

Determination of sialic acids in the nervous system of silkworm (*Bombyx mori* L.): effects of aging and development

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Abstract: Sialic acids mainly occur as components on cell surface glycoproteins and glycolipids. They play a major role in the chemical and biological diversity of glycoconjugates. Although sialic acids exhibit great structural variability in vertebrates, glycoconjugates with sialic acids have also been determined in small amounts in invertebrates. It has been suggested that sialic acids play important roles in the development and function of the nervous system. Despite *Bombyx mori* being a model organism for the investigation of many physiological processes, sialic acid changes in its nervous system have not been examined during development and aging. Therefore, in this study we aimed to determine sialic acid changes in the nervous system of *Bombyx mori* during development and aging processes. Liquid chromatography-mass spectrometry (LC-MS) and lectin immunohistochemistry were carried out in order to find variations among different developmental stages. Developmental stages were selected as 3rd instar (the youngest) and 5th larval instar (young), motionless prepupa (the oldest) and 13-day-old pupa (adult development). At all stages, only Neu5Ac was present, however, it dramatically decreased during the developmental and aging stages. On the other hand, an increase was observed in the amount of Neu5Ac during the pupal stage. In immunohistochemistry experiments with *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) lectins, the obtained staining was consistent with the obtained LC-MS results. These findings indicate that sialic acids are abundant at the younger stages but that they decrease in the insect nervous system during development and aging, similarly as in mammals.

Key words: Sialic acids; nervous system; capLC-ESI-MS/MS; lectin immunohistochemistry; development; aging

INTRODUCTION

Sialic acids (Sias) are negatively charged monosaccharides, which are found in higher animals and some microorganisms [1-4], as basic components of some proteins and lipids found in the cell membrane and in secreted macromolecules. Due to their various types (over 50 different types), Sias contribute to the enormous structural diversity of complex carbohydrates [5-7]. Generally, Sias are prominently positioned at non-reducing ends of oligosaccharide molecules. They present as terminal monosaccharides, which are mainly linked with galactose residues by α 2,3 or α 2,6 glycosidic bond [8-9]. Mammals also form α 2,8-linked sialic acid homopolymer known as polysialic acid (PSA), which is found in neural cell adhesion molecules (NCAM), and plays many roles in NCAM adhesion, neurite outgrowth and cell migration [10]. Sialic acids have received much attention to date be-

cause they participate in the pathogenesis of many diseases such as cancer [11-15], inflammatory diseases [16-19] and viral infections [20-25]. Our knowledge of this important carbohydrate family has improved with advances in the development of sialic acid analogs [11].

N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and 5-deamino-5-hydroxy-neuraminic acid or 2-Keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) are three common members of the Sia family (Fig. 1A-C). Among them, the most frequently observed type is Neu5Ac [26]. Only Neu5Ac is ubiquitous, while the others are not found in all species. The best investigated example next to Neu5Ac is Neu5Gc, which occurs frequently in the animal kingdom, but not in healthy human tissues (occurring only in some tumors in minute quantities [27]) and it has not been detected in bacteria.

KDN, which is a deaminated neuraminic acid, has been determined increasingly in lower vertebrates and bacteria [28]. Many studies related to analysis of Sias in animals have been carried out so far [1,2,5,6,29]. Although Sias have been found in large amounts and exhibit great structural variability in vertebrates [30-33], glycoconjugates that have sialic acids have been determined only in small amounts in insects. Remarkably, Neu5Ac was observed in a short period of *Drosophila melanogaster* embryogenesis [34-39], in the grasshopper *Philaenus spumarius* larvae [40], in hemolymph of the grasshopper *Docioctaurus maroccanus* [41], in the African migratory grasshopper *Locus migratoria* [42], in the prothoracic glands [43-45] and testes [46] of the greater wax moth *Galleria mellonella*, and the most recently in the midgut and salivary glands of the mosquito *Aedes aegypti* [47]. Due to the development of modern analysis methods, detection of Sias in other less developed animals would not be surprising in the future. Despite very detailed studies, there is no Sia found in plants. Although Shah et al. [48] found Neu5Ac and Neu5Gc in *Arabidopsis thaliana* suspended cell culture, this report was not accepted in subsequent studies [49].

Evidence has suggested that glycans play important roles in the nervous system development and function. For example, glycosylation affects various neuronal processes such as neurite outgrowth and morphology. It is suggested in previous studies that glycosylation contributes molecular events that underlie learning and memory [10,11,50-53]. In addition, glycosylation is an effective regulator in cell signaling and has a role in memory pathways [10,11,52-55]. In mammals, the central nervous system has the highest concentration of sialic acids [56]. The majority is present on gangliosides (65%), glycoproteins (32%) and the remaining 3% exist as free forms [26,57].

In order to determine the number, order and molecular structure of sugar units on glycoconjugates, specific analytical methods are required. One of the most important of these methods is liquid chromatography-mass spectrometry (LC-MS), which provides a rapid determination of the molecular weight and structure of trace amounts of monomers [58,59]. Since the biosynthesis of glycans depends on the concerted action of glycosyltransferases, the structures of glycans are much more variable than those of proteins

and nucleic acids. Therefore, the structures of glycans can be easily altered by the physiological conditions of the cells. Accordingly, age-related alterations of the binding type of monomers like sialic acids are relevant to the understanding of physiological changes found in aged individuals. It is important to determine the molecular events that occur in glycoconjugates during aging [60]. Binding properties of monomers could be identified with some carbohydrate-specific macromolecules called lectins. Lectins, which are protein- or glycoprotein-structured molecules, are found in many organisms and have two or more carbohydrate binding sites. They can be obtained from various sources like viruses, bacteria, plants and animals. Moreover, lectins can certainly have more than a single monosaccharide-binding specificity and can recognize internal residues as well as terminal ones. They are used in order to determine the location, intensity and binding properties of glycans in cells [61-65]. The expression of the Sia α 2-3Gal and Sia α 2-6Gal groups that are changed during aging is biologically significant for a number of reasons. First, because sialic acids are ubiquitous components of cell surface glycoconjugates; a change of sialic acid expression during aging may modulate molecular and cellular interactions by changing the electrostatic potential of cells. Second, a change in the expression of sialic acid during aging may disturb cell-cell recognition via various sialic acid-binding molecules. Third, a change in the sialic acid content of some glycoproteins during aging may reduce their normal function by changing their physical properties, their vulnerability to enzymatic digestion and the ability of lectins or antibodies to recognize their underlying structures [60,66].

Among insects, silkworm (*Bombyx mori* L.) has many advantages in the field of science such as being an economically important insect, the ease of its cultivation during experiments, and its well-known biology and physiology [67,68]. From the viewpoint of sialoglycobiology, silkworm eat mulberry leaves and this makes them suitable to work with Sias, since plants do not have sialic acids [49].

In this study, sialic acid changes in the central nervous system of *Bombyx mori* due to aging and development processes have been studied. We aimed to identify the presence, amount and bond-type changes of sialic acids during development and aging. Two

different methods were used in order to find variations among different developmental stages. The first was based on an analytical determination by capillary liquid chromatography-electrospray ionization-mass spectrometry (capLC-ESI-MS/MS) in order to find the quantitative changes of sialic acids during development and aging. The second was a lectin immunohistochemistry method based on fluorescent labelling in order to find qualitative changes in sialic acids and their glycosidic linkage types.

MATERIALS AND METHODS

Materials

Because silkworms eat mulberry leaves and plants do not have sialic acids, silkworms were selected as working material to avoid contamination from feeding material. Silkworm eggs were obtained from the Bursa Kozabirlik Company, Turkey. They were reared with fresh mulberry leaves in the laboratory at $25 \pm 1^\circ\text{C}$ and $75 \pm 5\%$ relative humidity under 14 h light/10 h dark cycles. Stages of insects were selected as 3rd instar (the youngest), 5th instar (young), motionless prepupae (the oldest) and 13-day-old pupae (adult development) in order to observe developmental changes.

All chemicals and solvents (LC-MS grade) are commercially available. Standard sialic acids, Neu5Ac, Neu5Gc, KDN (A9646, G9793 and 60714), and DMB (1,2-diamino-4,5-methylenedioxy-benzene dihydrochloride, D4784), were purchased from Sigma-Aldrich, USA. Sialic acid and DMB derivatization reaction is shown in Fig. 1D. FITC-labeled lectins

(*Maackia amurensis* agglutinin – MAA and *Sambucus nigra* agglutinin – SNA) were purchased from EY Lab Laboratories, San Mateo, USA.

Sample preparation

The central nervous system (CNS: including brain and ventral nerve cord) of insects was dissected under a stereo microscope ($n=50$ for each developmental stage and analysis) and collected in methanol for LC-MS analysis. For lectin immunohistochemistry, brains were collected in 4% paraformaldehyde in phosphate buffer saline (PBS), pH: 7.3 and stored at $+4^\circ\text{C}$ until embedding.

CNS tissues in methanol were homogenized with a homogenizer (Heidolph, Silent Crusher S) for tissue and cell disruption. Methanol was removed under nitrogen stream at 40°C , and then the dry matter was weighed. Sialic acid determination was conducted as described previously [69-70]. Briefly, dry matter was redissolved in $5 \mu\text{L}$ of 2 M acetic acid per mg of dry matter and kept at 80°C for 3 h in order to release sialic acids from glycoconjugates, including glycoproteins, glycolipids and proteoglycans. After acid hydrolysis, samples were centrifuged at 10000 g for 3 min and supernatants were mixed with an equal volume of DMB mixture (7 mM DMB, 18 mM sodium hydrosulfite and 0.75 M β -mercaptoethanol in 1 mL of 1.4 M aqueous acetic acid). The mixtures were kept at 60°C for 2.5 h in the dark for derivatization. Finally, after centrifugation at 16300 g for 10 min, supernatants were transferred to 250- μL HPLC (high pressure liquid chromatography) vials. $0.5 \mu\text{L}$ of each sample was injected and analyzed by capLC-ESI-MS/MS. The standards, Neu5Ac, Neu5Gc and KDN ($1.25 \mu\text{g}/\text{mL}$), were directly derivatized with DMB (Fig. 1D) and the reaction mixture directly injected into the capLC-ESI-MS/MS system.

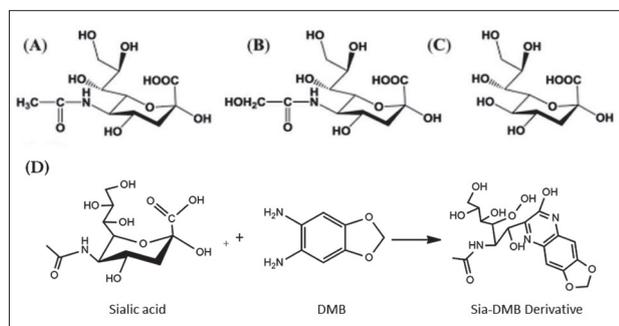


Fig. 1. The main sialic acid types and DMB-derivatization. **A** – Neu5Ac, **B** – Neu5Gc, **C** – KDN, **D** – Sialic acid derivatization reaction with DMB agent for determination of sialic acids.

CapLC-ESI-MS/MS analysis

An Agilent 1200 Capillary HPLC system with an ODS (Octadecylsilane, C18) capillary column (ACE C18 150 x 0.5 mm, $5 \mu\text{m}$) was used as a liquid chromatography system. A methanol-acetonitrile-water (7.5:5:87.5, v/v) mixture was used as mobile phase and elution was performed in the isocratic mode. Column temperature was maintained at 30°C during analyses. Samples were stored at 5°C in a refrigerated

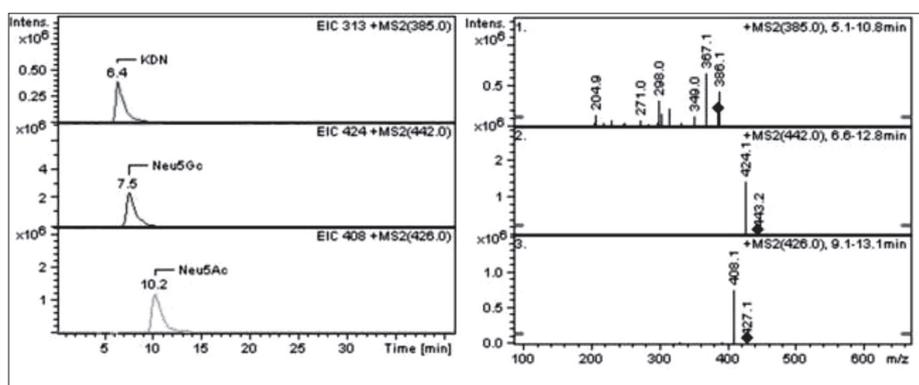


Fig. 2. LC-ESI-MS/MS mass spectrum and chromatogram of DMB-standard sialic acid species (KDN, Neu5Gc and Neu5Ac). m/z values of molecular ions $[M+H]^+$ and CID fragments $[M+H-H_2O]^+$ were monitored and labeled by software.

autosampler board (Agilent G1377A). Injection volume and flow rate were settled as 0.5 μ L and 20 μ L/min, respectively.

All mass spectrometry measurements were performed using an HCT Ultra ion trap mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization (ESI) source in positive mode. Spectrometric conditions such as ion optics voltages, nebulizer gas and dry gas flow rates, and dry gas temperature were controlled by Esquire Control software 6.1 (Bruker Daltonics). Nitrogen was used as nebulizer and dry gas; helium (He) (99.9 %) was used as a collision gas in the ion trap. Ion source settings were: dry temperature 300°C, nebulizer pressure 15.0 psi and dry gas flow 5 L/min. MS/MS spectra were carried out by collision-induced dissociation (CID) using a multiple reaction monitoring (MRM) system. All mass spectra were acquired in the mass range 200-600 m/z , with a scan speed of 26000 m/z per second. Data analyses were carried out by using Data Analysis software (v.3.4, Bruker Daltonics).

Lectin immunohistochemistry

After fixation in 4% paraformaldehyde, brain samples were dehydrated gradually in ethanol series and then embedded in Lowicryl HM20 (EMS, Catalog #14340). Lowicryl blocks were sectioned using an ultramicrotome at 0.75 μ m thickness and transferred onto lysine-coated glass slides. Fluorescein isothiocyanate (FITC)-labeled lectins were prepared in 0.01 M PBS (pH: 7.3). Glass slides were washed with PBS three times and then treated with PBS blocking buffer consisting of

0.01 M PBS, 0.1% Tween-20, 0.5% bovine serum albumin, 0.1% sodium azide and 0.1% gelatin for 30 min. After blocking, sections were incubated with lectins for 1 h and washed three times with PBS for 5 min. Sections without lectin staining were used as negative control. Finally, sections were covered with glycerin and then observed under a fluorescent microscope (Leica DM 4000B) [71].

Statistical analysis

Data are expressed as mean values \pm SEM (standard error of the mean) for the indicated number of independent determinations. All statistical analyses were performed by Graphpad software (GraphPad Software, Inc.). One-way analysis of variance (ANOVA) followed by a multiple comparison test was used to compare developmental stages; P values <0.05 were considered statistically significant.

RESULTS

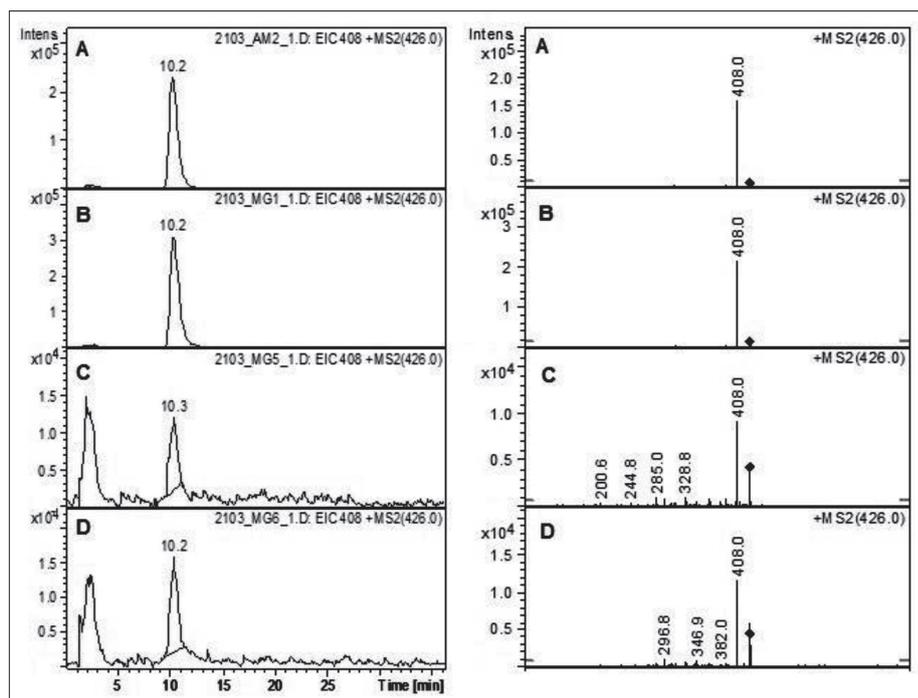
CapLC-ESI-MS/MS analysis

Retention times and m/z values of DMB-derivatized standard sialic acids are shown in Fig. 2. Retention times of KDN, Neu5Gc and Neu5Ac standards were 6.4, 7.5 and 10.0 min, respectively. $[M+H]^+$ and CID fragments of standards are shown in Table 1. Obtained data from standards were used for confirmation of working material.

In order to determine whether substituted sialic acids exist in the cells, a 5- μ L 2 M acetic acid treat-

Table 1. Characteristic ions and CID fragments of DMB-standard sialic acid derivatives.

Sialic acids	RT	[M + H] ⁺	CID Fragments	
			[M + H-H ₂ O] ⁺	Fragments (<i>m/z</i>)
KDN	6.4	385	313	205-271-298-367
Neu5Gc	7.5	442	424	313-295-283-229
Neu5Ac	10.0	426	408	313-295-283-229

**Fig. 3.** LC-MS chromatograms and spectra of Neu5Ac related with developmental stages. A – 3rd instar, B – 5th instar, C – Motionless prepupa, D – Pupa.

ment per mg of dry matter was used to release Sias from glycoconjugates without damaging the substitutions. Thus, quantification was normalized using this approach. Released sias were derivatized with DMB, and then separated by capLC-ESI-MS/MS, and retention times-CID fragments were compared with standards.

DMB-labeled Sias in all samples were analyzed using proton adducted pseudomolecular ion [M+H]⁺ by MS in positive ion mode. [M+H-18]⁺ is a CID fragment in the ion trap system. The ion trap fails to trap the ions at the lower end of the *m/z* range when using CID for tandem mass spectrometry (MS/MS) due to an inherently low mass cutoff (LMCO) [72]. Therefore, [M+H-18]⁺ ions obtained from MRM of sialic acids are very useful for screening the chromatogram and spectrum in a MS/MS system. Other small

ions obtained from fragmentation of the main ion, particularly the fragmentation of [M+H-18]⁺, were characterized using MS³ as described in our previous study [73].

Proton adducted pseudomolecular ions of DMB-labeled standards (Neu5Ac, Neu5Gc and KDN) were observed at *m/z* 426 [M+H]⁺, *m/z* 442 [M+H]⁺ and *m/z* 385 [M+H]⁺, respectively (Fig. 2). Fragment ions of Neu5Ac, Neu5Gc and KDN were observed at *m/z* 408 [M+H-18]⁺, 424 [M+H-18]⁺ and 385 [M+H-18]⁺, respectively.

When compared with standard Sias, the only detectable sialic acid type was Neu5Ac (Fig. 3). Proportional amounts of Neu5Ac during the developmental stages are shown in Table 2 and Fig. 4. According to the results, the highest Neu5Ac amounts were found in 3rd instar larvae, the youngest stage. During devel-

Table 2. Proportional amounts of Neu5Ac during development. Peak areas from LC-MS analysis were simplified according to the minimum value. Results are expressed as means \pm SEM of three determinations. One-way ANOVA followed by a multiple comparison test were used to compare developmental groups; $P < 0.05$.

	3 rd instar	5 th instar	MPP	Pupa
Neu5Ac	20	1	2	18

Table 3. Changes in intensity of MAA and SNA labelings. The number of "+" indicates the intensity of labelling.

Lectin	Lectin Specificity	Stages			
		3 rd	5 th	MPP	Pupa
MAA	Neu5Ac α 2,3Gal	++++	+++	-	+++
SNA	Neu5Ac α 2,6Gal/GalNAc	+++	+	-	+

opment in larval stages, the concentration of Neu5Ac dramatically decreased in 5th instar larval (young) and motionless prepupal (MPP, the oldest) stages. Among the larval stages, the amounts of Neu5Ac in 3rd instar larva were found to be 20 and 10 times higher compared to 5th instar and MPP, respectively. In the pupal stage where tissue remodeling happens for adult development, the amount of Neu5Ac increased and was determined as 18 and 9 times higher than amounts in 5th instar and MPP, respectively (Table 2 and Fig. 4).

Lectin immunohistochemistry

Glycosidic linkage types of Sias were determined by FITC-labeled lectins with a fluorescent microscope in *Bombyx mori* brain sections. MAA (recognizing Neu5Ac α 2,3Gal) and SNA (recognizing Neu5Ac α 2,6Gal/GalNAc) lectins were used to determine α 2,3- and α 2,6-linked sialic acids, respectively.

According to labelings, the most α 2,3- and α 2,6-linked Sias were found in the 3rd instar larva samples (Fig. 5-6). Labelings were observed as decreased in the MPP, but increased again in the pupa stage for both lectin experiments. Additionally, it was found that MAA labelings were more intense than SNA in all stages (Table 3). Considering the negative control sections (no lectin was applied), no labeling was observed in these sections (data not shown).

DISCUSSION

Regarding the nervous system, metamorphosis is a period in which some significant changes occur in the

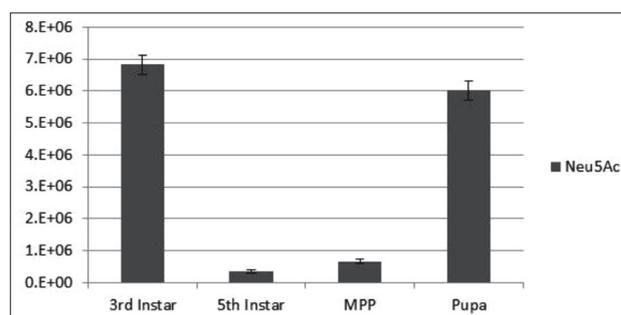


Fig. 4. Proportional amounts of Neu5Ac during development. Peak areas from LC-MS analysis were simplified according to the minimum value. Y-axis shows the peak area. Results are expressed as mean \pm SEM of three determinations. One-way ANOVA followed by multiple comparison test were used to compare developmental groups; $P < 0.05$.

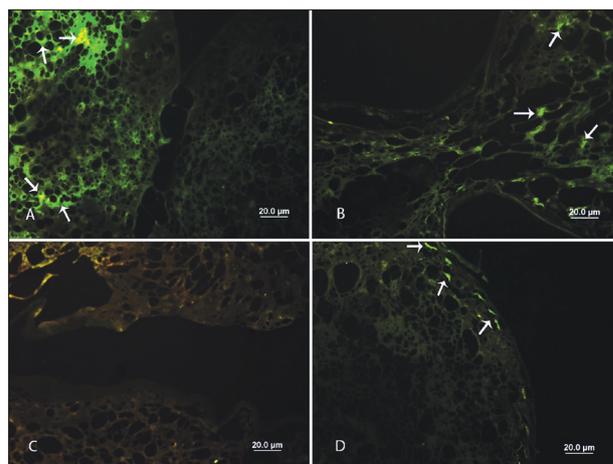


Fig. 5. FITC-labeled MAA lectin micrographs from *Bombyx mori* brain sections. A – 3rd instar, B – 5th instar, C – Motionless prepupa, D – Pupa. Arrows indicate the labelling.

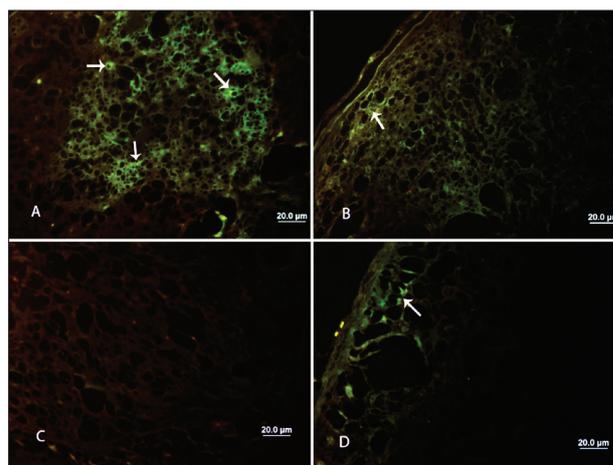


Fig. 6. FITC-labelled SNA lectin micrographs from *Bombyx mori* brain sections. A – 3rd instar, B – 5th instar, C – Motionless prepupa, D – Pupa. Arrows indicate the labelling.

ventral nerve cord, such as the merging of ganglia and shortening of connections between ganglia [74]. Developmental changes in glycans play an important role in molecular interactions. Moreover, determination of significant glycan units in insects is an important issue in solving the molecular mechanisms of aging.

We aimed to determine whether sialic acid types and amounts change in the *Bombyx mori* nervous system during development and aging. According to our results, sialic acid amounts were found to be the highest in the 3rd instar larval (the youngest) stage. In the young larval stage (5th instar) and in the oldest stage (motionless prepupa), Sias were observed to be 20- and 10-fold decreased, respectively. Since Sias represent complex glycosylation, these values indicated that complex glycosylation of glycoproteins presents mostly in younger mammal individuals [45,75-85].

Sialic acid alteration values obtained from capLC-ESI-MS/MS were supported with the fluorescent labeled lectin results. Maximum labelings were found in the youngest stage, and a significant reduction in fluorescence was observed during development and aging. The highest labelings observed in 3rd instar larvae support the analytical results and point out the abundance of complex glycosylation in this phase. Our results correspond with many articles showing sialic acid reduction with aging in vertebrates and invertebrates [86-88]. On the other hand, it was stated that the expression of α 2,3- and α 2,6-linked Sia is region-specific [87].

The existence of Sias in insects was shown by several methods in the developing nervous system of *D. melanogaster* embryos [34,36]. Also, they were shown in the prothoracic glands of *G. mellonella* by comparing gas chromatography (GC) mass spectrometric results with transmission electron microscopic results [44]. After this study, they were also identified in malpighian tubes of *Philaenus spumarius* [40]. Lastly, in 2015, an active α 2,6-sialyltransferase was found in *B. mori*. It was shown that it is expressed in different organs and in various stages of development [89]. All these findings suggest that insects have sialic acid metabolism.

From the viewpoint of aging, Karaçalı et al. [45] have shown in *G. mellonella* that sialic acids play a

masking role over the N-acetylgalactosamine receptor that hemocytes recognize. As in our study, the reduction of sialic acids was shown in the developing testes of insects [46] and prothoracic glands of *G. mellonella* during aging [45]. Despite the existence of sialic acids in the embryo and nervous system of *D. melanogaster* [35,36,90], *Danaus plexippus* and *Trichoplusia ni* eggs [91], there is no information about the changes in sialic acids in the insect nervous system associated with aging.

In summary, changes in the levels of sialic acids indicate that complex glycan types decrease during development and aging processes. The presence of α 2,3- and α 2,6-linked sialic acids was also found to decline in vertebrates [86,87,90]. An increase in sialic acids in mid-metamorphosis suggests that they have important roles during the remodeling of organs for adulthood. These findings are the first identification of sialic acid changes in the nervous system of *B. mori* and could contribute to our knowledge about sialic acid metabolism.

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