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Research article

QUANTITATIVE AND DYNAMIC EXPRESSION PROFILE OF PREMATURE AND ACTIVE FORMS OF THE REGIONAL ADAM PROTEINS DURING CHICKEN BRAIN DEVELOPMENT

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Abstract: The ADAM (A Disintegrin and Metalloprotease) family of transmembrane proteins plays important roles in embryogenesis and tissue formation based on their multiple functional domains. In the present study, for the first time, the expression patterns of the premature and the active forms of six members of the ADAM proteins – ADAM9, ADAM10, ADAM12, ADAM17, ADAM22 and ADAM23 – in distinct parts of the developing chicken brain were investigated by quantitative Western blot analysis from embryonic incubation day (E) 10 to E20. The results show that the premature and the active forms of various ADAM proteins are spatiotemporally regulated in different parts of the brain during development, suggesting that the ADAMs play a very important role during embryonic development.

Key words: ADAM, Gene expression, Protein, Brain development, Chicken

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Abbreviations used: ADAM – A Disintegrin and Metalloprotease; APP – amyloid precursor protein; BCA – bicinchoninic acid; CNS – central nervous system; ECM – extracellular matrix; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; ELISA – enzyme-linked immunosorbent assay; HE-EGF – heparin-binding EGF-like growth factor; kDa – kilodalton; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; LGI1 – leucine-rich glioma inactivated 1; RT-PCR – reverse transcription-polymerase chain reaction; SDS – sodium dodecyl sulfate; TACE – tumor necrosis factor alpha converting enzyme; TBST – tris-buffered saline-Tween; TGF-α – transforming growth factor-α; TNF-α – tumor necrosis factor-α

INTRODUCTION

Members of the ADAM family are type I transmembrane proteins and consist of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich and EGF-like domains, a transmembrane domain, and an intracellular domain [1-3]. The prodomain keeps the metalloprotease site of ADAMs inactive through a "cysteine switch" and is responsible for the latency of these enzymes. It can also chaperone the proper folding of ADAMs, particularly of the metalloprotease domain, in which most ADAMs contain a conserved sequence which binds to the zinc ions and is likely to function as an active protease for degrading substrate proteins. It is known so far that ADAM1, 8-10, 12, 13, 15-17, 19-21, 24-26, 28, 30, and 33-35 have proteolytic activity [4]. The disintegrin domain is involved in interaction with integrins, although most of the ADAMs do not contain the Arg-Gly-Asp sequence (apart from human ADAM15), which is found in most other integrin ligands [5, 6]. The cysteine-rich and epidermal growth factor (EGF)-like domains are involved in adhesion, e.g., by interacting with extracellular matrix (ECM) proteins and the cell-adhesive molecule syndecan. The cytoplasmic domain is highly variable both in length and in sequence and plays a role in intracellular signaling transduction [1, 5, 6-8].

ADAMs play a role in embryogenesis and tissue formation [4, 9-11]. For example, ADAM10 mutant mice as well as ADAM17- and ADAM19-deficient mice die of multiple cardiovascular and brain defects at embryonic stages or at birth [12, 13]. ADAM22 or ADAM23 mutant mice show ataxia and prominent hypomyelination of the peripheral nerves and tremor, respectively [14, 15].

During central nervous system (CNS) development, members of the ADAMs are widely expressed in distinct anatomical structures and play a pivotal role in neuronal proliferation and differentiation, cell migration, axon outgrowth and guidance, and synaptogenesis [4, 9, 10]. For example, several members of the ADAMs, including ADAM9, ADAM10, ADAM12, ADAM17, ADAM22 and ADAM23, are spatially and temporally regulated in distinct anatomical structures of the developing chicken brain and/or spinal cord [16, 17]. ADAM10 sheds Notch ligands and regulates Notch signaling, which plays a critical role in embryonic development [18]. ADAM2 contributes to the migration of neuroblasts to the olfactory bulb during mouse brain development [19]. ADAM21 contributes to neurogenesis and guides neuroblast migration by protein shedding and integrin binding [9]. Finally, ADAM10 guides the axons of retinal ganglion cells to project correctly to their target region in the tectum [20].

In our previous studies, we have shown that five members of the ADAMs are expressed in distinct structures of the developing brain by in situ hybridization at the mRNA level [16]. But little is known about how the latent and the active forms of the ADAM proteins are regulated in distinct parts of the developing brain. The ADAM active forms can be distinguished from their precursors by Western blotting according to the differences of their molecular weight. Therefore, in the present study, we continue to analyze the expression patterns of

different forms of the ADAM proteins ADAM9, ADAM10, ADAM12, ADAM17, ADAM22, and ADAM23 in different regions of the developing brain – the telencephalon, diencephalon, tectum, cerebellum, and the hindbrain. We chose these six proteins for investigation because of their involvement in brain development [4, 9, 10]. Our results show for the first time that the premature and the active forms of the ADAMs are spatially and temporally regulated in the developing brain, suggesting involvement of the ADAMs in brain development.

MATERIALS AND METHODS

Chicken embryos and antibodies

Fertilized eggs of White Leghorn chicken (*Gallus gallus domesticus*) were purchased from a local farm and incubated in a forced-draft incubator (Grumbach, Asslar, Germany) at 37.5°C and 65% humidity. The embryos were collected and studied at embryonic incubation day (E) 10, E12, E14, E16, E18 and E20 with at least three embryos per stage. After the embryos were anesthetized by cooling on ice, the embryos were removed from their eggs. Then distinct parts of the brain – the telencephalon, diencephalon, tectum, cerebellum, and the hindbrain – were separated, frozen in liquid nitrogen and stored at -80°C for quantitative Western blot analysis.

Antibodies against ADAM9, ADAM10, ADAM12, ADAM17, ADAM22 and ADAM23 were produced by a custom service (Eurogentec, Seraing, Belgium). All antibodies were generated to the two portions of chicken protein sequence with a high titer by enzyme-linked immunosorbent assay (ELISA) test. The amino acid sequences of the peptides used to immunize the rabbits for production of the chicken ADAM antibodies are summarized in Tab. 1. Pre-incubation of the antibody with its corresponding immunizing peptide abolished the specific bands measured by Western blot.

Tab. 1. Amino acid sequences of the peptides of the ADAMs used to immunize the rabbits for the production of chicken ADAM antibodies.

Name	Sequence 1	Sequence 2
ADAM9 (NP_001026567)	CKLGRERRETSNASST (in prodomain)	CNTKGYGGSIDSGPPY (in transmembrane)
ADAM10 (NP_989592.1)	CEEPTTEKPSEESDSD (in prodomain)	QRQRPRESYQMGHMRH (in cytoplasmic domain)
ADAM12 (NP_001136322.1)	CDPSHRSQGGRYKRET (in prodomain)	CVQKRDGPRRPLPYQI (in cytoplasmic domain)
ADAM17 (NP_001008682.1)	QDHQRMDTIQEDPSTD (in cytoplasmic domain)	CFKLQRQNRVDSKETE (in cytoplasmic domain)
ADAM22 (NP_001138700.1)	CYRRQRQIPQGDYVKK (in cytoplasmic domain)	CGNRKKVRGKRFRPRS (in cytoplasmic domain)
ADAM23 (NP_001138702)	ASMQLQDHETESSSEW (in prodomain)	CIRDTGNKKDEGPKGP (in EGF-like domain)

Quantitative Western blot analysis

The quantitative Western blot analysis method was used according to the previous protocol described by Hoffrogge et al. [21]. Briefly, the separated brain tissues were homogenized in RIPA buffer (100 mg tissue in 400 µl buffer) with a sonicator. The protein was determined by the bicinchoninic acid (BCA) protein assay reagent kit (Therma Scientific, Rockford, USA) and the concentrations of the total proteins in samples were measured by a plate reader (Tecan, Crailsheim, Germany) [22]. After the samples were heated at 95°C for 5 min in 5x Laemmli buffer, the proteins (60-100 µg per lane) were electrophoretically separated on 4-15% gradient SDS-polyacrylamide gels (Biorad, München, Germany), followed by transfer to nitrocellulose membrane with a semi-dry electro-blotting cell (Biorad, München, Germany). After blocking with 3% milk in tris-buffered saline-Tween (TBST) buffer (TBS buffer with 0.1% Tween 20, pH 7.6) for 1 hour, the membranes were incubated with primary polyclonal rabbit antibodies raised against the different ADAMs (Eurogentec, Germany) and/or mouse monoclonal antibody raised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, UK) at 4°C overnight. After the blots were rinsed 3 times with TBST buffer, the secondary AlexaFluor680-coupled goat anti-rabbit IgG antibody (Molecular Probes, Karlsruhe, Germany) for ADAMs detection and the IRDye800-coupled goat anti-mouse IgG antibody (Rockland, Gilbertsville, USA) for GAPDH detection were added on the same blotting membrane. Pre-stained peqGOLD marker IV (PeqLab, Erlangen, Germany) was used as a molecular weight marker. Protein visualization and quantification were performed with Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). The band intensities of ADAM proteins were normalized to the reference protein GAPDH and the relative amount of the ADAMs was compared at different stages.

Statistical analysis

Results were reported as mean ± SEM from at least three independent experiments. Statistical evaluation was carried out using the two-tailed Student's t-test. A difference was considered to be statistically significant when the p-value < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

Identification of specificity of the chicken ADAM antibodies

To investigate the specificity of the ADAM antibodies, the Western blot was performed in the whole chicken E14 brain lysate (Fig. 1) using the pre-immune serum of the individual rabbit (lane 1), the coupling of the ADAM antibody together with its corresponding immunizing peptide (lane 2), only the secondary antibody (lane 3), and using the individual ADAM antibody (lane 4). Generally, no specific positive bands were found in lane 1 and lane 3 under different situations. Only the individual chicken ADAM antibody detected the strong signal bands (Fig. 1, lane 4), which were almost abolished by adding

corresponding immunizing peptide (Fig. 1, lane 2), suggesting that the antibodies used here can specifically detect the corresponding ADAM proteins.
ADAM9 antibody reveals a signal band at 60 kDa (Fig. 1A, lane 4). Based solely on the amino acid sequence, the mature chick ADAM9 protein lacking the signal peptide and prodomain is predicted to be about 55.5 kDa. Therefore, the band of 60 kDa detected by the ADAM9 antibody in the Western blot is likely to be the glycosylated mature form of the ADAM9 protein [23].
ADAM10 antibody detects two bands at 114 kDa and 67 kDa by Western blotting (Fig. 1B, lane 4). According to the amino acid sequence of ADAM10, the mature ADAM10 protein lacking the signal peptide and prodomain is predicted to be about 60 kDa; therefore, the band of 67 kDa detected by the ADAM10 antibody in the Western blot is likely to be the glycosylated mature form of the ADAM10 protein. The 114 kDa band is likely the premature full-length protein with glycosylation, as suggested by Hall and Erickson [24].

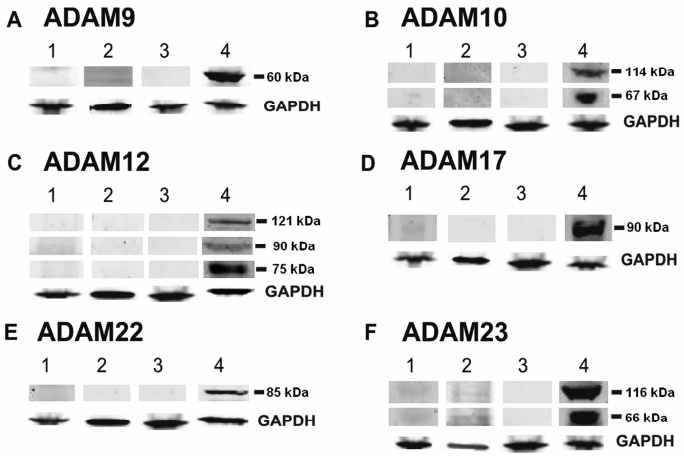

Fig. 1. Specificity of the antibodies analyzed by Western blotting. To investigate the specificity of the antibodies, different parts of the brain lysates were analyzed by Western blotting. Lane 1 shows the result with pre-immune serum blot (lane 1), lane 2 with the couple of antibody together with the antigen, lane 3 without the first antibody as the negative control, and lane 4 with the produced antibody. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is used as the internal loading control. Cerebellar lysate at E14 was used for detection of ADAM9 (A), ADAM10 (B), ADAM22 (E) and ADAM23 (F), and cerebellar lysate at E10 for detection of ADAM17 (D). Diencephalon lysate at E14 was used for ADAM12 (C).

In the case of the ADAM12 antibody, three bands with 121 kDa, 90 kDa and 75 kDa were found (Fig. 1C, lane 4) in a similar manner to mouse C2 cells [25]. The predicted full-length amino acid sequence of ADAM12 is about 102 kDa; the amino acid sequence lacking the signal peptide but with the prodomain is about 97 kDa, and the one lacking both the signal peptide and the prodomain is about 76 kDa. Therefore, the two bands of 121 kDa and 75 kDa are most likely the

glycosylated full-length and the mature form without the prodomain, respectively (Fig. 1C, lane 4). Recently, a short splice isoform of chicken ADAM12 (ADAM12-S) was found and is weakly expressed in the developing brain measured by reverse transcription-polymerase chain reaction (RT-PCR) [17]. The predicted full-length amino acid sequence of this ADAM12-S is about 76 kDa. Because the 90 kDa protein was only found in the developing diencephalon (see below), this band may represent the glycosylated full-length form of the premature ADAM12-S (Fig. 1C, lane 4). The ADAM17 antibody can only detect one specific band at 90 kDa (Fig. 1D, lane 4), which may belong to the mature, active form without the signal peptide but with the partial prodomain (predicted 91 kDa). Both ADAM22 and ADAM23 belong to the non-proteolytic ADAM proteins [4]. For ADAM22, only one band (85 kDa) was detectable by ADAM22 antibody (Fig. 1E, lane 4) and this band is most likely the ADAM22 protein without the prodomain (predicted 79 kDa). For ADAM23, two bands at 116 kDa and 66 kDa were found by ADAM23 antibody (Fig. 1F, lane 4). These two bands are most likely the glycosylated full-length protein (predicted 84 kDa) and the protein without the prodomain (predicted 59 kDa).

Regional ADAM expression patterns in distinct parts of developing brain

The aim of this study was to analyze the expression patterns of six members of the ADAM proteins in different parts of the developing brain – the telencephalon, the diencephalon, the tectum, the cerebellum and the hindbrain – by quantitative Western blot analysis using antibodies raised against the chicken ADAMs. Because separation of the distinct parts of the brain before E10 is difficult, the embryos from E10 to E20 were chosen for investigation. Expression patterns of each ADAM protein are shown according to the anatomical regions of the brain and developmental stages for ADAM9 (Fig. 2), ADAM10 (Fig. 3), ADAM12 (Fig. 4), ADAM17 (Fig. 5), ADAM22 (Fig. 6), and ADAM23 (Fig. 7). Pre-immune serum and water were used as negative controls (e.g., Fig. 1). In general, each of the ADAM proteins investigated shows a spatially restricted and temporally regulated expression pattern during brain development.

ADAM9

The maturely active ADAM9 protein of 60 kDa was measured by Western blot analysis in different parts of the developing chicken brain (Figs. 1A and 2A). The results show that ADAM9 protein in the telencephalon seems to decrease from E10 to E18, and a small increase of the amount is found at E20 (Fig. 2C). In the diencephalon, ADAM9 expression is constant from E10 to E20, except for a small increase at E16 (Fig. 2C). In the tectum, the ADAM9 signal is increased from E10 to E12, followed by a decrease from E14, and is constant to E20 (Fig. 2D). In the cerebellum, ADAM9 protein translation decreases from E10 to E20 with a small increase at E16 compared to E14 and E18 (Fig. 2E). In the hindbrain, ADAM9 expression is constant from E10 to E20 with a small decrease at E16 (Fig. 2F).

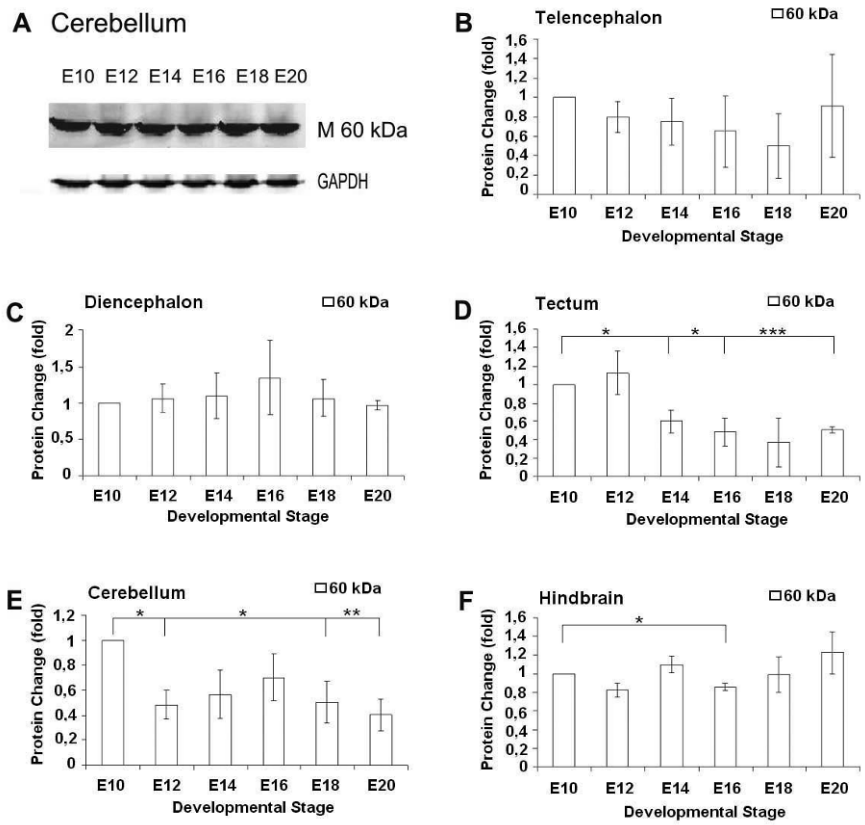

Fig. 2. ADAM9 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the cerebellum at different stages are shown in (A). Expression patterns of the active ADAM9 form (white) are demonstrated in (B) for telencephalon, in (C) for diencephalon, in (D) for tectum, in (E) for cerebellum, and in (F) for hindbrain. M in (A) indicates the mature ADAM10 protein form of 60 kDa.

ADAM10

The two different forms of ADAM10 proteins of a 114 kDa premature form and a 67 kDa mature form are changed greatly during brain development (Figs. 1B and 3A). In the telencephalon, the protein of the premature form maintains a constant amount from E10 to E20 (Fig. 3B), but the signal of the mature form increases strongly by 10-fold at E14, and by about 5-fold from E16 to E18, and then decreases to a level similar to E12 at E20. In the diencephalon, the amount of the premature form decreases gradually from E10 to E20 (Fig. 3C), while the expression of the mature form increases about 2.5-fold and then decreases gradually to E20. From E14 to E20 the amount of the mature form is clearly higher than that of the premature form at the same stages (Fig. 3C). In the tectum only the premature ADAM10 protein is detectable in a similar amount between

E10 and E12 (Fig. 3D). In the cerebellum, the protein of the premature form remains relatively high at E10 and then decreases slightly and is maintained from E12 to E20 (Fig. 3E), whereas the mature ADAM10 protein is only found from E14 to E18. In the hindbrain, the premature protein maintains a constant amount (Fig. 3F), but the mature form is only detectable from E14 to E18 and increases gradually.

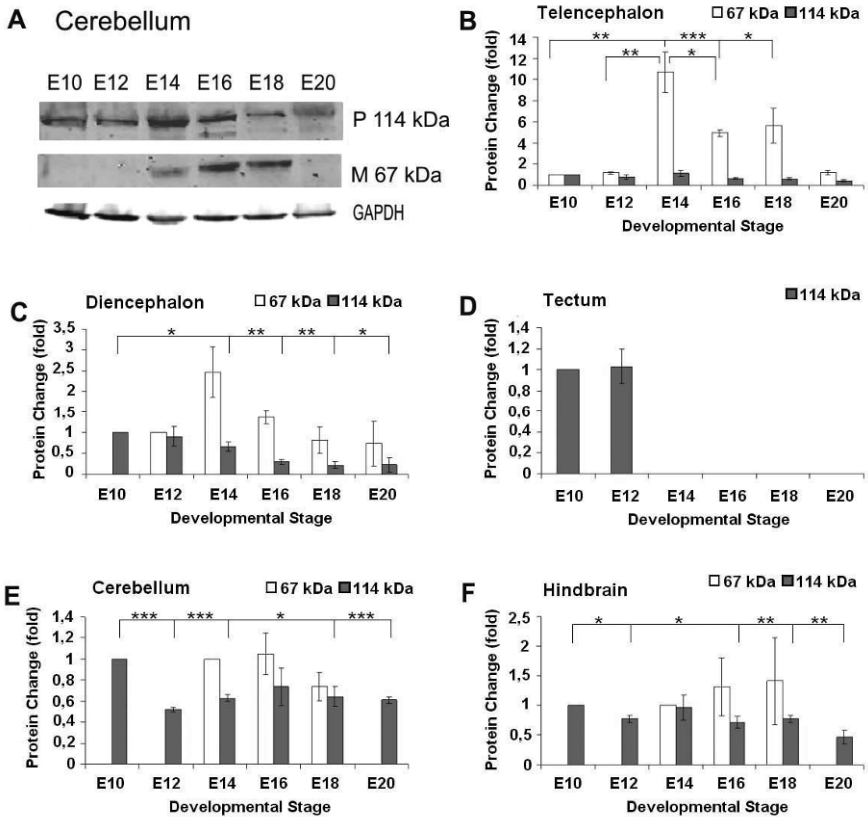

Fig. 3. ADAM10 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the cerebellum are shown in (A). Expression patterns of the active (white) and the premature form (gray) of ADAM10 are shown in the telencephalon (B), diencephalon (C), tectum (D), cerebellum (E), and hindbrain (F). P and M in (A) indicate the premature (114 kDa) and the mature (67 kDa) forms of ADAM10 protein.

ADAM12

In the developing brain, two protein forms of ADAM12 of 121 kDa and 75 kDa and a 90 kDa ADAM12-S were found (Figs. 1C and 4A). Generally, the mature form of 75 kDa was found in all detected parts of the brain, but the premature forms of 121 kDa and 90 kDa were only detectable in certain regions and stages

(Fig. 4). In the telencephalon, the amount of premature protein increases from E10 to E16, followed by a decrease until E20 to a level similar to E12 (Fig. 4B), while the premature 121 kDa was only found from E10 to E14 and is not detectable from E16. In the diencephalon, the premature 121 kDa protein is expressed only at E10 and E12 (Fig. 4C), while the expression of the mature ADAM12 protein increases from E10 to E16 and then decreases until E20 (Fig. 4C). The 90 kDa ADAM12-S protein was only seen in the diencephalon and it increases from E12, reaching a maximal amount at E14, followed by a gradual decrease to E20 (Fig. 4C). In the tectum, the premature 121 kDa protein was only found from E10 to E14 (Fig. 4D), while the mature form is constant from

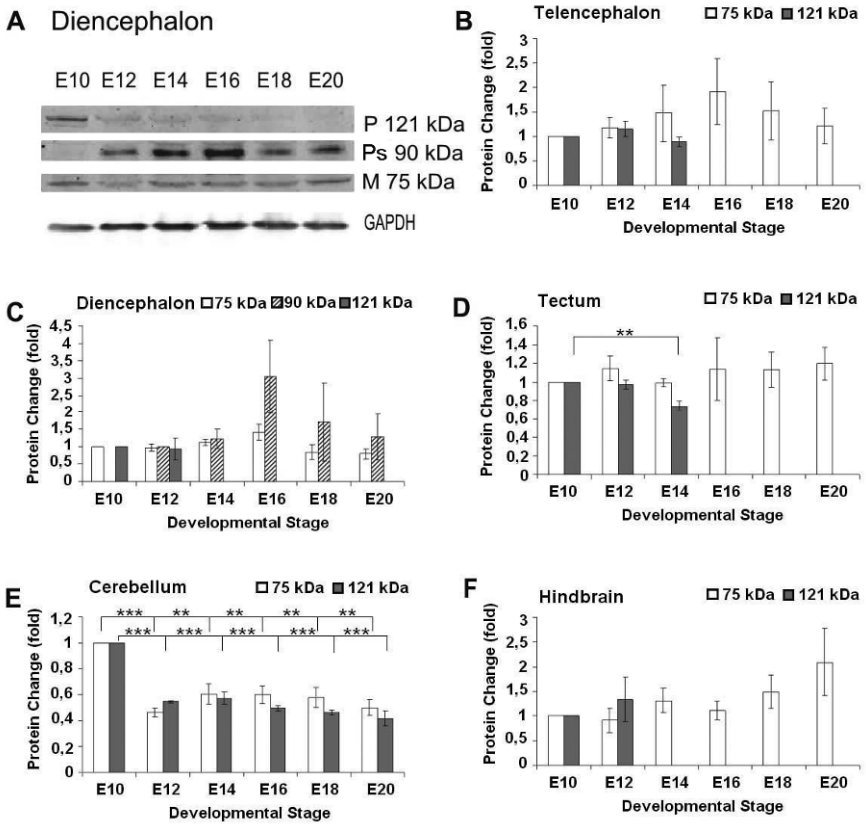

Fig. 4. ADAM12 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the diencephalon are shown in (A) with premature form (P; 121 kDa), the mature form (M; 75 kDa) and a premature form of ADAM12-short isoform (Ps; 90 kDa). Diagrams of telencephalon (B), diencephalon (C), tectum (D), cerebellum (E), and hindbrain (F) show the expression patterns of the active form (white), the premature form (gray) and the premature ADAM12-short isoform (diagonal lines; only in C) of ADAM12 protein.

E10 to E20. In the cerebellum, the premature 121 kDa and the mature 75 kDa proteins show a similar expression pattern: a relatively high level at E10 and then a decrease of 50% from E12 to E20 (Fig. 4E). In the hindbrain, the premature 121 kDa protein is detectable only between E10 and E12, while the mature form increases from E10 to E20 (Fig. 4F).

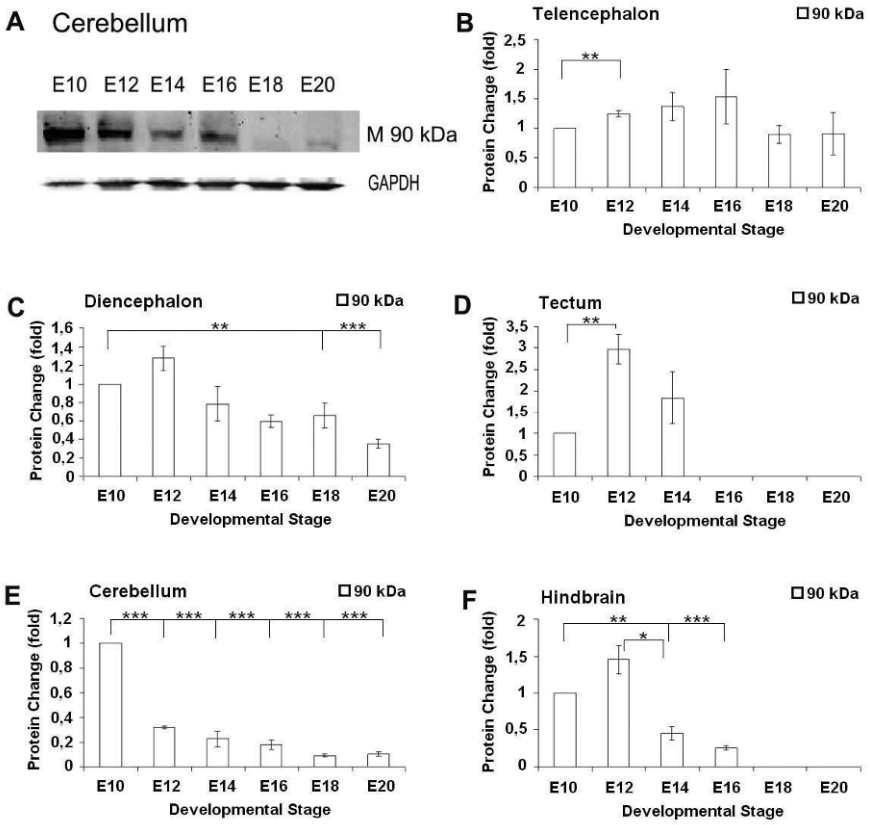

Fig. 5. ADAM17 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the cerebellum are shown in (A). M in (A) indicates the mature ADAM17 protein (90 kDa). Diagrams of the telencephalon (B), diencephalon (C), tectum (D), cerebellum (E), and hindbrain (F) show the expression patterns of the active form (white) of ADAM17.

ADAM17

The mature ADAM17 protein of 90 kDa was found in the developing brain with considerable variation. In the telencephalon the protein increases from E10 to E16, followed by a decrease to E20 (Figs. 1D and 5B), while in the diencephalon it increases from E10 to E12, followed by a decrease to E20, when the protein amount is at a level of 30% of that at E10 (Fig. 5C). In the tectum and the

hindbrain, the protein shows a significant increase from E10 to E12, followed by a decrease to E14 in the tectum (Fig. 5D) or to E16 in the hindbrain (Fig. 5F). The protein is no longer detectable anymore from E16 in the tectum (Fig. 5D) or from E18 in the hindbrain (Fig. 5F). In the cerebellum, ADAM17 protein was found in a relatively high amount at E10, followed by a decrease of the amount by about 20% of that at E10 (Fig. 5E).

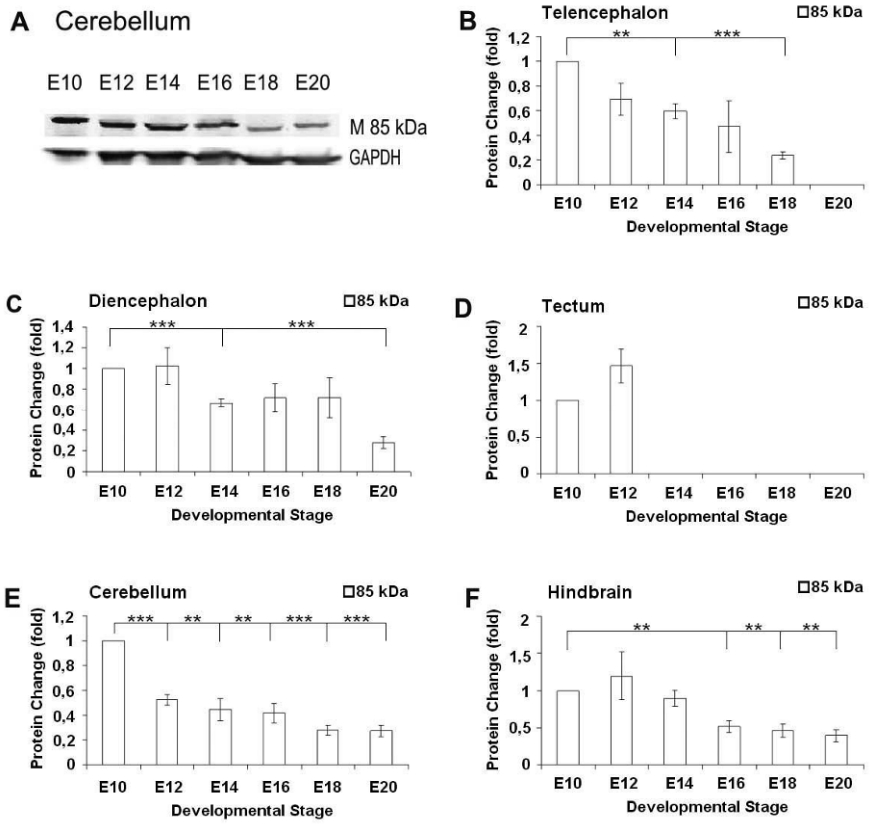

Fig. 6. ADAM22 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the cerebellum at different stages are shown in (A). M in (A) indicates the mature ADAM12 protein (85 kDa). Diagrams of the telencephalon (B), diencephalon (C), tectum (D), cerebellum (E), and hindbrain (F) show the expression patterns of the active form (white) of ADAM22.

ADAM22

In developing chicken embryo the mature form of ADAM22 is measurable at 85 kDa, which shows a decrease over time in all parts of the brain (Fig. 1F and 6A). In the telencephalon the amount of the mature protein decreases continuously from

E10 to E18 and is no longer detectable at E20 (Fig. 6B). In the diencephalon, it decreases from E12 to E20 (Fig. 6C). At E20 the protein level is only 30% of that at E10 (Fig. 6C). In the tectum, the mature form is only detectable at E10 to E12 (Fig. 6D). In the cerebellum the protein amount decreases continuously from E10 to E20 and at E20 is only 50% of the level at E10 (Fig. 6E). In the hindbrain the protein increases from E10 to E12, followed by a continuous decrease from E12 to E20 (Fig. 6F).

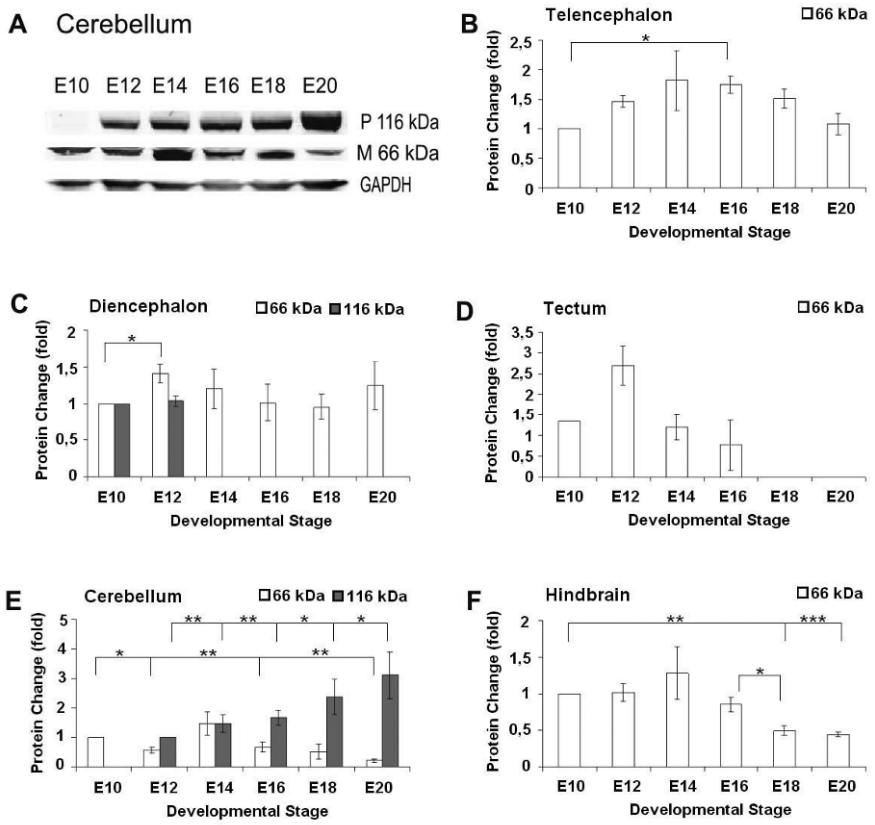

Fig. 7. ADAM23 expression patterns in different parts of the brain during embryonic development. Different parts of the brain were analyzed by Western blotting and quantified by Odyssey Infrared Imaging System (LiCOR – Odyssey program). Representative Western blot bands in the cerebellum at different stages are shown in (A). P and M in (A) indicate the premature (116 kDa) and the mature (66 kDa) forms of ADAM23 protein. Diagrams of the telencephalon (B), diencephalon (C), tectum (D), cerebellum (E), and hindbrain (F) show the expression patterns of the active (white) and the premature (gray) form of ADAM23.

ADAM23

ADAM23 is expressed as two forms in the developing chicken brain with a premature form of 116 kDa and a mature form of 66 kDa (Figs. 1G and 7A). In

the telencephalon only the mature form is measurable. The protein increases from E10 to E14, followed by a decrease until E20 (Fig. 7B). In the diencephalon the premature protein is only detectable at E10 and E12, whereas the mature protein shows an increase from E10 to E12 and then a stable amount from E14 to E20 (Fig. 7C). In the tectum, the mature protein increases from E10 to E12, followed by a rapid decrease until E16 (Fig. 7D), while the premature protein is not detected. In the cerebellum the premature protein increases fromE12 to E20 (Fig. 7E), while the mature protein is expressed at lower levels from E10 to E20. In the hindbrain only the mature form is measurable, with an increase from E10 to E14 followed by a decrease from E14 to E20 (Fig. 7F).

DISCUSSION

In the present study, the expression patterns of the regional ADAM proteins with the premature and the active forms in distinct parts of the brain were investigated. Our results show that the premature and the mature forms of the investigated individual ADAM proteins are expressed and regulated spatiotemporally during chicken brain development (Figs. 2-7).

Generally, members of the ADAM family are expressed as zymogens with their prodomains, which is required for inhibition of proteolytic activity and enzyme secretion [4, 26]. The inhibition of the prodomain on the metalloprotease domain is dependent on the specific sequence of the prodomain [27]. In several members, e.g., ADAM12 and ADAM17, the prodomain is also essential for efficient transport, folding and expression [28-30]. The removal of the prodomain of the ADAMs by, e.g., furin, results in the activity of their proteolytic function, which sequentially plays an important role in embryonic development [9]. Therefore, the investigation of the amount of the active mature forms of the ADAM proteins in distinct parts of the developing brain will help us to understand the regulation of the ADAMs and their functions in embryonic development.

ADAM9

During embryonic development, the active mature form of ADAM9 of 60 kDa is found predominantly in distinct parts of the brain with different expression patterns (Fig. 2). In general, active ADAM9 protein is found to be stably expressed at all time points except in the tectum and the cerebellum, where there is a decrease (Fig. 2). Our results extend the finding described by Lin et al. [16] that ADAM9 is expressed in different parts of the brain using *in situ* hybridization.

ADAM9 is a membrane-anchored protein known to process amyloid precursor protein (APP), insulin B-chain, pro-tumor necrosis factor-α (TNF-α), gelatin, beta-casein and fibronectin [31-33]. Furthermore, ADAM9 is responsible for release of growth factors such as heparin-binding EGF-like growth factor (HB-EGF) from the cell membrane by ectodomain shedding [34]. Weskamp et

al. [35] showed that the function of ADAM9 can be replaced by other members of the ADAM family in ADAM9-deficient mice. Furthermore, ADAM9 affects cell adhesion by binding to the disintegrin domain of integrin α6β1 [36].

ADAM10

During chicken embryo development, the premature and active forms of ADAM10 are spatiotemporally regulated in different parts of the brain (Fig. 3). Hall and Erickson [24] reported that an inactive form of about 110 kDa and an active form without a signal domain of 82 kDa have been found in chicken embryo from E2-E6. Our data show that the inactive form of about 114 kDa and the active form without a signal domain and prodomain of 67 kDa are present in the developing brain from E10 to E20 (Fig. 3), suggesting that during different stages of development, the form of the ADAM10 protein is dynamically regulated. Similar to our data, Lin et al. [16] described weak expression of ADAM10 throughout the whole chicken brain at embryonic stage E12. The active form of the 67 kDa ADAM10 protein is expressed mainly in distinct parts of the brain from E12 onward (Fig. 3). Interestingly, Zamenhof et al. [37] reported that the differentiation of neurons is predominant from E10 and ADAM10 is responsible for the later development of neuronal tissue [38, 39]. Therefore, the expression of the active form of ADAM10 protein may be involved in neural differentiation. Remarkably, previous studies showed that ADAM10 plays important roles in embryonic development. For example, ADAM10 is required for axon extension and controls neurogenesis in *Drosophila* development [20, 40, 41]. ADAM10 can shed different substrates such as amyloid precursor protein (APP), Notch and its ligand Delta 1, HB-EGF, EGF receptor, and cadherins including E-cadherin, N-cadherin, protocadherin C3 and VE-cadherin. All of them are involved in embryonic development [12, 16, 42-48]. Therefore, the spatial and temporal regulation of the active form of the ADAM10 protein suggests a role of ADAM10 in development of the embryonic brain.

ADAM12

Two splice isoforms of chicken ADAM12, the normal ADAM12 and ADAM12-S, have been found [16, 17]. ADAM12-S is weakly expressed at the mRNA level measured by RT-PCR [17]. In the present study, our data show that the premature form of 121 kDa and the mature form of 75 kDa of the ADAM12 protein are detectable in all parts of the developing brain, whereas the premature form of 90 kDa of ADAM12-S is only detected in the diencephalon from E12 onward (Fig. 4), suggesting that the expression patterns of ADAM12 are regulated exactly and the different ADAM12 splice isoforms play a distinct role in brain development. During embryonic development, ADAM12 is expressed by oligodendrocytes [49]. It can provoke myogenesis in vivo [50] and is implicated in cell fusion during C2C12 differentiation in vitro [25], suggesting a role of ADAM12 in myoblast fusion [51]. In chicken embryo, ADAM12 is

expressed in several regions of the neuroepithelium; it seems to play a role in brain regionalization [16].

ADAM17

ADAM 17, also called tumor necrosis factor alpha converting enzyme (TACE), is known to cleave TNF-α and transforming growth factor-α (TGF-α) [52-55]. In addition, TACE activates the epidermal growth factor receptor (EGFR) by cleaving TNF-α, and through this mechanism ADAM17 is also involved in the processes regulating cell growth, proliferation and migration [56, 57]. By processing amphiregulin, ADAM17 plays a further role in activation of stoma EGFR [58]. Besides the cleavage of TNF-α and TGF-α, ADAM17 is necessary for cell proliferation in T4-2 cells [54]. In the present study, the active form of ADAM17 of 90 kDa was detected. As reported in the literature [52], we could only detect the mature form of ADAM17 in the chicken brain. Our data show that from E10, ADAM17 is downregulated in many parts of the chicken brain (Fig. 5). Because cell proliferation in chicken brain is predominant before E10 [37, 54], the downregulation of ADAM17 is in accordance with its role in proliferation, suggesting that ADAM17 plays an important role in cell proliferation during development of the brain.

ADAM22

ADAM22 is closely related to ADAM23 and is highly expressed in the brain [59]. ADAM22-deficient mice show ataxia and peripheral nerve hypomyelination [15], suggesting that ADAM22 is necessary for normal brain development and nervous system function [42]. In addition, ADAM22 is a receptor for leucine-rich glioma inactivated 1 (LGI1), which regulates synaptic transmission in cells [60]. The cytoplasmic domain of ADAM22 interacts with 14-3-3ζ protein, which is required for cell adherence and cell spreading [61]. Our results show that ADAM22 is expressed in high amounts in the chicken brain from about E12 to E20 (Fig. 6). Lin et al. [16] reported that ADAM22 plays diverse roles in several brain structures, which was confirmed by our results. Although Lin et al. [16] describe weak expression in the cerebellum at E14 and E16, and a strong signal at E19, in contrast, our results show a continuous decrease in the cerebellum (Fig. 6E). This difference suggests that ADAM22 expression may be distinctly regulated at mRNA and protein levels.

ADAM23

ADAM23 is essential for normal brain development and function of the nervous system [59, 9]. ADAM23 appears during the differentiation of P19 cells and is involved in the formation of neurites, where ADAM23 is asymmetrically distributed [62]. ADAM23-deficient mice show tremor and strict ataxia and die within two weeks after birth [14]. Like the previous studies [62, 63], we found that ADAM23 is expressed in two forms and its expression is regulated at different developmental stages (Fig. 8). Lin et al. [16] found a strong homogeneous ADAM23 signal in the cerebellum and a weak signal in the

hindbrain from E12 to E19 at the mRNA level, and we also detected the ADAM23 protein in the cerebellum and the hindbrain over this period (Fig. 7), suggesting that ADAM23 is involved in embryonic development.

CONCLUSION

Our data show that the six different proteins of the ADAM family are expressed variously in distinct parts of the developing brain. ADAM9 is expressed at a similar level during the whole development from E10 to E20, while ADAM12, ADAM17, ADAM22 and ADAM23 show a decreasing tendency over this period. Furthermore, the active form of ADAM10 is only detectable from E14 to E18. These findings are consistent with the fact that ADAM9 is essential for cell survival as well as for some basic physiological processes, whereas the other ADAMs are mainly involved in specific processes during brain development.

Competing interests. The authors declare that they have no competing interests.

Authors' contributions. A.M. carried out the Western blot, participated in data analysis and wrote the manuscript. X.Y. participated in data analysis. A.R. conceived the study and contributed to drafting the manuscript. J.L. participated in the design of the study and wrote the manuscript. All authors read and approved the final manuscript.

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