the molecules involved in the control of surface expression, by expression, assembly, trafficking, and degradation of nAChRs are poorly known. Also, modulators and downstream effectors of cholinergic signaling remain to be characterized.

1.2 *Caenorhabditis elegans* as a model organism

One powerful in system for studies of nAChRs is the 1 mm long nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans* offers a valuable organism to study the cellular and molecular responses of the nervous system *in vivo*, due to its simplicity and advantageous genetics, its completely sequenced genome, and the numerous tools available for behavioral and molecular analysis. The nematode has a simple nervous system consisting of exactly 302 neurons with well-characterized positions and synaptic connectivity. As in vertebrate nervous systems, *C. elegans* uses the classical neurotransmitters, and its general molecular machinery is similar to higher organisms. *C. elegans* is also very agreeable for genetics due to its small genome, short generation time of 3 days, capability of self- and cross-fertilization and convenient germline transformation. Mutant strains are produced at the North American *C. elegans* Gene Knockout Consortium and the Japanese National Bioresource Project, and are available for all researchers. *C. elegans* can easily be used for powerful techniques as RNA interference (see below), and its transparency makes tracking of reporter molecules, such as Green Fluorescent Protein (GFP) and rhodamine, possible without dissection.

1.3 The anatomy of *C. elegans*

The general anatomy of C. *elegans* consists of an exterior tube covered by a collagenous, extracellular cuticle, and an inner tube, containing the pharynx and the intestine. The pharynx is the eating organ of the nematode, which grinds food and pumps it into the

intestine. The intestine connects to the anus near the tail. Most of the body volume constitutes of the reproductive system (Fig 2). The hermaphrodite consists of 959 somatic cells, of which 81 are muscle cells and 302 nerve cells. The body musculature is arranged in four longititudal strips, which are attached to the cuticle through a thin layer of hypodermis. *C. elegans* undergoes 4 larval stages before reaching adulthood and sexual maturity. At each of the larval stages, a new cuticle is secreted and the old cuticle is discarded.

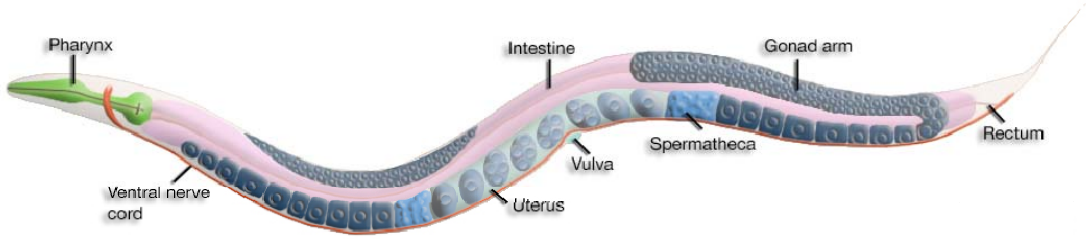

Figure 2. General hermaphrodite anatomy of *C. elegans* (from http://www.wormbase.org).

A great deal is known about the connectivity and structure of the nervous system of *C. elegans*. Most of the neuron cell bodies are arranged around in a ring around the pharynx, and along the ventral cord and in the tail. The principal chemosensory organs in nematodes are the amphids, consisting of twelve neuron pairs in the head. Many of the amphid neurons have ciliated extensions from the nerve ring to the tip of the head. The nerve ring receives sensory information, integrates it, and connects to motor neurons in the head and the nerve cord, which synapse to muscle and regulate motor output.

1.4 Nicotinic acetylcholine receptors in *C. elegans*

Acetylcholine is the primary excitatory neurotransmitter in *C. elegans*, and controls motor functions and locomotion by acting at the neuromuscular junctions of the body wall muscle, pharyngeal muscle and vulva muscles. Ligand binding and opening of nAChRs in neuromuscular junctions cause depolarization of the postsynaptic muscle cell and subsequent contraction. Many of the genes coding for subunits and proteins involved in the regulation of the subunits have been identified through the uncoordinated locomotion phenotype resulting from defective cholingergic signaling. In example, knock-out mutations in UNC-29 and UNC-63, which encode two different subunits of the nAChR, and UNC-50, which is involved in the receptor assembly, all give rise to an uncoordinated phenotype [4].

Over fifty genes coding for nAChR subunits have been predicted in the *C. elegans* genome, and 27 of these have been shown to form functional receptors. The subunits have been classified in five groups according to their sequence similarity. The ACR-16 like subunits have most resemblance to vertebrate alpha subunits. UNC-29 subunits are most similar to vertebrate muscle non-alpha subunits. The UNC-38 like subunits are

alpha like and bear resemblance to insect alpha subunits. The two other groups, DEG-3 and ACR-8 seem to be unique to nematodes, but could be similar to nAChR subunits in other spices. [5]. Two nAChRs are present in the neuromuscular junction of *C. elegans*; the levamisole receptor (named so because it is sensitive to the anthelmitic drug levamisole) consisting of the UNC-29, UNC-38 and LEV-1 subunits [6], and a second receptor suggested to consist of ACR-16, ACR-8 and perhaps other subunits [7]. In addition, an inhibitory GABA receptor, UNC-49, is present, and these three receptors generate locomotion. The opening of nAChRs cause depolarization of the body wall muscle and subsequent contraction, while opening of GABA receptors cause an inflow of chloride ions, hyperpolarization of the body wall and muscle relaxation. The alternation of these two events produces the characteristic sinusoidal movement of *C. elegans* [8].

1.5 Screening for regulators of nicotinic acetylcholine receptors

C. elegans is strongly affected by nicotine, which binds the receptors, forcing them to open, and causing paralysis by continuous contraction of the muscles. This gives a rigid phenotype, allowing studies of mutations and RNA interference leading to resistance to the effect of nicotine (Fig 3). Several different factors can lead to nicotine resistance by decreasing the number of functional nAChRs on the surface. Expression of the receptors can be transcriptionally or translationally regulated. Trafficking, maturation and assembly defects can lead to a reduction in the number of receptors. Increases in endocytosis and desenzitisation of the receptors can give less response to nicotine.

RNA interference

C. elegans can easily be used for RNAi interference (RNAi). RNAi is a process in which targeted gene silencing is achieved by the introduction of double-stranded RNA (dsRNA). The dsRNA triggers the degradation of homologous mRNA, shutting down the effect of specific genes. The introduced dsRNA is cleaved by Dicer, an enzyme with RNaseIII activity, into double stranded small interfering RNA (siRNA). The siRNAs are incorporated by a multicomponent nuclease, RISC, and unwinded to allow recognition and cleavage of homologous target mRNAs.

RNAi allows rapid gene inactivation, and has become an important method for studying gene function *in vivo*. In most species, dsRNA can only be introduced by microinjection, electroporation or transfection. In *C. elegans*, dsRNA can be supplied by soaking or feeding, making it agreeable to use RNAi for large scale screens.

An RNAi screen for genes involved in nicotine resistance

An RNAi screen of the entire *C. elegans* chromosome I, and additional neuropeptides and GPCRs, has recently identified a set of genes whose loss results in nicotine resistance. The screen was performed by allowing worm embryos to develop to adult stage on NGM plates seeded with bacteria expressing the dsRNA. The adult worms were transferred to liquid 0.45% nicotine for 1 hour and scored for nicotine resistance by counting the moving (non-paralyzed) worms. 1800 genes were tested in triplicate, resulting in 47 candidate genes. These genes include putative transcriptional and translational regulators, components of G-protein signaling and protein degradation pathways, various

kinases and phosphatases, and structural proteins. A major problem when performing screens is the obtaining of false positives. In this case resistance to nicotine paralysis can be caused by other factors than the function of nAChRs. The dsRNA could knock-down several different genes which are highly homologous to the target gene, which could give rise to a stronger phenotype. Also, the permeability of the cuticle varies during the development of the nematode, with lower permeability during the molt occurring between the last larval stage and adulthood than during the young adult stage. Knock-down of genes could potentially lead to developmental delays of a few hours, causing a less permeable cuticle and a decreased influx of nicotine into the body cavity, leading to nicotine resistance without influencing the nAChRs. To exclude false positives, that is genes that do not alter the cholinergic signaling, a number of rigid secondary screens must be performed.

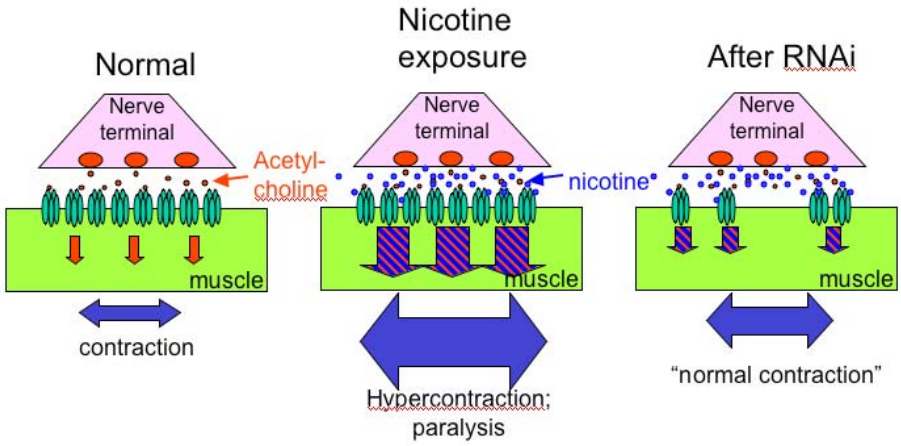

Figure 3. RNAi mediated nicotine resistance (with permission by Alexander Gottschalk, Johann Wolfgang-Goethe University, Frankfurt). Normally, the endogenous ligand ACh binds to nAChRs in the neuromuscular junction, leading to contraction of the muscle. Exposure to the agonist nicotine results in chronic opening of nAChRs, hypercontraction of the muscle and paralysis. RNAi mediated knock-down of genes involved in regulation of nAChRs results in a decrease of functional receptors, and nicotine resistance.

2. AIM OF PROJECT

One approach to gain information about the candidate genes is to study the expression pattern of the gene by using the reporter Green Fluorescent Protein (GFP). GFP is a fluorescent protein which absorbs ultraviolet light and emits it as green light by energy transfer. The protein naturally occurs in the Pacific jellyfish *Aequoria victoria*, where it absorbs blue chemiluminescence from the protein aequorin, and converts it to a green light which lights up the jellyfish. Since the molecular cloning and engineering of GFP, GFP has become an important tool in molecular biology, allowing detection and tracking

of tagged proteins in vivo. GFP can be used as a reporter of gene expression by generating expression constructs, in which the promoter region of a gene is fused to GFP. This approach will allow the expression of GFP to be driven by the promoter and reveal which cells the gene is expressed in. The use of expression constructs is advantageous in *C. elegans* due to the easily obtained germline transformation, and the transparency of the nematode, making visualization possible without dissection.

3. MATERIALS AND METHODS

3.1 *C. elegans* handling and strains

Nematodes were cultured at 20°C on nematode standard growth media (NGM) with *Escherichia coli* (strain OP50) as food source according to standard protocols. Wildtype worms were Bristol N2. A deletion mutant for *nicr-42, (ok1387)* (Fig 4), was obtained from the North American *C. elegans* Gene Knockout Consortium.

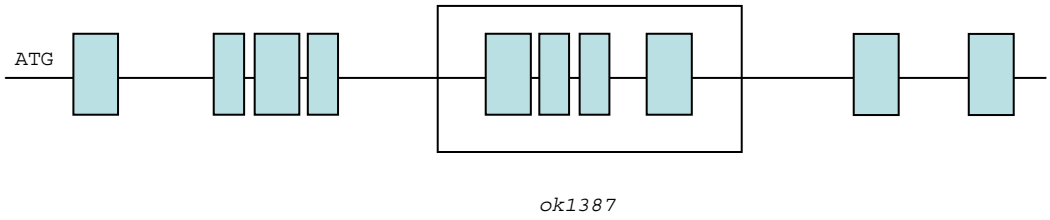

Figure 4. Deletion mutant of *nicr-42*. The deletion includes four of the 10 exons and spans 1/3 of the gene.

3.2 DNA sequences

All sequences used in the study were acquired from the *C. elegans* database Wormbase (http://www.wormbase.org). The genomic regions were selected according to the predicted start sites and coding regions annotated in the database. Identity and homology of genes was obtained from Wormbase.

3.3 Expression analysis

To determine the expression patterns of 9 of the candidate genes, expression constructs were generated by fusing the upstream regulatory regions, and in some cases part of the coding sequences, of the candidate genes to GFP (table 1). The constructs were used for for transformation by microinjection, resulting in extragenomic GFP expression under the

gene promoter in the progeny. This approach allows visualization of cells in the intact worm expressing the candidate protein, and makes it possible to identify genes expressed in neuronal and muscle cells.

Construct	**Putative function/Homology of gene**	**Regions inserted in GFP construct**
pnicr-3::GFP	Calcineurin-like phosphesterase	Upstream 984 bp
pnicr-10::GFP	GPCR, chemosensory receptor	Upstream 1253 bp
pnicr-13::GFP	Prion-like, (Q/N-rich)-domain	Upstream 850 bp, Ex1, In1
pnicr-26::GFP	Gamma-interferon inducible thiol reductase	Upstream 800 bp
pnicr-31::GFP	Guanine exchange factor	Upstream 808 bp
pnicr-33::GFP	F-box	Upstream 315 bp
pnicr-37::GFP	transcription factor, alpha-NAC	Upstream 549 bp
pnicr-40::GFP	neuropeptide receptor	Upstream 2008 bp
pnicr-42::GFP	GPCR, neuromedin U receptor 2	Upstream 1940 bp, Ex1, In1

Table 1. Candidate genes for nicotine resistance

Cloning of expression constructs

The predicted promoters of the 9 candidate genes (table 1) were cloned in the Fire lab vector pPD95.75. The vector has a series of restriction sites upstream Green Fluoerscent Protein (GFP), allowing the insertion of DNA sequences to drive the expression of GFP driven by the inserted sequences.

Expression constructs for *nicr-3*, *nicr-5*, *nicr-26*, *nicr-33*, *nicr-37* and *nicr-40* were created by inserting a region upstream of the start codon of the gene in the vector (Fig 5A). *nicr-10* is annotated in Wormbase as forming an operon (CEOP1320) with *pnk-1*, the upstream neighboring gene. An operon is a transcription unit consisting of several co-transcribed genes. An operon is transcribed as one polycistronic pre-mRNA, which is processed to monocistronic mRNAs resulting in multiple peptides. Genes in an operon are often related in function. To obtain the expression pattern of *nicr-10*, the region directly upstream of *pnk-1* was chosen to drive expression of GFP (Fig 5B). *nicr-31* is predicted to form an operon (CEOP1712) with the upstream gene W09C5.1, an uncharacterized gene containing a domain related to ribosomal protein S8E. The upstream region of W09C5.1 was inserted in the expression construct (Fig 5B).

Constructs for *nicr-13* and *nicr-42* were obtained by inserting the upstream region, the first exon, the first intron and a few bases of the second exon in frame with GFP (Fig 5C). The sequences were amplified from *C. elegans* genomic DNA by PCR with primers designed to include restriction sites for ligation to the vector. Ligated vectors were used to transform Subcloning Efficiency DH5alpha cells (Invitrogen, Carlsbad, CA) and the DNA was extracted with GenElute^{TM} HP Plasmid Midiprep Kit (Sigma, Saint Louis, MO).

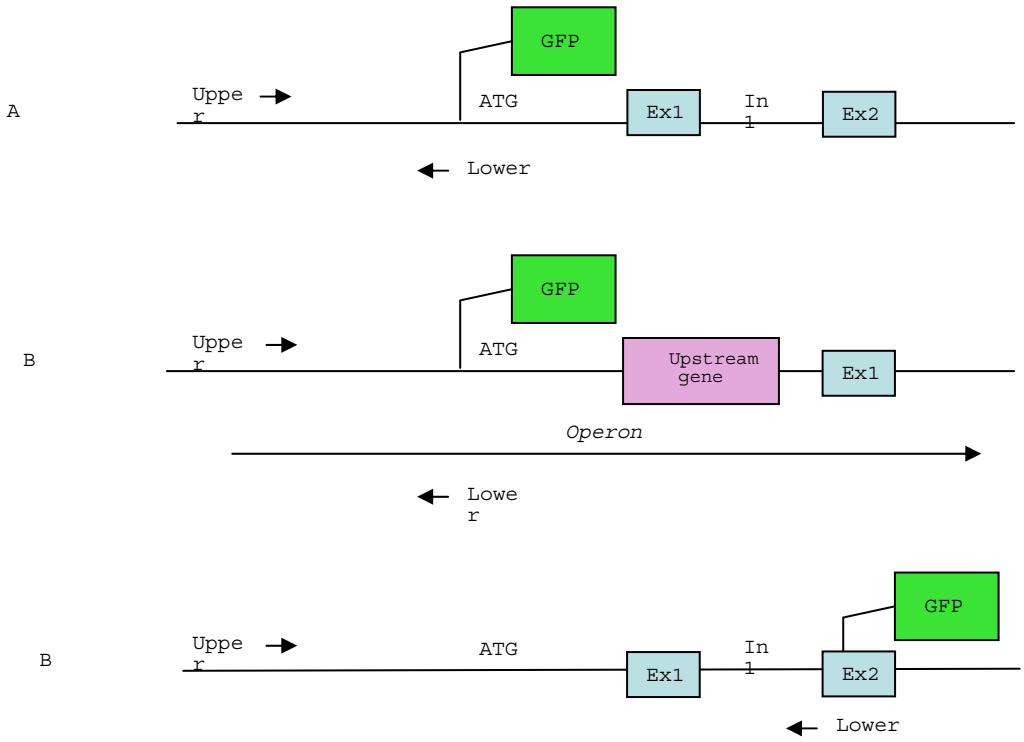

Figure 5. Design of GFP constructs. Two different approaches were used to design expression constructs. A) The region upstream of the start codon was inserted in the GFP vector pPD95.75. B) The region upstream of first gene in the operon. The candidate gene is the second gene in the operon. C) The upstream region, exon 1, intron 1 and a few bases of exon 2 were fused in frame with GFP in pPD95.75.

Microinjection of GFP fusions

Transformation of *C. elegans* was done by microinjection of the GFP construct, resulting in the formation of a transmissible extrachromosomal array [9]. Young adult hermaphrodites were mounted on agarose pads under halocarbon oil, and injected in the gonad cytoplasm. After injection, the animals were allowed to recover in physiological saline (M9 buffer) and transferred to NGM plates seeded with OP50. The constructs were coinjected with the transformation marker *rol-6(su1006)*. *rol-6* encodes a cuticle collagen required for normal cuticular morphology, and transformation with the dominant *rol-6(su1006)* results in a helically twisted body, giving an easily identified rolling phenotype. *rol-6(su1006)* was injected at a concentration of 30 ng/µl, and the GFP promoter constructs were injected at concentrations of 30 or 50 ng/µl.

Visualization of GFP expression

Obtained transgenic lines were identified by visualization of GFP expressing cells. The nematodes were paralyzed in 40 mM NaN_3 and mounted on 2% agar before imaging. MetaVue 6.1 was used to obtain and analyze images.

3.4 Identification of amphid and phasmid neurons

Sensory neurons in head and tail expressing GFP were identified by fluorescent dye-filling experiments using DiI dye (Molecular Probes, Eugene, OR). DiI is a red fluorescent membrane stain that labels cell membranes by inserting its two hydrocarbon chains into the lipid bilayers. When DiI is applied to intact worms, the dye fills 6 of the 12 amphid neurons in the head (Fig 6A) and two of the phasmid neurons in the tail (Fig 6B) by entering the cilia exposed to the external medium.

pnicr-13::GFP, *pnicr-40*::GFP and *pnicr-42*::GFP worms of all stages were soaked in DiI (dilution 1:100) for 20 minutes and allowed to crawl on bacteria for 30 minutes to wash out excess dye. The worms were imaged, and the green fluorescence from the GFP and the red fluorescence from the DiI were compared for each worm.

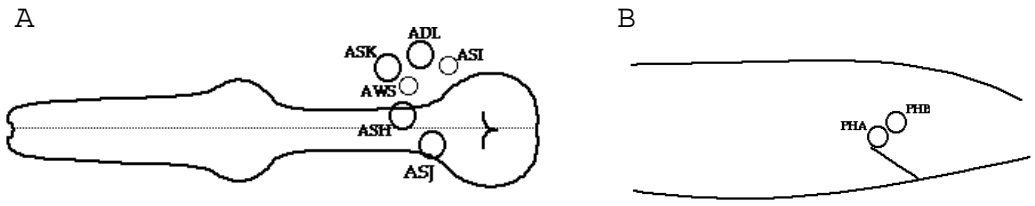

Figure 6. Neurons which are stained by DiI (from http://www.wormatlas.org). A) Amphid neurons in the head. B) Phasmid neurons in the tail.

3.6 Rhodamine take up by coelomocytes

To determine the identity of GFP expressing cells suspected to be coelomocytes, the worms were injected with rhodamine-conjugated α-bungarotoxin in the pseudocoeleom. Coelomocytes are scavenger cells, which are continuously taking up molecules from the pseudocoeleom by endocytosis. When high amounts of rhodamine is circulating in the body cavity, it will be taken up by the coelomocytes and accumulate, and the intense fluorescence allows identification of the cells.

pnicr-42::GFP worms were injected with a 1:200 dilution of rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) in the pseudocoeleom and allowed to recover on NGM plates seeded with OP50. After 6 hours, the worms were visualized as described above. To determine if *nicr-42* is essential for endocytosis in coelomocytes, *nicr-42(ok1387)* was injected as described above and compared to wildtype.

4. RESULTS

4.1 Expression patterns

Extragenomic lines were successfully made for all constructs except *pnicr-3*::GFP and *pnicr-26*::GFP (expression patterns are summarized in table 2 and Fig 7). *nicr-10* is a predicted chemosensory GPCR, which is positioned in a predicted operon with *pnk-1*, a gene observed to reduce fat accumulation in the intestine. The region directly upstream of *pnk-1* was chosen to drive expression of GFP. *pnicr-10*::GFP was expressed expression in the intestine, as expected for *pnk-1*. *nicr-13* is a gene of unknown function containing a polyglutamine rich domain and a zink-finger domain. *pnicr-13*::GFP showed strong expression all stages, including embryos. Expression was observed in head neurons (Fig 7A), tail neurons, ventral cord (Fig 7B), cuticle and seam cells (Fig 7C). In adults, expression was occasionally seen in vulva muscles (not shown). *nicr-31* is a predicted guanine nucleotide exchange factor, and *pnicr-31*::GFP showed strong embryonic expression (Fig 7D). In early larval stages, a diffuse expression could be detected in the anterior part of the animal (not shown), but no expression was seen in adult worms. *nicr-33* is a predicted F-box protein, and the expression of *pnicr-33*::GFP was observed only in pharyngeal muscle (Fig 7E). *nicr-37* is homologous to a human gene reported to code for alpha-NAC, and is also reported to function as a transcriptional coactivator, potentiating c-Jun in osteoblasts. *pnicr-37*::GFP showed expression vulval muscle (Fig 7F), pharyngeal muscle (Fig 7G), head neurons and tail neurons. *nicr-40* is homologous to a human neuropeptide receptor Y. Expression of *pnicr-40*::GFP observed in head neurons (Fig 7H), tail neurons, ventral cord (Fig 7I) and intestine. *nicr-42* is homologous to human neuromedin U receptor 2, and expression of *pnicr-42*::GFP was seen in head

Gene	Function/Homology	Expression pattern
nicr-3	Calcineurin-like phosphesterase	Not acquired
nicr-10	GPCR, chemosensory receptor	Intestine
nicr-13	Prion-like, (Q/N-rich)-domain	Head neurons, amphids, phasmids, nerve cord, vulval muscle, seam cells, cuticle
nicr-26	Gamma-interferon inducible thiol reductase	Not acquired
nicr-31	Guanine exchange factor	Strong embryonic, weak larval head
nicr-33	F-box protein	Pharynx
nicr-37	Transcription factor, alpha-NAC	Pharynx, head neurons, tail neurons, vulval muscle
nicr-40	Neuropeptide receptor	Head neurons, Ventral cord, Intestine
nicr-42	GPCR, neuromedin U receptor 2	Head neurons, Phasmids, Nerve cord, Coelomocytes

Table 2. *In vivo* expression patterns obtained from extrachromosomal arrays.

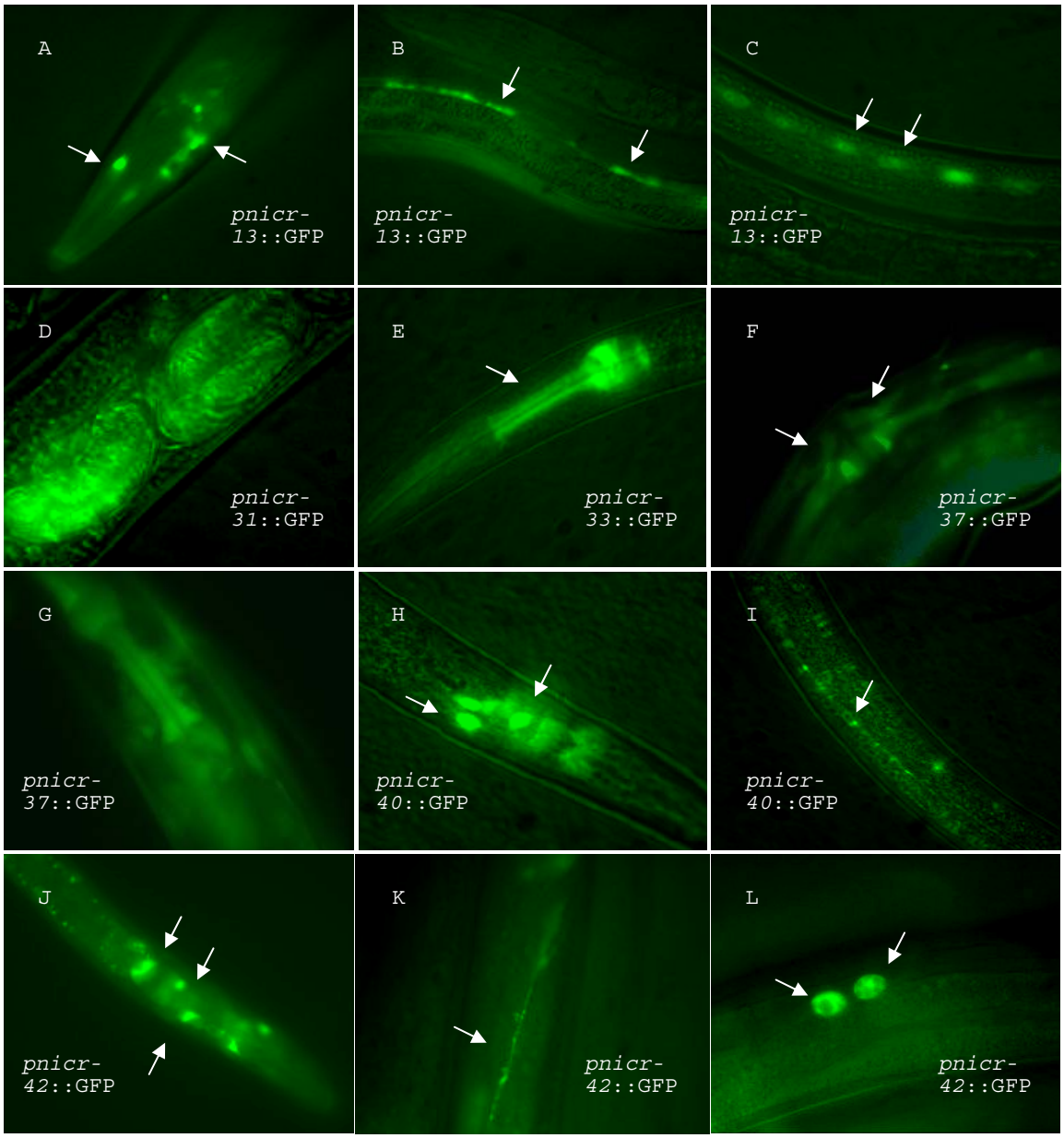

Figure 7. Expression patterns of GFP promoter constructs. Arrows indicate the cell types mentioned in the legend. A) *pnicr-13*::GFP expressed in head neurons around the pharyngeal bulb. B) *pnicr-13*::GFP expressed in neurons in the ventral cord. C) *pnicr-13*::GFP expressed in seam cells. D) *pnicr-31*::GFP expressed in embryos. E) *pnicr-33*::GFP expressed in pharyngeal muscle. F) *pnicr-37*::GFP expressed in vulva muscle. G) *pnicr-37*::GFP expressed in pharyngeal muscle. H) *pnicr-40*::GFP expressed in head neurons. I) *pnicr-40*::GFP expressed in ventral cord. K) *pnicr-42*::GFP expressed in ventral cord. L) *pnicr-42*::GFP expressed in coelomocytes.

neurons (Fig 7J), ventral cord (Fig 7K) and coelomocytes (Fig 7L). *pnicr-3*::GFP and *pnicr-26*::GFP were injected several times without obtaining GFP expression. The injections did result in *rol-6(su1006)* phenotype, indicates that the injections were successful, and that the GFP constructs did not contain the regulatory sequences necessary to drive GFP expression.

4.2 Identification of amphid and phasmid neurons

Colocalization of GFP expression and DiI was seen in all three tested lines (figure 7). In *pnicr-13*::GFP, four of the stained amphids expressed GFP (Fig 8A). In *pnicr-40*::GFP colocalization of GFP and DiI was observed in three neurons (Fig 8B) and in *pnicr-42*::GFP in four neurons (Fig 8C). In all tested worms the two stained phasmids PHA and PHB expressed GFP (not shown). All lines had head neurons which were not stained by DiI. In conclusion, the GFP expression in at least some of the neurons in *pnicr-13*::GFP, *pnicr-40*::GFP and *pnicr-42*::GFP occurs in the sensory amphid and phasmid neurons. The exact identity of the amphids remains to be clarified.

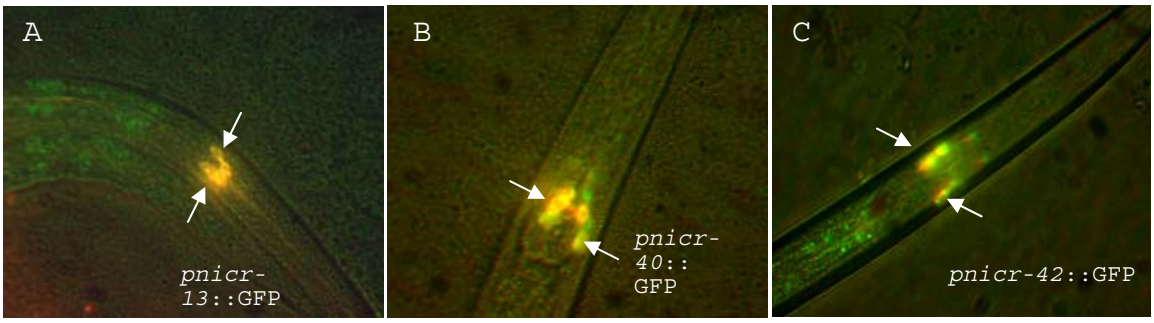

Figure 8. Identification of amphids and phasmids by dye-filling with DiI. The figures show overlay of GFP expression (green) and DiI staining (red). Arrows indicate colocalization of the two fluorophors (yellow). A) *pnicr-13*::GFP, B) *pnicr-40*::GFP and C) *pnicr-42*::GFP.

4.3 Rhodamine take-up by coelomocytes

In *pnicr-42::*GFP, GFP expression colocalized perfectly with red fluorescence from the rhodamine taken up by the coelomocytes (figure 8A), confirming the identity of the GFP expressing cells as coelomocytes. Comparison of rhodamine take-up in N2 (Fig 8B) and *nicr-42(ok1387)* (Fig 8C) h after injection showed no obvious difference.

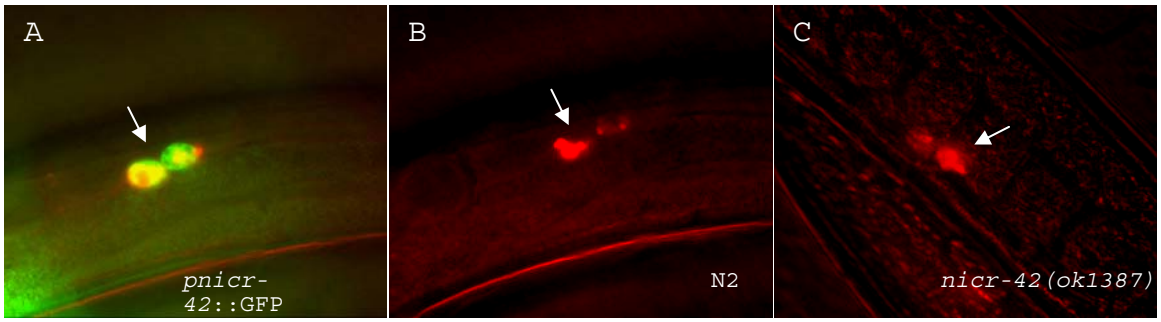

Figure 9. Rhodamine take-up experiments. Arrows indicate coelomocytes. A) Overlay of GFP expression and rhodamine fluorescence in *pnicr-42*::GFP. B) Rhodamine take up after 6 h in N2. C) Rhodamine take up after 6 h in *nicr-42(ok1387)*.

5. DISCUSSION

When investigating the function of a gene, an important clue is which cells the gene is expressed in. Expression patterns can give indications about which processes and mechanisms a gene is involved in, and if the gene product is specific for certain cell types or more generally present in several different tissues. When studying genes associated to a certain phenotype, the expression pattern can be used to gain knowledge of how the phenotype is generated. For example, genes can affect nicotine resistance by specifically regulating or modulating nAChRs in neurons and/or muscle, but also by altering cuticle permeability, development or the general cellular machinery. Neuronal and muscle expression of a candidate indicates a possible role in regulation in cholinergic signaling, while exclusive expression in other tissues implies that the gene is involved in separate mechanisms.

nicr-10, a putative chemosensory GPCR, is in a predicted operon (CEOP1320) with *pnk-1*, a pantothenate kinase suggested to reduce fat accumulation in the intestine. In the promoter construct, the region upstream of *pnk-1* was chosen to get the expression pattern for the entire operon. GFP expression was only observed in the intestine, the tissue expected for expression of *pnk-1*. If *nicr-10* truly is expressed only in intestine, an underlying factor for the nicotine resistance could be that the dsRNA has knocked down several molecules homologous to *nicr-10*, of which some might be involved in cholinergic signaling. Since many GPCRs are known to modulate neurotransmission, and GPCRs have a high degree of homology, this explanation is not unlikely. It is possible that the operon is also expressed in other tissues in low, non-detectable levels, or that other sequences important for the regulation of the gene, such as intronic regions, are missing in the construct. Possibly, the two genes are not in an operon, implying that the assumed promoter region is incorrect and that the obtained expression pattern only reflects the expression of *pnk-1*. Full-length protein fusions of NICR-10 with a reporter could be used under the two possible promoters to detect the true expression pattern of *nicr-10*.

nicr-13 is a gene of unknown function containing a C2H2 zink finger domain and a glutamine/asparagine-rich tract. *nicr-13* is by gene ontology predicted to bind nucleic acids and to localize to the nucleus, suggesting a transcriptional role. The observed expression pattern in neurons, hypodermal and seam cells, opens several possible explanations for the observed nicotine resistance. One is modulation of nAChRs in neurons, by e.g. transcription. Defects in synaptic morphology could also cause nicotine resistance. Seam cells can under development act as stem cells to generate ventral cord neurons, and malfunctioning in these cells could lead to defects in synaptic transmission at the neuromuscular junctions. The expression in hypodermis points to the possibility of some kind of cuticle defect, leading to less permeability to nicotine. Another explanation is that developmental delays caused by knock-down of *nicr-13* in one or several of the expressing tissues give rise to the observed nicotine resistance. Nicotine resistance caused by a decreased cuticle permeability in knock-downs of *nicr-13* is supported by the observation that *nicr-13*(*ok486)* is more resistant to cuticle disruption by bleaching (25% bleach solution) than N2 and *nicr-13*(*tm883).* 100 worms of each strain were tested, and after 10 minutes in the solution, 85% of *nicr-13*(*ok486)* were still intact, while 9% of *nicr-13*(*tm883)* and no N2s were undamaged by the bleach. Further experiments with proper controls and statistical analysis are needed to confirm the observed cuticle permeability, but these results indicate that *nicr-13*(*ok486)* is a loss of function allele resulting in decreased cuticle permeability, possibly by causing a developmental delay. To gain more understanding of the function of *nicr-13*, a full-length fusion with GFP could be constructed to show the subcellular localization of the protein. Localization of the gene product in nucleus would indicate a transcriptional role for *nicr-13*.

nicr-31 contains a Sec-7 domain, which is highly homologous to human Brefeldin A-inhibited guanine nucleotide-exchange protein 1 (GEP1). GEP1 activates ADP-ribosylation factor 1 and 3 (ARF1/3) by catalyzing a GDP/GTP exchange on the the target. Activation of ARF1/3, is necessary for membrane budding to occur in the Golgi apparatus, and for vesicular trafficking of proteins. GEP2, another guanine nucleotide exchange factor, has recently been suggested to be involved in trafficking of assembled GABA receptors from the ER to the endosome or cell membrane in HEK2932 cells [10]. In *C. elegans*, *nicr-31* is embryonic lethal (reported from the Japanese National Bioresource Project), suggesting that the gene either functions in development, or is involved in essential cellular mechanisms. *nicr-31* has by RNAi been reported to be involved in embryonic and larval development, growth and locomotion. Even though the homology to GEP1 indicates a possible role in trafficking of nAChRs, the restrictive embryonic expression pattern of the operon suggest that the nicotine resistance was caused by a developmental delay rather than a direct effect on cholinergic signaling.

The function of *nicr-33* has not been determined, but the gene contains an F-box, a domain which mediates protein-protein interactions with Skp1. Skp1, the F-box protein and a third protein, Cullin, form SCF complexes, which act as E3 ubiquitin ligases in the ubiquitin-proteasome system. E3 ligases target substrates for degradation, and thereby control the abundance of the targets. Ubiquitination plays an important role in overall activity of receptors, not only regulating the number of receptors in the cell surface, but also the proportion of active receptors, making components of the ubiquitin system

possible players in nicotine resistance. The obtained expression of *pnicr-33*::GFP is restricted to the pharynx, suggesting a muscle specific function of the gene and potential involvement in regulation of nAChRs. However, modification of nAChR activity in pharyngeal muscle does not explain how the phenotype was generated, since nicotine induced paralysis mainly occurs in body wall muscle. A possible explanation is that the dsRNA has targeted several F-box proteins in other tissues than the pharynx, or that the knock-down of *nicr-33* results in development delays. Further studies of a loss of function mutant of *nicr-33* could resolve if the gene is involved in cholinergic signaling or development.

nicr-37 consists of two domains, one 99.5% homologous to human alpha-NAC, and another homologous to ubiquitin associated protein (UBA). alpha-NAC is one of the first cytosolic proteins to interact with the nascent polypeptide as it is being translated by the ribosome. By binding mistranslated and misfolded peptides, it prevents the peptides from binding the ER membrane and being translocated, thereby protecting the cell from defect proteins. [11]. The UBA domain does not influence the NAC activity and is suggested to be involved in cotranslational degradation of the defect peptide [12].

alpha-NAC is also reported to function as a bone-specific transcription factor, and coactivate c-Jun mediated transcription in osteoblasts. Acting in transcriptional regulation, alpha-NAC and has been suggested to be involved in apoptosis, development and cell proliferation [13]. Both proposed roles suggest that the gene might have a severe effect on viability and development of organisms. It remains to be explored if the observed expression of *pnicr-37*::GFP in pharynx, vulva and neurons signifies specific regulation of cholinergic signaling, or if the observed nicotine resistance is due to unspecific effects, caused by developmental delays or by defect proteins not being properly degraded.

nicr-40 is a GPCR belonging to the rhodopsin superfamily and related to the mammalian neuropeptide Y receptor. The function of *nicr-40* is not known, and the ligand has not been determined. Proteins with clear homology to neuropeptide Y have not been described in *C. elegans*, indicating a different ligand than in mammalian systems. Expression of *pnicr-40*::GFP was observed both in head and tail neurons, including amphid and phasmid neurons, and in the nerve cord, supporting a neuronal function of *nicr-40*. Expression of *pnicr-40*::GFP was also observed in the intestine, and it would be of interest to clarify if *nicr-40* functions both in neurons and intestine, or if the absence of important regulatory sequences might have given an incorrect pattern. A full-length protein fusion to GFP could be used to further examine the expression of *nicr-40*, and deletion mutants could be tested for nicotine resistance to establish if the gene is involved in cholinergic signaling.

nicr-42 is an orphan GPCR related to the mammalian neuromedin U receptor 2. The expression pattern is restricted to head neurons, including amphids, the phasmids, nerve cord, and surprisingly coelomocytes. The expression in neurons suggests that *nicr-42* is involved in neuronal signaling, and possibly modulates cholinergic signaling. The expression in the coelomocytes could imply a different function. The coelomocytes are three pair of scavenger cells which continuously take up molecules from the

pseudocoeleom by endocytosis for degradation. The coelomocytes have been suggested to function as a "liver", and to clean up the body fluid from hazardous molecules, but are not necessary for viability of nematodes in laboratory conditions. The expression of *pnicr-42*::GFP in coelomocytes implies a possibility that *nicr-42* is involved in endocytic trafficking, and that the RNAi mediated knockdown of *nicr-42* results in defect endocytosis in neurons or coelomocytes, or both. The rhodamine-uptake assay showed that endocytosis in the coelomocytes was functional in *nicr-42(ok1387)*, suggesting that *nicr-42* is not essential for endocytosis. However, the rhodamine uptake in *nicr-42(ok1387)* and wildtype was observed 6 h after rhodamine injection, so subtle delays or differences would not be detected. Careful time-course experiments to study uptake of rhodamine could more accurately determine if *nicr-42* affects endocytosis in coelomocytes.

Defective endocytosis in neurons could affect cholinergic signaling, either by influencing the number of active nAChRs on the cell surface through trafficking, or by acting upstream or downstream of nAChRs. Recent studies have shown that expression of nAChR in HEK293 cells is induced by endocytosis of ErbB proteins in response to neuregulin [14] and that rapid SNARE-dependant endocytosis of nAChRs in chick ciliary ganglion neurons is required for downstream signaling of the receptors [15]. *cup-4*, an orphan receptor in *C. elegans* homologous to a nicotinic receptor subunit, has been shown to be necessary for endocytosis in coelomocytes [16]. This opens up for the idea that *nicr-42* could have a function related to nAChRs in coelomocytes, which would explain the otherwise unexpected expression of *pnicr-42*::GFP in cells not involved in neuronal cholinergic signaling. Examination of nicotine resistance and endocytosis function of *nicr-42(ok1387)* will clarify if *nicr-42* is involved in regulation of nAChRs in neurons, and in endocytosis in the coelomocytes.

No expression patterns were obtained from *pnicr-3*::GFP and *pnicr-26*::GFP in spite of successful injections. Most likely, enhancer elements and/or intronic and exonic sequences were needed for expression.

Expression patterns generated from promoter regions should be regarded as approximations. *C. elegans* has a compact and simple genome with promoter regions no longer than 2 kb, and few enhancers and suppressors compared to other organisms. Many genes investigated through expression constructs include a few thousand or hundred bases of the upstream region, and have given patterns confirmed by other methods. However, since the constructs lack enhancers, repressors, and elements in the introns, exons and 3' ends, which can have a role in transcriptional and translational regulation of the gene, an obtained expression pattern may not be completely correct. Ideally, the entire gene with its promoter should be fused to GFP, resulting in a properly expressed full-length protein fusion. This approach would not only give a more accurate expression, but also show subcellular localization of the protein, giving further indication of the function of the protein. Because of the large construct size that such full-length protein fusions generate, this method may not be worthwhile for screening a larger number of genes. An acceptable compromise for screening is to use the promoter region and include part of the gene in the reporter construct, which increases the specificity of the GFP expression, and keeps a reasonable size of the construct. For genes that seem interesting from several

different aspects, such as its putative function, phenotype and expression pattern, full-length protein fusions can be generated.

Another drawback with the method is the mosaicism of transgenic lines. Some of the cells may not contain the extrachromosomal array, and may therefore not express the reporter. This problem can be overcome by integration of the array in the genome, but this time consuming approach is not suitable for screening. Obtaining and studying several independent lines generally gives a good picture of the expression pattern.

In summary, transformation of expression constructs is an important experimental tool for *in vivo* studies of gene expression in *C. elegans*. In spite of its limitations, the method gives important clues about the function of a gene. Expression patterns were obtained for seven of the nine constructs. The exclusive embryonic expression *pnicr-31*::GFP, the broad expression pattern of *nicr-13* and decreased cuticle permeability of *nicr-13(ok486),* and the putative function of *nicr-37,* suggests that the RNAi mediated nicotine resistance of these gene was due to developmental or general cellular defects. The obtained neuronal expressions of *pnicr-40*::GFP and *pnicr-42*::GFP propose a possibility of involvement of *nicr-40* and *nicr-42* in regulation of neuronal nAChRs. To establish the role of these genes, the phenotype generated by RNAi should be confirmed with mutants. *nicr-10* does from the expression pattern not seem to be involved in cholinergic signaling, but could be further explored due to the doubt of whether the right promoter region was used.

6. FUTURE PROSPECTS

Since RNAi does not always completely eliminate the target gene product, and also may target several homologous molecules, it is essential to confirm the generated phenotype with knock-out mutants of the candidate genes. This will confirm which genes truly imply nicotine resistance. The candidate gene mutants that are available are currently being tested in the lab.

An important piece of information when characterizing the candidate genes, is if the surface abundance of the nAChRs is influenced when the gene is knocked down or mutated. This can be studied by the use of a strain with epitope tagged nAChR subunits. By injecting rhodamine-conjugated antibodies a staining pattern reflecting the surface expression of the tagged nAChR subunit will be obtained. By comparing staining patterns of knockdowns or mutants with wildtype, effects on surface expression caused by knock-down of the candidate genes can be detected and quantified.

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