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Nicotine affects protein complex rearrangement in *Caenorhabditis elegans* cells

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**Abstract**

Nicotine may affect cell function by rearranging protein complexes. We aimed to determine nicotine-induced alterations of protein complexes in *Caenorhabditis elegans* (*C. elegans*) cells, thereby revealing links between nicotine exposure and protein complex modulation. We compared the proteomic alterations induced by low and high nicotine concentrations (0.01 mM and 1 mM) with the control (no nicotine) *in vivo* by using mass spectrometry (MS)-based techniques, specifically the cetyltrimethylammonium bromide (CTAB) discontinuous gel electrophoresis coupled with liquid chromatography (LC)–MS/MS and spectral counting. As a result, we identified dozens of *C. elegans* proteins that are present exclusively or in higher abundance in either nicotine-treated or untreated worms. Based on these results, we report a possible network that captures the key protein components of nicotine-induced protein complexes and speculate how the different protein modules relate to their distinct physiological roles. Using functional annotation of detected proteins, we hypothesize that the identified complexes can modulate the energy metabolism and level of oxidative stress. These proteins can also be involved in modulation of gene expression and may be crucial in Alzheimer’s disease. The findings reported in our study reveal putative intracellular interactions of many proteins with the cytoskeleton and may contribute to the understanding of the mechanisms of nicotinic acetylcholine receptor (nAChR) signaling and trafficking in cells.

**Keywords**

Nicotine, insulin signaling, fatty acid metabolism, oxidative stress, hormesis, Alzheimer’s disease, epigenetics

**History**

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**Introduction**

Most of us are affected by nicotine, not only because of the common use of tobacco products; nicotine is also included in many popular vegetables of the family Solanaceae, such as tomatoes and peppers (Siegmund et al., 1999). However, these two sources provide the body with radically different doses of nicotine (Matta et al., 2007). Nicotine, contained in tobacco, is a common drug of addiction and smoking is a leading cause of preventable deaths in developed countries (WHO, 2012). This alkaloid interacts with nicotinic acetylcholine receptors (nAChRs), with varying affinities that can act on a variety of downstream effectors (Schuller, 2009; Sobkowiak & Lesicki, 2011). Nicotine competes with the hormone/neurotransmitter acetylcholine for binding to its receptors. Nicotinic receptors appeared in evolution long before the development of the nervous system. Further, cholinergic signaling appeared in the early stages of evolution and seems to be conserved; therefore, the current study employed the *C. elegans* model to investigate nicotine-induced changes in protein complex patterns in cells and to gain better insight into cellular responses to nicotinic receptor activation in humans (Skok, 2009).

The free-living nematode *C. elegans* is one of the best-studied multicellular model organisms. *C. elegans* has emerged as a powerful experimental system to study the molecular and cellular aspects of human disease *in vivo*. It has been estimated that approximately 42% of human disease genes have an ortholog in the genome of *C. elegans*, including genes associated with Alzheimer’s disease, Parkinson’s disease, spinal muscular atrophy, hereditary nonpolyposis colon cancer and many others age-related disorders (Markaki & Tavernarakis, 2010).

Due to its short lifespan, transparent body and completed genome sequence, *C. elegans* has become an ideal model system in basic biological research. It is also an excellent model organism for the study of drug action (Engleman et al., 2016; Musselman et al., 2012; Schafer, 2004; Wolf & Heberlein, 2003), including studies of the response to nicotine (Feng et al., 2006; Sellings et al., 2013; Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2011; Wonnacott et al., 2005). Mammals have 17 genes encoding nAChR subunits, in contrast to the nematode *C. elegans*, which has 29 nAChR subunits (Millar, 2003).

In previous studies, worms showed acute responses to nicotine exposure and after no more than 60 min in the
presence of nicotine, most of the worms completely changed their behavior; depending on the nicotine concentration, they move faster than in control conditions or are paralyzed (Sobkowiak et al., 2011). The reaction in some conditions is quick and takes less than 10 min. Therefore, it could not be explained by protein synthesis de novo, but rather by a disturbance of cholinergic signaling and dysfunction of motor protein complexes.

In C. elegans, proteomic approaches are part of the essential toolbox for the study of gene function. It is well-known that protein function is often dependent on its interactions with other molecules, by formation of specific, macromolecular protein complexes. Thus, the same proteins may be present in different types of complexes (Fonslow et al., 2014). Some techniques enable high-throughput screening of a large number of proteins in a cell, such as yeast two-hybrid, tandem affinity purification, mass spectrometry (MS), protein microarrays, synthetic lethality and phage display (Fonslow et al., 2014). We used the protocol based on the cationic detergent cetyltrimethylammonium bromide (CTAB) (Akins et al., 1992), which is the most effective agent for the solubilization of highly hydrophobic proteins and integral membrane proteins (Polati et al., 2009). Rozema & Gellman (1995) in the early 90s used CTAB as an artificial chaperon to prevent the misfolding and aggregation of recombinant proteins. CAT electrophoresis allows the separation of proteins to be carried out with the retention of native activity (Akins et al., 1992). Many proteins separated on CAT gels may remain in native complexes, and under the conditions presented in our study, CTAB may be considered as non-denaturing detergent. The combination of CAT gel electrophoresis and tandem MS is a simple method for revealing many potential interacting proteins associated directly or indirectly with a protein of interest. The use of CAT electrophoresis allowed comparison of the number and intensity of bands in the control versus nicotine-treated nematodes.

Since the isolation of protein complexes from native tissue enables the analysis of interactions within the normal cellular milieu, it provides a significant advantage over traditional protein interaction screens, such as yeast two-hybrid and tandem affinity purification. The interaction discovery techniques, like the two-hybrid system or affinity purifications, suggest physical associations between proteins (Aloy & Russell, 2004). On the other hand, direct physical interactions of proteins might be rather limited, covering perhaps <1% of the theoretically possible interaction space (Franceschini et al., 2013). Probably, proteins do not necessarily need to undergo a stable direct interaction to have a specific, functional interplay. Together with direct, physical interactions, such indirect interactions constitute the larger superset of functional protein–protein associations (Franceschini et al., 2013).

Other methods focus on monitoring and characterizing specific biochemical and physicochemical properties of a protein complex (Shoemaker & Panchenko, 2007a, 2007b). In the C. elegans community, the MS-based proteomic studies of the protein–protein interactions are becoming increasingly common (Fonslow et al., 2014).

Four proteomic studies have considerably expanded the number of proteins predicted to be associated with nAChRs (Kabbani et al., 2007; Paulo et al., 2009, 2013; Yeom et al., 2005). Kabbani et al. (2007) used matrix-assisted laser desorption ionization time-of-flight tandem MS to identify 21 proteins that were either pulled down with the TM3–TM4 loop of mouse β2 or co-immunoprecipitated with β2. Paulo et al. (2009) used MS to analyze brain proteins, affinity-immobilized by α-bungarotoxin (which binds to α7 nAChRs), from wild-type mice and mutant mice lacking α7. A comparison of the results identified 55 proteins in wild-type samples that were not present in comparable brain samples from α7 nAChR knockout mice (Paulo et al., 2009). Paulo et al. (2013) compared the proteomic alterations induced by nicotine treatment in cultured pancreatic cells by using MS-based techniques, specifically SDS-PAGE coupled with liquid chromatography (LC)–MS/MS and spectral counting (Paulo et al., 2013). In their study, out of thousands of proteins in pancreatic cells, hundreds were identified exclusively or in higher abundance in either nicotine-treated or untreated cells. Using two-dimensional electrophoresis, Yeom et al. (2005) presented the proteomic analysis of nicotine addiction-associated proteins in the striatum of rat brains. Most proteomic studies investigating nicotinic receptors focused on neuronal receptors, in contrast, we did not select for proteins expressed specifically in neurons.

Due to the limited number of studies on nicotine’s effects on rearrangements of protein complexes, we initiated a study on proteomic effects of nicotine on C. elegans. The nicotine dosages were selected based on our preliminary study as well as previous reports (Feng et al., 2006; Matta et al., 2007; Sobkowiak & Lesicki, 2009, Sobkowiak et al., 2011, 2014, Taki et al., 2014; Waggoner et al., 2000), in which nicotine treatment had a biphasic response. A high dose of nicotine (1 mM) is genotoxic (our unpublished data), while a reasonably low (0.01 mM) concentration has a protective effect (Sobkowiak & Lesicki, 2009). Nicotine did not evoke cytotoxic effects in any of our experiments (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014). Sudheer et al. (2007a,b) revealed that nicotine did not exert any cytotoxic effects even up to the 1000-mM concentration. On the other hand, abstracting from details, C. elegans behavior in the presence of 0.01 mM nicotine is very similar to worm behavior in the presence of 1 mM nicotine and in the control conditions (Sobkowiak et al., 2011), although the acute locomotor effects were revealed to be one of the most sensitive indicators of drug effects (Leung et al., 2008). The scientific data exploring the acute response to nicotine at the inner cellular protein complexes level are still scarce. Thus, like in our previous research, acute effects are described as those resulting from a 60-min exposure.

Here, we report identification of protein complexes associated with nicotine treatment. For the first time in C. elegans, we used the CAT gel system to observe protein complexes, and identified interacting proteins by MS, during the acute response to nicotine. The potential role of nicotine in Alzheimer’s disease, insulin signaling, fatty acid metabolism, oxidative stress, hormesis and chromatin modulation is discussed. To our knowledge, this study for the first time
demonstrates a putative role of nicotine in modulation of protein complex composition.

**Experimental section**

**Materials**

Tricine, CTAB, arginine and free-base (−)-nicotine (≥99%, GC, liquid) (O. No. N3876) were purchased from Sigma-Aldrich, St. Louis, MO. Acrylamide and bis-acrylamide were obtained from Fluka, distributed by Sigma-Aldrich, St. Louis, MO.

*C. elegans* maintenance

All tests were performed on the wild-type Bristol N2 strain of *C. elegans* obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (Minneapolis, MN). Standard methods were used for the maintenance and manipulation of strains (Stiernagle, 2006). Nematodes were maintained at 22°C on 5 cm nematode growth medium (NGM) agar plates seeded with *Escherichia coli* (OP50).

All the experimental procedures presented in this paper were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

*C. elegans* synchronization

L1 larvae were prepared by egg synchronization. The hermaphrodites were lysed in 20% bleach (Clorox), 0.5 M NaOH, until fragmented. The resulting eggs were incubated in M9 buffer (Stiernagle, 2006) without food overnight (14–18 h) at 22°C with agitation (150 rpm), to allow eggs to hatch, and reach arrested development due to starvation (Stiernagle, 2006). Resulting larvae were used to obtain synchronous cultures of *C. elegans*. Large quantities of *C. elegans* were grown in 100 mL of liquid S-medium, by using concentrated *E. coli* OP50 as a food source (Sobkowiak et al., 2011; Stiernagle, 2006). A synchronous population of worms was needed in order to eliminate variation in results due to age differences.

**Experimental workflow**

Young adult worms (42 h of growth in S-medium at 22°C) were fully developed but were not egg-laden, as nicotine has been shown to affect egg laying behavior (Waggoner et al., 2000). The 100-mL liquid cultures of young adult *C. elegans* were divided into three equal parts. The first part was collected as a control (no nicotine), the second was treated with 1 mM (−)-nicotine for 60 min and the third one was treated with 0.01 mM (−)-nicotine for 60 min. The animals were treated with nicotine in liquid culture without food under rotary agitation on a shaker at 150 rpm at 22°C, so that the cultures were well-oxygenated. The nicotine was washed away from the animals prior to testing: worms were collected from each condition at 60 min following nicotine exposure, washed 5 times with S-medium on a coarse sintered-glass funnel through paper filter. The experimental workflow is summarized in Figure 1. The experiments were performed in parallel and we repeated the experiment (four independent experiments) for both the nicotine-exposed and control groups.

**Protein extraction**

Young adult worms were homogenized with a pestle for about 1 min then placed into 1.5 ml microcentrifuge tubes in 900 mL of CAT sample buffer (10 mM Tricine–NaOH pH 8.8, 1% CTAB, 10% glycerol, 5 mM NaF and 0.01% phenylmethanesulfonyl fluoride) (Akins & Tuan, 2002). The samples were spun in a microfuge for 0.5 min at 16 000 g to pellet debris or insoluble material prior to loading the gel. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Appleton, WI).

**CAT gel analysis**

The proteins were analyzed by CTAB discontinuous gel electrophoresis (CAT gel system) (Akins & Tuan, 1994; Walker, 2002). A Hoefer SE600 vertical slab gel unit (Hoefer Scientific Instruments, Holliston, MA) was used with stacking and separating gels consisting of 4 and 10% acrylamide, respectively. The samples containing the same amount of proteins (100 μg of protein per line) were separated at 40 V in CAT gel for 18 h at 22°C. After the tracking dye had exited the separating gel, the gel was removed and stained with Coomassie blue R-250 (Sigma-Aldrich, St. Louis, MO).
Protein identification

Rebuilding of protein complexes due to nicotine presence results in changes in protein complex patterns visualized by staining of the CAT gel. Any changes detected in the whole gel for protein complexes band localization or intensity would be selected for analysis. However, in our experiment the most pronounced effect of nicotine influence was observed in the top area of the gel. Thus, this subregion of the protein gel was included for further analysis. Three bands from each lane at the top of the gel were selected and excised.

Separation of peptides with high pressure liquid chromatography and subsequent tandem mass spectrometry analysis was performed at the Mass Spectrometry Laboratory of Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Peptides mixtures were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS/MS) using Nano-Acquity (Waters) LC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA). Prior to the analysis, proteins were subjected to standard “in-solution digestion” procedure (Przewloka et al., 2011) during which proteins were reduced with 200 mM dithiothreitol (for 60 min at 60 °C), alkylated with 200 mM iodoacetamide (45 min at room temperature in darkness) and digested overnight with trypsin (sequencing Grade Modified Trypsin – Promega V5111). The peptide mixture was applied to RP-18 precolumn (nanoACQUITY Symmetry® C18 – Waters 186003514) using water containing 0.1% TFA as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18 – Waters 186003545) using an acetonitrile gradient (5%–35% AcN in 70 min) in the presence of 0.05% formic acid with the flowrate of 250 nL/min. The column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. Other MS parameters were the following: m/z range: 300–2000 Th; MS1 resolution: 35 000; MS2 resolution: 15 000; number of precursor ions for fragmentation: 10; intensity threshold for tandem MS: 10⁵; precursor selection mass window: 2 Th; charge state screening parameters: 2 + and above; relative CE of 40%; dynamic exclusion: disabled; APEC searching: enabled. A blank run (injection of Milli-Q water) ensuring cross contamination monitoring from previous samples preceded each analysis.

Bioinformatics and data analysis

Acquired raw data were processed by Mascot Distiller v. 2.4.2 followed by Mascot Search v. 2.4.1 (Matrix Science, London, UK, on-site license) against NCBI nr database v. 20130325, 23 919 380 entries restricted to Eukaryota. Search parameters for precursor and product ions mass tolerance were: 20 ppm and 0.6 Da, respectively; enzyme specificity: trypsin; missed cleavage sites allowed: 1; fixed modification of cysteine by carbamidomethylation and variable modification of lysine carboxymethylation and methionine oxidation.

Standard methods built into Mascot were used to determine if peptides significantly exceeded threshold values (Perkins et al., 1999). The peptides with Mascot scores exceeding the threshold value, corresponding to <5% expectation values (in this particular case peptides exceeding Mascot Score of 30, see supplemental data for details) were considered as positively identified.

All data generated from the gel sections were searched by BLAST against the WormBase database (http://www.wormbase.org/). The interactions among the identified proteins were analyzed using as reference the STRING database of known and predicted protein interactions including both validated physical protein–protein interaction and putative interaction inferred from co-expression, gene fusion and text mining, etc. (http://string-db.org/) (Franceschini et al., 2013). The open-source software Cytoscape ver. 3.0.2. (http://www.cytoscape.org/) was used to visualize the molecular interaction networks and biological pathways as well as to integrate these networks with annotations, gene expression profiles and other state data. C. elegans proteins were annotated using terms from the Gene Ontology (GO) project (http://www.geneontology.org).

Venn diagrams

The eulerAPE software (drawing area-proportional Euler and Venn diagrams using ellipses) was employed to illustrate the distribution of protein appearance after nicotine treatment, compared with control samples (http://www.eulerdiagrams.org/eulerAPE/).

Estimation of protein abundance

Exponentially modified protein abundance index (emPAI) – an approximate, label-free, relative quantization – was used for the estimation of absolute protein abundance in samples (Ishihama et al., 2005). Since emPAI is highly dependent on peptide sampling in MS, only high-quality emPAI values were reported (i.e. containing more than 100 spectra) as described in MASCOT’s emPAI calculation procedure. The mean value was calculated using data from four biological replications.

Results

CAT gel electrophoresis of C. elegans proteins in different nicotine concentrations compared with control conditions

In the gel area where the proteins formed large complexes, we observed changes in the position and intensity of the bands in the presence of nicotine, compared with control conditions (see enlarged view in Figure 2). The pattern of bands in the control was more similar to that of the bands from samples treated with 1 mM nicotine than to that of the bands observed in the 0.01 mM nicotine group. The patterns were almost similar in all the replications of experiments (Figure 2).

Core proteins identified in complexes across all bands in both nicotine-treated and untreated C. elegans

Four biological replicates were tested and a protein was taken into the consideration if it was detected in all replicates in the same band. Using this criteria, MS analysis identified 597 proteins. We found that approximately 15% (n = 91) of the proteins appeared in all gel bands (see Figures 2 and 3). These proteins, shared among control and both nicotine treatments, are listed in Table 1 (see supporting information). The complexes migrate on CAT gels as three closely spaced...
bands (Figure 2). Most of the determined proteins would be considered as “high abundance proteins”. Overall, the identified proteins include cytoskeletal components (e.g. actin, myosin and tubulin), proteins involved in vesicular transport (clathrin, kinesin), and others: spectrins, heat shock proteins, vitellogenin, ribosomal proteins, translation elongation factors, transitional endoplasmic reticulum ATPase (CDC-48), mitochondrial proteins, etc. (Table 1 in supporting information).

Proteins specific to nicotine-treated worms

The proteins unique to the nicotine-treated *C. elegans* cells were compared with the proteins unique to the untreated samples.
Generally about 5% of the total proteins identified were exclusive to untreated worms (see Figure 3, Venn VII “only 1”: 29 proteins), while about 6% (see Venn VII “only 2”: 38 proteins) and 20% (see Venn VII “only 3”: 118 proteins) of the total proteins identified were exclusive to high and low nicotine conditions, respectively. About 45% (see Venn VII common to 1, 2 and 3: 271 proteins) of the identified proteins were found in both nicotine-treated and untreated worms (Figure 3).

Proteins identified exclusively in nicotine-treated worms are listed in Tables 2–4 (see supporting information). We identified 53 proteins unique to both 0.01 mM and 1 mM nicotine-treated worms (Table 2) as well as 118 and 38 proteins unique only to the 0.01 mM nicotine-treated worms (Table 3) and only to the 1 mM nicotine-treated worms (Table 4), respectively. Proteins identified exclusively in untreated worms (control) are listed in Table 5. Proteins unique to the high, intermediate and low molecular weight bands specifically are shown in Tables 6–8, respectively (see supporting information).

**Protein complexes**

By using Cytoscape and protein interaction data retrieved from the STRING database, we visualized the interaction networks of the identified proteins in different experimental conditions. Most forms of proteins are included in the complex (Figure 4). In Figure 5, we show the core complex of proteins shared by all experimental conditions.

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**Venn diagrams No.:**

I 1a vs. 1b vs. 1c
II 2a vs. 2b vs. 2c
III 3a vs. 3b vs. 3c
IV 1a vs. 2a vs. 3a
V 1b vs. 2b vs. 3b
VI 1c vs. 2c vs. 3c
VII 1 vs. 2 vs. 3
VIII a vs. b vs. c
IX 1a1b1c vs. 2a2b2c vs. 3a3b3c
X 1a2a3a vs. 1b2b3b vs. 1c2c3c

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Figure 3. Area-proportional Venn diagrams comparing proteins from *C. elegans* treated with 0.01 mM and 1 mM nicotine and from the control (0 mM nicotine). For example, 1a1b1c means proteins common to the 0 mM nicotine condition, occurring simultaneously in all its bands, while “only 1a” means proteins unique to the band 1a only occurring there. The number of proteins detected in each of the set is given in parentheses. Venn diagrams marked by roman numerals. To identify and locate proteins clearly in different lanes, throughout the article the following notation was used: 1 = control worms (no nicotine); 2 = worms exposed to the high concentration of nicotine (1 mM); 3 = worms exposed to the low concentration of nicotine (0.01 mM). Letters indicate the position of bands: a = highest molecular weight; b = intermediate molecular weight; c = lowest molecular weight. As a result, each band is marked by a number followed by letter, e.g. 2c denotes the lowest band in the 1 mM nicotine group/condition. When comparing the composition of the various bands, e.g. 2a2c means the proteins common to bands 2a and 2c. The number in brackets indicates the number of identified proteins. “Only 2c” means a specific protein, found only in the specified set.
We calculated the important topological features of networks. These features include average shortest path, clustering coefficient, degree distribution and closeness centrality. To see networks features, please download Cytoscape from http://www.cytoscape.org and download file core.cys (http://goo.gl/MCSFMN) and all.cys (http://goo.gl/OHi2Q). Details of the core network consisting of 1235 interactions among 91 proteins and the entire identified 597 proteins network consisting of 18 711 interactions can also be viewed.

Quantitative proteomic differences among all experimental conditions

The majority of the proteins identified are common to both nicotine-treated and untreated C. elegans cells. Quantitative proteomic analysis was performed to investigate differences in the abundance of these proteins. The protein abundance differed among the control (0 mM nicotine) and two nicotine concentrations (0.01 and 1 mM) (Figure 3). The differences in staining intensity (Figure 2) reflect the relative abundance of proteins. MS analysis revealed dozens of proteins that were present in significantly different relative abundances in the nicotine-treated worms than in the control (see supporting information). For example, dimethylglycine dehydrogenase was more abundant in the nicotine-treated worms, the remaining proteins of this group were more abundant in the control. Table 9 includes 182 differentially abundant proteins that were identified in all experimental conditions.

Discussion

General remarks

We investigated nicotine-induced changes in protein complex patterns in cells of C. elegans. Several proteome studies of C. elegans, using various methods of separation, detection and quantification were reported earlier (for a review, see Schrimpf & Hengartner, 2010), but to our best knowledge, we used for the first time the CAT electrophoresis and MS to identify protein complexes.

We compared the proteomic alterations induced by low and high nicotine concentrations (0.01 mM and 1 mM) with the control (no nicotine). Proteins identified in C. elegans were present exclusively or in higher abundance in either nicotine-treated or untreated worms. We report a putative network that captures the key protein components of nicotine-induced protein complexes, and identified complexes that can modulate the energy metabolism and level of oxidative stress. These proteins can also be involved in modulation of gene expression and may be crucial in Alzheimer’s disease. The findings reported in our study reveal intracellular interactions of many proteins with the cytoskeleton. Many of the listed proteins – such as profilin, filamin, myosin and plectin – are common cytoskeletal proteins that bind actin, which may be associated with the protein pattern reorganization observed after nicotine treatment, as shown in Figure 2.

We observed differences in the protein-banding patterns on gels, which are not clearly noticeable in the presence of nicotine in other proteomic analyses (Paulo et al., 2013). Paulo et al. (2013) observed only minor visible differences in the protein banding patterns within same cell type replicates and similarly, when comparing nicotine-treated and untreated cells of the same type. Such a result would indicate that SDS-PAGE used by Paulo et al. (2013) indeed separates polypeptides and subsequent MS analysis would identify common proteins regardless of nicotine treatment because SDS destroy most of the complex structure of proteins (Walker, 2002). A potential explanation why the results from our study with nicotine are somewhat different from those using SDS gels is that acute response to nicotine is triggered by protein complex rearrangements which are not possible to detect using SDS-PAGE.

The most visible differences in the protein complex pattern were observed in the presence of low nicotine concentrations, compared with the controlled conditions and high nicotine concentration (Figure 2). In the presence of 0.01 mM nicotine, a lower intensity of bands was observed. This may be due to the fact that in the analyzed protein complexes several peptidases were found, e.g. NEPrIlysin metallopeptidase, aspartyl protease (ASP-1, ASP-2) and elements of proteasome RPN-10 (supplemental Table 3). Surprisingly, a plethora of other proteins unique to the low nicotine concentration associated with the core complex described above was revealed.

Our MS analysis of proteins separated by CAT gel electrophoresis has identified many proteins that were not among the proteins listed by other authors (Kabbani et al., 2007; Paulo et al., 2009, 2013). Among the identified peptides, there are also many novel proteins, often referred to as “unnamed”, that have not yet been described in the context of cellular response to nicotine.

C. elegans responds to nicotine, a major component of cigarette smoke, in a manner similar to that of mammals. Further, it converts nicotine to cotinine, showing that it breaks down nicotine in a similar manner to humans (Green et al., 2009). Under physiological conditions, the cell is exposed to a number of external factors, of which only a few induce adaptive responses. The process begins at the moment in which the cell membrane protein receptor receives an extracellular signal and begins transduction of the signal across the plasma membrane, and the final cell reaction is initiated. An acute response of cells to a signal is mainly based on a change in the activity of the enzymes and protein complex rearrangements. It is known from yeast studies that most of these complexes are composed of several core proteins and several proteins that may temporarily connect to them (Gavin et al., 2006).

The proteins that together formed the bands were excised for analysis. The presence of the proteins in the same band does not necessarily mean they are in a physical complex with each other (Figure 2). The bioinformatic analysis, using existing databases, showed that many of the proteins identified here form complex networks with permanent interactions (see Figure 5). We suppose that the retention of native protein activity noted by Akins et al. (1992) is at least partially due to the fact that proteins remain in native complexes. The existing data contained in large databases of protein interactions, such as STRING (Franceschini et al., 2013), confirm this hypothesis. Almost all proteins
identified by us in the bands interact with each other, suggesting that indeed they may form complexes (see Figures 4 and 5). In our experiments, we identified the core proteins, present in all tested bands. The core complex contains 91 proteins (Figure 3, Table 1). The complex binds other proteins and is involved in many processes. In our opinion, it is most important to discuss here the aspects of cell stress and changes in proteins involved in Alzheimer’s disease.

Nicotine and cell stress

In the presence of 0.01 mM nicotine, we found a 4-5-fold decrease in vitellogenin (VIT-1 and VIT-5), which is predicted to function as a lipid transport protein (Zhang et al., 2013). It is well-known that nicotine may influence the fat stores, as an increase in body weight is observed in many people after they quit smoking (Filozof et al., 2004). Smoking stimulates lipolysis in vivo (Andersson & Arner, 2001). Lipolysis appears to be the most sensitive system to nicotine stimulation among various metabolic processes (Andersson & Arner, 2001). Nicotine can also stimulate lipolysis directly, by binding to nicotinic cholinergic receptors in adipose tissue (Andersson & Arner, 2001). The increase in the rate of lipolysis leads to increased concentrations of free fatty acids. In humans, nicotine decreases food intake and increases thermogenesis, increases the delivery of free fatty acids to the liver and skeletal muscle, and decreases muscle glucose uptake (Bajaj, 2012). These effects of nicotine are associated with increased lipoprotein secretion and intramyocellular lipid saturation as well as peripheral insulin resistance (Bajaj, 2012).

Figure 4. The protein complex network of C. elegans: view of the entire identified 597 protein dataset. It is not possible to clearly show all the data on the printed figures. Due to the high complexity of the protein network and the multiplicity of interactions between proteins the best way is analyzing the data in a special program – Cytoscape. It is an open source software platform for visualizing complex networks. Please download Cytoscape from http://www.cytoscape.org and download file all.cys (http://goo.gl/OhH2Q) to see in details all protein network. The data are complemented with interactions obtained from the STRING database, including coexpression (solid), cooccurrence (dots), database and textmining (backward slash), experimental (parallel lines), fusion (sinewave) and neighborhood (separate arrow) data. Also discussed proteins involved in Alzheimer’s disease (magenta), insulin signaling (blue), lipid storage and metabolism (violet), oxidative stress (khaki), chromatin modulation (dark green), structural constituent of cytoskeleton (red), nicotine signal transduction, kinases and phosphatases (green) were marked. For details, see also supplemental data. This figure is in color in the on-line version.
Among the proteins unique to the samples treated with the low concentration of nicotine, we found protein phosphatase PPM-1. According to WormBase, the best human ortholog for this protein is protein phosphatase 1B (PTP1B). PTP1B has emerged as a key regulator of signaling networks that are implicated in metabolic regulation, in particular, insulin signaling, endoplasmic reticulum stress response, cell–cell communication, energy balance and vesicle trafficking. Thus, protein-tyrosine phosphatase 1B is involved in metabolic diseases, such as obesity and type 2 diabetes (Bakke & Haj, 2014).

In the presence of the low nicotine concentration, we found additionally protein phosphatase 2A (PPTR-1). This phosphatase also connects nutrient levels to stress, metabolism, development, longevity and behavior, specifically through the insulin/IGF-1 signaling pathway (Murphy & Hu, 2013).
A PPTR-1 regulatory subunit regulates *C. elegans* insulin/IGF-1 signaling by modulating AKT-1 phosphorylation (Padmanabhan et al., 2009). AKT-1 is a crucial element in signaling of nAChRs and changes in PPTR-1 levels affect the activity of AKT-1 (Schuller, 2009; Sobkowiak & Lesicki, 2011). Activation of AKT by nicotine occurred within minutes at concentrations achievable by smokers (West et al., 2003). PPTR-1 also regulates DAF-16-dependent outputs of the insulin/IGF-1 signaling pathway, such as fat storage (Padmanabhan et al., 2009).

We observed fatty acid-binding proteins (FAR-6, FAR-1), very low-density lipoprotein receptor (F44E2.4) and many mitochondrial proteins. Nicotine probably contributes to an increase in the demand for ATP produced by mitochondria, which are transported by kinesin to the areas with an increased demand for energy (Yan et al., 2013). ATP can be consumed by the ABC transporter, SPG-7 metalloprotease, ATP-dependent RNA helicase and vacuolar H-ATPase (Fuller-Pace, 2013; Juhola et al., 2000; Lee et al., 2010; Sheps et al., 2004). We found all these enzymes in the presence of 0.01 mM nicotine (supporting information Table 3). This may suggest that low nicotine concentration may contribute to the modulation of ATP turnover.

In the samples treated with 0.01 mM nicotine, we found a 7-fold decrease in talin-1 (TLN-1), as compared with the control conditions. TLN-1 is predicted to have insulin receptor-binding activity, actin-binding activity, and is predicted to be a structural constituent of cytoskeleton. Talin-1 expression is correlated with reduced invasion and migration as well as decreased malignancy in human liver cancer cell lines; the suppression of talin-1 promotes invasion and migration (Fang et al., 2014).

Among the many proteins, we found also acyl-coenzyme A oxidase and 3-ketoacyl-CoA thiolase, which are involved in degradation pathways, such as fatty acid beta-oxidation (Poirier et al., 2006). Two substrates of acyl-CoA oxidase are acyl-CoA and O₂, whereas its two products are trans-2,3-dehydroacyl-CoA and H₂O₂. Reactive oxygen species (ROS), e.g. H₂O₂, are assumed to be detrimental to many biological processes. Catalase (CTL) is a very important enzyme in protecting the cell from oxidative damage by ROS, decomposing H₂O₂ (Nicholls, 2012). In our experiments, CTL joined within no more than 60 min to the complex core, and was present in the complex only after nicotine treatment (see supporting information Table 2). This suggests a very fast and efficient cell response to the presence of nicotine. CTL activity was found to be significantly increased in nematodes, with restriction of glucose metabolism only after several days (Schulz et al., 2007). In the presence of nicotine, we found all three *C. elegans* CTLs (CTL-1, CTL-2 and CTL-3).

The increase in fatty acid beta-oxidation and respiration causes increased ROS formation leading to mitohormentically increased catalase activity and stress resistance (Schulz et al., 2007). Induction of mitochondrial metabolism might cause a positive response to increased formation of ROS and other stressors, leading to a secondary increase in stress defense, cumulating in reduced net stress levels (Schulz et al., 2007). Schulz et al.’s (2007) findings are in accord with the hormesis theory of aging, hormesis being defined as a short-lasting and nonlethal stressor that induces the stress response mechanisms of an organism and thereby increases not only stress resistance. Also, hormesis has been observed in *C. elegans* under different conditions of stress, such as direct oxidative and thermal stress (Cypser & Johnson, 2002). This is in line with our previous work: we found that nicotine in some concentrations is genoprotective and in others it is neutral or genotoxic (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014). We observed in human leukocytes and *C. elegans* cells that treatment with a low concentration of nicotine results in a lower level of DNA damage than in control conditions (Sobkowiak et al., 2014). We therefore hypothesize that it was due to the presence of CTL in core proteins in places where hydrogen peroxide was generated. High concentrations of nicotine limited the capabilities of cells to defend against ROS and induced damage greater than under control conditions. The low concentrations of nicotine efficiently stimulated the cells’ ROS defense mechanism, as the amount of ROS was even smaller than in control conditions, resulting in a reduced amount of DNA damage in the presence of the low nicotine concentration, as compared with the control (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014).

Another symptom of stress was the appearance of proteins that are produced by cells in response to exposure to stressful conditions. Interestingly, the heat shock protein hsp-60 (HSP60) was about 7.5-fold more abundant at the low nicotine concentration in comparison to the control or high nicotine concentration. HSP60 is a mitochondrial chaperonin that is typically responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix. It has also been shown to be involved in the cell stress response: heat shock response is a homeostatic mechanism that protects cells from damage (Karunanithi & Brown, 2015). Calabrese et al.’s (2007) data are in favor of the importance of HSP-60 in the heat shock signal pathway as a basic mechanism of defense against neurotoxicity elicited by free radical oxygen produced in neurodegenerative disorders.

**Nicotine and proteins involved in Alzheimer’s disease**

Nicotine treatment, by stimulation of brains of Alzheimer’s disease patients through desensitization and upregulation of nicotinic acetylcholine receptors, has been shown to attenuate the decline in some of the cognitive deficits symptomatic of the disease and particularly effective in reversing attentional deficits (Pogocki et al., 2007). Amyloid beta (Aβ) is a peptide whose misfolding and aggregation in abnormal neural tissue has been implicated as a cause of Alzheimer’s disease (Walsh & Teplow, 2012). The following proteinases have the ability to degrade the Aβ peptide in Alzheimer’s disease: NEPrilysin; insulin-degrading enzyme; plasmin; endothelin converting enzymes; cathepsin; glutamate carboxypeptidase II; angiotensin-converting enzyme; etc. (Hong-Qi et al., 2012; Li & Buxbaum, 2011). Deficiency of NEPrilysin and insulin-degrading enzyme caused increased cerebral accumulation of endogenous Aβ in transgenic models of Alzheimer’s disease *in vivo* (Li & Buxbaum, 2011). In our experiment in the presence of a low concentration of nicotine, we found proteins which showed a high degree of homology to the NEPrilysin metallopeptidase family: NEP-22, NEP-2, F44E7.4 and cathepsin.
These findings seem to confirm the beneficial effects of nicotine in the course of Alzheimer’s disease treatment. They may be additionally supported by the fact that we found also F44E2.4. According to WormBase, its best human ortholog is a very low-density lipoprotein receptor. Interestingly, low-density lipoprotein receptor-related protein (LRP) regulates Aβ clearance (Li & Buxbaum, 2011).

It has been thought that prion proteins play an important role in the pathogenesis of Alzheimer’s disease (Kellett & Hooper, 2009). The cellular form of one prion protein interacts with and inhibits β-secretase (BACE1), the rate-limiting enzyme in the production of Aβ (Kellett & Hooper, 2009). β-secretase is a kind of aspartyl protease. In the presence of 0.01 mM we found prion-like proteins PQN-85, PQN-27 and aspartyl protease (ASP-1 and ASP-3). In all experimental conditions (0, 0.01 and 1 mM nicotine), we observed prion-like-(Q/N-rich)-domain-bearing protein (PQN-87). However, in the presence of 0.01 mM nicotine a decrease was observed in the abundance of this protein to 64%, as compared with control conditions. This strengthens the relationships of nicotine with prevention of Alzheimer’s disease.

In the presence of both concentrations of nicotine, we found transthyretin-like protein (TTR-1). Transthyretin (TTR) is thought to have beneficial side effects, by binding to the beta-amyloid protein, thereby preventing beta-amyloid’s natural tendency to accumulate into the plaques associated with the early stages of Alzheimer’s disease (Li & Buxbaum, 2011). Failures in the treatment of Alzheimer’s disease by using nicotine may result from the fact that in the presence of 1 mM nicotine we observed only a 27% increase in the abundance of this protein, as compared with the control.

Additionally, in the presence of 0.01 mM nicotine we found TTR-18 and TTR-16, which are transthyretin-like proteins. TTR is also the carrier of the thyroid hormone thyroxine. A single in vivo study in C. elegans suggested that wild-type human TTR could suppress the abnormalities seen when Aβ was expressed in the muscle cells of the worm (Link, 1995). Increased neuronal synthesis of TTR may favorably impact on Alzheimer’s disease because TTR has been shown to inhibit Aβ aggregation and detoxify cell-damaging conformers (Wang et al., 2014).

Our results may indicate that indeed nicotine contributes to the emergence of Alzheimer’s-related peptidase and prion-like proteins in the complex, and that these proteins are involved in the response to alkaloid. Thus, nicotinic drug treatment probably should be taken into account as a putative novel protective therapy in Alzheimer’s disease, but in a strictly controlled dose, suited to a given patient.

Other effects of nicotine on protein complexes

Binding of nicotine to the nicotinic receptor starts the signal transduction process. It has been shown, using confocal fluorescence microscopy and immunogold labeling in electron microscopic studies, that actin is co-localized with the nAChR, which is regarded as a crucial element in the response to nicotine (Shoop et al., 2000). Results from in vitro cotransfection experiments suggest that acetylcholine receptors are tethered to the actin cytoskeleton via several adapter proteins (Antolik et al., 2007). Here it is worth noting that among the proteins unique to the low molecular weight band (see supporting information Table 8), we found in all experimental conditions (0, 0.01, 1 mM nicotine concentrations) the NRA-4 nicotinic receptor-associated protein. NRA-4 may control nAChR subunit composition or allow only certain receptor assemblies to leave the endoplasmic reticulum (Almedom et al., 2009). NRA-4, evolutionarily conserved proteins forming a protein complex in the ER, affect synaptic nAChR subunit composition in C. elegans (Almedom et al., 2009). This nicotinic receptor-associated protein altered in vivo sensitivity to cholinergic agonists like nicotine, and could affect nAChR properties by influencing the representation of particular subunits in the mature receptors (Almedom et al., 2009). NRA-4 could act as a “nucleation center” for nAChR assembly and regulate inclusion of particular subunits during pentamer assembly. Only in the presence of low nicotine concentrations did we find nicotinic acetylcholine receptor ACR-21 (supporting information Table 3), which may indicate that it is only in low concentrations that the nicotinic receptor is strongly, physically connected to the protein complex observed. ACR-21 belongs to ACR-16 group which encodes an alpha-7-like homomer-forming subunit of nAChR superfamily orthologous to human nicotinic cholinergic receptor alpha 7. We hypothesize that the nicotinic receptor is not always directly, physically connected to the protein complex involved in the response to nicotine. This may explain some different results of experiments designed to detect proteins that form a complex with the nicotinic receptor (Kabbani et al., 2007; Paulo et al., 2009).

In our experiment, we also found seventeen of histone H3 forms (see supporting information Table 4). Nucleosome remodeling is associated with histone modifications (Tessarz & Kouzarides, 2014). It is noteworthy that we have identified 17 different histone H3 variants, showing highest similarity to human histone H3.2, deposited in transcriptionally silent areas that can be reversibly activated (Hake & Allis, 2006). Histone H3 is the most extensively modified of the five histones. It is an important protein in the field of epigenetics, where its sequence variants and variable modification states are thought to play a role in the dynamics and long-term regulation of genes (Tuesta & Zhang, 2014). Alterations in gene expression are implicated in the pathogenesis of several neuropsychiatric disorders, including drug addiction (Plazas-Mayorca & Vrana, 2011). Increasing evidence indicates that changes in gene expression in neurons, in the context of animal models of addiction, are mediated in part by epigenetic mechanisms that alter chromatin structure on specific gene promoters (Renthal & Nestler, 2009). Exposure to drugs of abuse induces changes within the brain’s reward regions in several modes of epigenetic regulation – histone modifications such as acetylation and methylation (Nestler, 2014). A recent genetic screening strategy has also identified histone H3 lysine 9 (H3K9) methylation as a key determinant for peripheral tethering of silenced heterochromatin at the nuclear envelope (Towbin et al., 2012). Histones were observed by us in the protein complex in the presence of 1 mM nicotine. In both nicotine conditions we discovered histone-arginine N-methyltransferase (PRMT-1) and S-adenosylmethionine (SAM). SAM generates the universal
concentration of nicotine both in C. elegans forming a DNA bulge. This observation was made in the low concentrations of nicotine both in C. elegans cells and in human leukocytes (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014). The increase in head radius was probably due to the size of the comet head that is formed in the comet assay in place of the nucleus (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014). The increase in head radius was probably due to the size of the comet head that is formed in the comet assay in place of the nucleus (Sobkowiak & Lesicki, 2009; Sobkowiak et al., 2014).

It should be stressed that our results indicate an approximate 23-fold and 7.7-fold increase in putative mitochondrial dimethylglycine dehydrogenase in the presence of 0.01 mM and 1 mM nicotine, respectively. Dimethylglycine dehydrogenase is a mitochondrial matrix enzyme involved in the metabolism of choline, converting dimethylglycine to sarcosine (Binzak et al., 2001). We suppose that nicotine induces imbalance in cholinergic signalization by stimulation of only the nAChR branch. Activation of only half of the cholinergic pathway by nicotine is detected by cells and in response the cells remove choline – the substrate used for the synthesis of acetylcholine. This is a probable explanation of the huge increase in the quantity of dimethylglycine dehydrogenase.

Conclusions

A good understanding of the remodeling of protein complexes will make it possible to create a map showing the interactions between all or some of the proteins of the proteome. This will be an important step in combining proteomics with cell biology. This process resembles constructing a three-dimensional puzzle composed of several two-dimensional puzzles stacked on top of one another, requiring the assembly of, often oddly shaped, interlocking pieces.

Simultaneous stimulation of nAChRs and mAChRs by acetylcholine may be required to synchronize and balance ionic and metabolic events in a single cell, and the net biologic response is determined by a unique combination of nAChRs and mAChRs expressed by an individual cell. Nicotine can imbalance these systems modifying only one branch of the feedback loop in cholinergic signalization system. Overstimulation nAChRs branch by high nicotine concentration likely results in reduction of cholinergic system signalization by degradation/stopping production of acetylcholine. Thus, the physiological effect of high nicotine concentrations was more similar to control conditions than the effect evoked by low nicotine concentration. The foregoing hypothesis may offer only one of a number of possible explanations for why the response to low doses of nicotine is different from the response to high doses of this alkaloid.

The results of this study may be of great importance for the understanding of the functioning of a single cell under normal conditions, and under conditions of various diseases or drug dependence. The associated protein complexes, in addition to nAChRs, might represent targets for the development of drugs to treat various diseases, like Alzheimer’s disease, obesity or diabetes. There is increasing interest in finding small molecules, e.g. nicotine and its derivatives, that affect protein–protein interactions, and this strategy could be used to identify the compounds that either disrupt or strengthen interactions between nAChRs and an associated protein as part of developing novel therapeutics. We have discovered a number of unnamed proteins in the complex core and in the presence of the low nicotine concentration, of which almost nothing is known. The role of these proteins in the cell’s response to nicotine should be taken into account and needs to be confirmed in future experiments. Verification of the proteins that we identified as a potential nicotine response interactome will need to be performed via traditional biochemical methods, such as Western blotting and coimmunoprecipitation, before more concerted efforts are devoted to the detailed study of these interactions. The compiled list of proteins in the interaction network obtained from this study is not meant to be comprehensive, nor have we validated the identified proteins via orthogonal assays. Nevertheless, we think that our work has laid a solid foundation for further exploration of the nicotine response interactome in both neuronal and non-neuronal tissues. Furthermore, the CAT electrophoresis, together with the proteomic MS analysis procedure, could also be applied to the investigation of the multiprotein complexes.

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Declaration of interest

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Supplementary materials available on line
Supplementary Tables 1-9