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# Acetylcholinesterase and agrin: Different functions, similar expression patterns, multiple roles

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ABSTRACT

Acetylcholinesterase (AChE) and agrin play unique functional roles in the neuromuscular junction (NMJ). AChE is a cholinergic and agrin a synaptogenetic component. In spite of their different functions, they share several common features: their targeting is determined by alternative splicing; unlike most other NMJ components they are expressed in both, muscle and motor neuron and both reside on the synaptic basal lamina of the NMJ. Also, both were reported to play various nonjunctional roles. However, while the origin of basal lamina bound agrin is undoubtedly neural, the neural origin of AChE, which is anchored to the basal lamina with collagenic tail ColQ, is elusive. Hypothesizing that motor neuron proteins targeted to the NMJ basal lamina share common temporal pattern of expression, which is coordinated with the formation of basal lamina, we compared expression of agrin isoforms with the expression of AChE-T and ColQ in the developing rat spinal cord at the stages before and after the formation of NMJ basal lamina. Cellular origin of AChE-T and agrin was determined by in situ hybridization and their quantitative levels by RT PCR. We found parallel increase in expression of the synaptogenetic (agrin 8) isoform of agrin and ColQ after the formation of basal lamina supporting the view that ColQ bound AChE and agrin 8 isoform are destined to the basal lamina. Catalytic AChE-T subunit and agrin isoforms 19 and 0 followed different expression patterns. In accordance with the reports of other authors, our investigations also revealed various alternative functions for AChE and agrin. We have already demonstrated participation of AChE in myoblast apoptosis; here we present the evidence that agrin promotes the maturation of heavy myosin chains and the excitation–contraction coupling. These results show that common features of AChE and agrin extend to their capacity to play multiple roles in muscle development.



## 1. Introduction

Components of the neuromuscular junction (NMJ) are usually viewed as members of the two functional groups. One set of these components, responsible for the synthesis (choline acetyl transferase), functioning (nicotinic receptor) and elimination (acetylcholinesterase; AChE) of acetylcholine (ACh) is involved in the cholinergic signaling (reviewed in [1]). Another set of NMJ components is responsible for the development of the NMJ and its maintenance. Essential representatives of this group are agrin and muscle specific kinase (MuSK) with its downstream signaling cascade (reviewed in [2]). According to this traditional view, the synaptogenetic apparatus is responsible for the appropriate expression and targeting of the cholinergic set of NMJ components. However, this division of NMJ components does not extend to their functioning at the molecular level. For example, although AChE and nicotinic receptor are both cholinergic NMJ components and both bind ACh, they have completely different genetic pattern, protein structure and even ACh binding site. On the other hand, AChE shares several common features with agrin which is a nerve derived factor inducing postsynaptic NMJ differentiation at its contact with the sarcolemma [3]. Both, agrin and AChE are polymorphic: they are transcribed from a single gene while their targeting and functioning are determined by alternative splicing. Unlike most other NMJ components they are expressed in both, muscle and motor neuron and both reside on the synaptic basal lamina of the NMJ. Both, AChE [4] and agrin [5] were also found at several other non-cholinergic sites where they have various functions unrelated to NMJ.

Since AChE and agrin are both synthesized in the motor neurons and since both reside on the synaptic basal lamina of the NMJ, one would expect that their common features extend to their expression and targeting. However, while the origin of the basal lamina bound agrin in the NMJ is undoubtedly neural, the neural origin

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of AChE at this site is elusive. In our previous study [6] we approached this question on the experimental model in which human myotubes are innervated with the motor neurons extending from the embryonic rat spinal cord [7–9]. Detecting synaptic AChE by species-specific anti-rat and anti-human AChE antibodies we found that both, muscle and motor neuron contribute AChE to the synaptic basal lamina of the NMJ [6]. In continuation of this research we tested the hypothesis that motor neuron proteins targeted to the NMJ basal lamina follow common temporal pattern of expression, which is coordinated with the basal lamina formation. We therefore compared the expression of AChE with the expression of agrin in the developing rat spinal cord at the stages before and after the formation of basal lamina in the developing NMJ. Besides catalytic AChE subunit we also followed the expression of structural subunit ColQ [10], a collagenic tail which anchors three tetramers of catalytic subunits of the asymmetric, A12 (16S) AChE form, to the NMJ basal lamina. AChE expression was compared to the expression of agrin isoforms 8, 19 and 0. Numbering refers to the Z splicing site isoforms which either have (agrin 8 and 19) or do not have (agrin 0) a domain of 8 aminoacids essential for the synaptogenetic activity [11–13]. Expression was followed at the mRNA level in the developing rat spinal cord at the developmental stages corresponding to the periods before (embryonic days E15 and E21) and after (adult) the formation of the NMJ basal lamina in the cocultures used in our previous studies [[6]; reviewed in [14]]. Cellular origin of mRNAs was determined by in situ hybridization and their levels by RT PCR.

One common feature of AChE [4] and agrin [5] is also their capacity to serve various functions unrelated to NMJ. We have been testing whether these alternative functions can also be found in the cultured human muscle. In accordance with the reports of Park et al. [15], Zhang et al. [16] and Toiber et al. [17] we already demonstrated participation of AChE in myoblast apoptosis [18]. We also reported stimulatory actions of agrin on the maturation of the excitation–contraction (e–c) coupling mechanism [19] and membrane excitability [20] in human myotubes in vitro. Here, we report new results of the experiments in which we tested the influence of agrin on the maturation of the e–c coupling and on the maturation of the myosin isoforms.

## 2. Methods

### 2.1. Experimental animals

All studies reported here were approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (permit No: 63/01/99). Timed-pregnant Wistar rats at 15 or 21 days of gestation and adult rats (1–3 months old) were sacrificed by exposure to $CO_2$ narcosis. Further steps of the preparation of the spinal cord sections for the RT PCR analyses and in situ hybridization were performed as described [21]. Human skeletal muscle myotubes were used to assess alternative effects of agrin. Cultures were prepared as described before [8,9,22].

### 2.2. RT-PCR and in situ hybridization of AChE and agrin mRNA

AChE-T (splice variant T) and ColQ mRNA expression was measured by competitive RT-PCR; primers sequences for AChE-T and ColQ and the detailed protocols are provided in [21] and [6], respectively. The same technique was used for determination of agrin mRNA transcripts 0, 8 and 19. Primers spanned alternative splicing site Z for agrin mRNA enabling us to detect all splice variants at this position; the following primers were used: forward 5′-CATCGAATACCTCAATGCTGTGATTGAG-3′ – exon 31, reverse 5′-GCAGAACGTGCAGATTACATGGCC-3′ – exon 34. The motor neuron origin of AChE and agrin mRNAs was verified by non-radioactive in situ hybridization using digoxigenine-labeled RNA probe as described before [21]. AChE probe extended exons 4 and 6 of rat AChE-T cDNA (nucleotides 1471–1801). Agrin probe was overlapping Z – splicing site of Z19 variant of agrin cDNA; this probe therefore detected agrin 19 and agrin 8 splice variants [11–13].

### 2.3. Maturation of e–c coupling and myosin heavy chain (MyHC) expression in the cultured human skeletal muscle myotubes

The effects of agrin 8 on the maturation of the e–c coupling were determined in human myotubes differentiated for 12 days. Detailed description of the experimental techniques were provided before [19,20]. Briefly, $Ca^{2+}$ responses elicited by 40 mM caffeine and expression of voltage-dependent $Ca^{2+}$ channels (T- and L-type) was measured by patch-clamp. Protein expression of slow and fast isoforms of MyHC was determined by Western Blot.

## 3. Results

### 3.1. Time courses of expression of mRNAs encoding AChE-T catalytic subunit, ColQ and agrin isoforms 0, 8 and 19 in the developing rat spinal cord

Quantitative measurements of the mRNA levels of AChE-T, ColQ and agrin isoforms 0, 8, and 19 in the rat spinal cord homogenates at the embryonic day 15 (E15), at birth (E21) and in the adult (1–3 months after birth) revealed three distinct temporal patterns of expression (Fig. 1A–C). Expression of mRNAs encoding AChE-T variant and agrin 19 increased during the third week of embryogenesis and reached their peak level at E21. By contrast, agrin 8 and ColQ remained lowly expressed throughout embryonic development, but were markedly up-regulated after birth when NMJ basal lamina starts to form. Agrin 0 which lacks synaptogenetic activity was, surprisingly, prominently expressed at all developmental stages. Expression patterns are schematically presented in Fig. 1D. In situ hybridization (Fig. 1E; see also [21] for precise AChE mRNA localization in the adult rat spinal cord) confirmed expression of AChE and synaptogenetic agrin isoforms (transcripts 8 and 19) in the motor neurons in the rat spinal cord.

### 3.2. The effects of agrin on e–c coupling and expression of MyHC in cultured human skeletal muscle myotubes

Electrophysiological measurements of e–c coupling and myosin heavy chain (MyHC) expression were assessed after the 11–12 days treatment of differentiating human skeletal muscle myotubes with agrin 8. Upon stimulation with 40 mM caffeine $Ca^{2+}$ release was detected in 31 ± 6% ($n$ = 20 fields) and 70 ± 7% ($n$ = 17 fields) of control and agrin-treated myotubes, respectively (Fig. 2A, left panel). We next determined whether agrin 8 affects expression of voltage-dependent $Ca^{2+}$ channels (T- and L-type). In agrin-treated myotubes, the peak density of T-type ($I_{Ca,T}$) and L-type ($I_{Ca,L}$) $Ca^{2+}$ currents was lower and higher, respectively ($P < 0.0001$) (Fig. 2A, right panel). For further details see [19]. To further investigate the involvement of agrin 8 in maturation of contractile apparatus, MyHC expression was determined by Western Blot (Fig. 2B). Agrin-treated myotubes displayed higher expression of slow and fast MyHC isoforms compared to control myotubes ($P < 0.05$).

## 4. Discussion

In the experimental model of the in vitro innervated human muscle [8,9,22] used in our previous investigations in which we found that not only muscle but also motor neurons contribute