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Heterogeneity in Synaptogenic Profile of Astrocytes from Different Brain Regions

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Abstract Astrocytes, the most abundant glial cells in the central nervous system (CNS), comprise a heterogeneous population of cells. However, how this heterogeneity impacts their function within brain homeostasis and response to injury and disease is still largely unknown. Recently, astrocytes have been recognized as important regulators of synapse formation and maturation. Here, we analyzed the synaptogenic property of astrocytes from different regions of the CNS. The effect of conditioned medium derived from astrocytes (astrocyte-conditioned medium (ACM)) from cerebral cortex, hippocampus, midbrain and cerebellum, in synapse formation, was evaluated. Synapse formation was analyzed by quantification of pre- and postsynaptic proteins, synaptophysin, and postsynaptic density protein 95 (PSD-95). ACM from the four regions increased significantly the number of synaptophysin/PSD-95 puncta on neurons from the same and different brain regions. Differences on astrocytic synaptogenic potential between the regions were observed according to ACM protein concentration. Thus, cerebellar astrocytes have higher synaptogenic effect when ACM is less concentrated. Also, heterotypical co-culture assays revealed that neurons from cerebral cortex and midbrain equally respond to ACM, indicating that differences in synapse effect are unlike to be neuron-autonomous. The expression profile of the synaptogenic molecules secreted by astrocytes from distinct brain regions was analyzed by qPCR. Gene expression of glypicans 4 and 6, hevin, and secreted protein-acidic and rich in cysteine (SPARC) greatly varies between astrocytes from different brain regions. Furthermore, in vivo analysis of hevin protein confirmed that variance. These findings highlight the heterogeneity of astrocytes and suggest that their synaptogenic potential may be different in each brain region, mainly due to distinct gene expression profiles.

Keywords Astrocyte · Synapse · Growth factors · And heterogeneity

Introduction

Astrocytes constitute the most abundant glial cells in the human brain [1]. Since their discovery in the end of the nineteenth century, astrocytes were considered a morphologically heterogeneous group of cells [2]. During the last decade, however, in vivo and in vitro evidences accumulated showing that those cells are much more heterogeneous in their function than previously thought, which led to the identification of several subpopulation of astrocytes within the brain [3] for review. Astrocytes differ in their morphology, developmental origin, neurogenic and proliferative potential, gene expression profile, physiological properties, and response to injury and disease [4–8] for review.

In the central nervous system (CNS), synapses can be ensheathed by astrocyte protrusions, usually referred as perisynaptic astroglial processes (PAPs) [9]. Thus, a single astrocyte can communicate with several synapses. In the hippocampus, it is estimated that an astrocyte can be in close contact with up to 140,000 synapses [10], while in the cerebral cortex, an astrocyte cover approximately 20,000–120,000 synapses [11]. Interestingly, PAPs can detect and integrate synaptic activity [12, 13], preventing neurotransmitters

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spillover and shaping extracellular neurotransmitter diffusion and uptake. However, the mechanisms through which astrocytes perform these functions and if these are common to all subgroups of astrocytes are still poorly understood.

The intimate relation of PAPs with neurons provides indispensable factors for the formation of neural circuits. In this environment, astrocytes ensure potassium ion homeostasis and extracellular pH regulation, regulation of intracellular calcium concentration, neurotransmitters uptake, release of neuroactive molecules known as gliotransmitters (e.g., glutamate, d-serine, ATP) [14–16], express receptors for several neurotransmitters, release trophic factors and synaptogenic molecules, such as thrombospondin (TSP), hevin, secreted protein-acidic and rich in cysteine (SPARC), glypicans, tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), brain-derived neurotrophic factor (BDNF), and others [17–22]. However, whether this plethora of functions can be performed by all astrocytes or are restricted to distinct subpopulations remains elusive. Evidences have been accumulating supporting the idea that molecularly distinct astrocytes perform unique functions.

The way that glial signals promote the formation of synapses varies with the neuronal type [23]: whereas hippocampal and cerebellar neurons form synapses in culture in the absence of glial cells [24], retinal ganglion cells, and subplate neurons do not [25–27]. Further, the proportion of perisynaptic astroglia might differ among brain regions [28, 29]. It remains unclear whether glia-driven synapse is a general principle that applies to all neuronal cell types and brain regions. Although astrocytes have been increasingly recognized as regulators of synapse formation, little is known about whether astrocytes within different brain regions have the same synaptogenic potential.

Here, we took advantage of the cell culture system to compare the ability of astrocytes from distinct regions to induce synaptogenesis in homotypic and heterotypic regions. We showed that astrocytes from cerebral cortex, hippocampus, midbrain, and cerebellum are powerful regulators of excitatory synapses and have different gene expression patterns of synaptogenic factors. These findings highlight the heterogeneity of astrocytes and suggest that their synaptogenic potential may be different in each brain region, mainly due to distinct gene expression profiles.

Materials and Methods

Animals: For astrocyte cultures, newborn (post natal day 0, P0) Swiss mice were used. For cerebral cortex, hippocampus, and midbrain neuronal cultures, embryonic (E14) Swiss mice were used. For cerebellum neuronal cultures post natal day 7 (P7), Swiss mice were used. All animal use protocols were approved by the Animal Use Ethics Committee of the Federal University of Rio de Janeiro.

Neuronal Cultures: neuronal cultures from cerebral cortex, hippocampus, and midbrain were prepared as described previously [22]. Brain tissues from Swiss mice at E14 were dissociated and plated at a density of 50,000–150,000 neurons/well of 13-mm diameter, previously coated with polylysine (50 μg/ml; Sigma Chemical Co., St. Louis, MO). Neurons were maintained in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27, penicillin, streptomycin, L-glutamine, Fungizone, and cytosine arabinoside (0.65 μM; Sigma Chemical Co., St. Louis, MO). This protocol generates a neuron-enriched culture (98 % of neurons and 2 % of astrocytes). Granule neurons were isolated from P7 Swiss mouse cerebellum as described previously [30]. Briefly, cerebella were dissected and cells were dissociated and platted at a density of 0.5×10^6 cells/well on a 24-well plate previously coated with polylysine (50 μg/ml; Sigma Chemical Co., St. Louis, MO). Cells were maintained in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27, penicillin, streptomycin, L-glutamine, Fungizone, and cytosine arabinoside (0.65 μM; Sigma Chemical Co., St. Louis, MO), at 37 °C in a humidified incubator with 5 % CO_2 and 95 % air chamber overnight.

Secondary Astrocyte Cultures: primary astrocyte cultures were prepared from cerebral cortex, hippocampus, midbrain, and cerebellum of newborn Swiss mice as previously described [22]. The brain structures were removed and the meninges were gently stripped off. Tissues were dissociated and cells were plated onto 25-cm^2 culture plates previously coated with polylysin (50 μg/ml; Sigma Chemical Co., St. Louis, MO) in Dulbecco's minimum essential medium with nutrient mixture (DMEM/F-12; Invitrogen, Carlsbad, CA) enriched with L-glutamine, penicillin, streptomycin, Fungizone, and 10 % fetal bovine serum (Invitrogen, Carlsbad, CA). The cultures were incubated at 37 °C in a humidified 5 % CO_2, 95 % air chamber for 7–10 days until reaching confluence. After that, the cells were submitted to one passage, generating merely pure astrocytic cultures. This protocol engenders astrocyte-enriched cultures constituted by 60 to 98 % glial fibrillary acidic protein (GFAP)-positive cells.

Astrocyte-Conditioned Medium: after reaching confluence, secondary astrocyte cultures were washed to eliminate residual serum and the cultures were incubated with DMEM/F-12 without fetal bovine serum, for 24 h. The medium was collected and centrifuged to remove cellular debris. To analyze the effect of astrocyte-conditioned medium (ACM) in neuronal synapse formation, neurons were cultured for 12 days in vitro (DIV), and then treated with ACM or with DMEM/F-12 without serum (control), for 3 h, followed by fixation with 4 % paraformaldehyde for 15 min and immunostaining of synaptic proteins.

Immunocytochemistry: cultures were fixed with 4 % paraformaldehyde for 10 min, washed three times in phosphate-buffered saline (PBS), permeabilized with 0.2 % Triton X-100

for 5 min at room temperature and nonspecific sites were blocked with 10 % bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 1 h. After blocking, coverslips were incubated overnight at 4 °C with the following primary antibodies: mouse anti-synaptophysin (Millipore Corporation, Darmstadt, Germany; 1:1000); rabbit anti-PSD-95 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:200); rabbit anti-GFAP (Dako Cytomation, Glostrup, Denmark; 1:1000); mouse anti-SPARCL1 (R&D Systems, Inc., Minneapolis, MN; 1:500). After incubation with primary antibody, coverslips were washed three times in PBS and incubated with the following secondary antibodies for 2 h, at room temperature: Alexa Fluor 546 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes; 1:1000) or Alexa Fluor 488 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes; 1:400). Nuclei were stained with 4′,6-diamidino-2-phenyindole (DAPI, Sigma Chemical Co., St. Louis, MO) and the coverslips were mounted on glass slides. The images were obtained with a TE 2000 Nikon microscope.

Synaptic Analyzes: to analyze synapse formation, neurons that were at least two cell diameters from their nearest neighbor were randomly selected. Approximately 15–20 digital images of both fluorescence emission 546 and 488 nm were recorded for each selected neuron. Colocalized puncta were identified with the plug-in "Puncta Analyzer" for the NIH image processing package ImageJ 1.29×, as previously described [22].

Immunohistochemistry: P15 mice were transcardially perfused with saline to clear out the blood, fixed with 4 % PFA for 24 h, and the brains were cryoprotected in sucrose (10, 20, and 30 %). The tissues were embedded in optimum cutting temperature compound (OCT) and cryosectioned (12 μm). Slices were blocked with 10 % bovine serum albumin (Sigma Chemical Co., St. Louis, MO) with 0.5 % Triton X-100 in PBS solution for 1 h. Primary antibodies incubations were then performed overnight at 4 °C: mouse anti-SPARCL1 (R&D Systems, Inc., Minneapolis, MN; 1:50) and rabbit anti-GFAP (Dako Cytomation, Glostrup, Denmark; 1:1000). After incubation with primary antibodies, tissues were washed three times with PBS and then incubated with secondary antibodies for 2 h: Alexa Fluor 546 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes; 1:1000) or Alexa Fluor 488 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes; 1:400). Nuclei were stained with 4′,6-diamidino-2-phenyindole (DAPI, Sigma Chemical Co., St. Louis, MO). The slices were mounted and imaged on a Leica SP5 confocal microscope. Levels of hevin in astrocytes (in vivo) were quantified by measuring the intensity of hevin immunostaining in GFAP-positive cells in each region. In order to do that, at least 15 cells were randomly selected around its extremities and the colocalization of hevin and GFAP was quantified in each cell, using region of interest (ROI) option in ImageJ (FIJI).

Quantitative RT-PCR: total RNA was Trizol® (Invitrogen, Carlsbad, CA) extracted from cortical, hippocampal, midbrain, and cerebellar astrocytes cultures according to manufacturer's protocol and RNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized from messenger RNA (mRNA) with a high-capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster, CA). Sense and antisense specific oligonucleotides for: TGF-β1 (F) TAC CAT GCC AAC TTC TGT CTG GG A, (R) ATG TTG GAC AAC TGC TCC ACC TTG; TSP-1 (F) CAT GGT CCT GGA ACT GAA GG, (R) TCC ATT GTG AAA GCA GAG GG; Glypican 4 (F) ACA ACC CAG AAG TCC AGG TTG ACA, (R) ACT CCG AAG GGC ACT GCT GAT ATT; Glypican 6 (F) GTC CGG ACC TAC GGG ATG CTG TAC, (R) TCT TCC ATT TCT GTG GTG CAG CAG G; Hevin (F) ACC GTG TCC ACT TCC TAT, (R) CCT CTT CTT CTT CCT CTT CTT C; SPARC (F) AAT TTG AGG ACG GTG CAG AG, (R) AAG TGG CAG GAA GAG TCG AA; TNF-α (F) CCT CCC TCT CAT CAG TTC TAT, (R) AGC CTT GTC CCT TGA AGA; BDNF (F) GAG CCT CCT CTA CTC TTT CT, (R) GGA TAC CGG GAC TTT CTC TA, and β-actin (F) TGG ATC GGT TCC ATC CTG G, (R) GCA GCTCAG TAA CAG TCC GCC TAG A were used. Quantitative real-time RT-PCR was performed using a Maxima SYBR Green quantitative RT-PCR (qPCR) Master Mix, including Maxima Hot Start *Taq* DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Reactions were performed on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method [31]. The amount of target genes expressed in a sample was normalized to the average of the endogenous control.

Statistical Analysis: one-way analysis of variance (ANOVA) followed by Tukey's test was applied to evaluate differences among conditions using the GraphPad Prism software version 5.0 (GraphPad Software, USA). Results are expressed as mean ± error and were considered to be statistically significant if $p < 0.05$.

Results

Astrocytes Induce Synapse Formation in Different Brain Regions

To investigate the effect of distinct subpopulations of astrocytes in synapse formation in vitro, neuronal cultures were treated with DMEM/F12 (control) (Fig. 1a–d) and with

astrocyte-conditioned medium (ACM) (Fig. 1e–h). Synapse formation was evaluated by immunostaining of the pre- and postsynaptic proteins, synaptophysin, and postsynaptic density protein 95 (PSD-95) and the quantification of colocalized punta (Fig. 1i–k).

The treatment with ACMs increased more than two times the number of synapses structurally formed at the cerebral cortex, hippocampus, midbrain, and cerebellum (Fig. 1k). We did not observe significant differences between the synaptogenic potential between ACMs from these regions. It is interesting to note that whereas ACMs from cerebral cortex and hippocampus increased the number of synaptophysin and PSD-95 puncta, ACMs from midbrain and cerebellum did not (Fig. 1i, j), suggesting different mechanisms underlying induction of synapse formation in these regions.

Neuron-astrocyte interactions are essential to synapse formation in some regions; however, if this is cell-autonomous (astrocyte or neurons or both) is still a matter of discussion. In order to address whether the synaptogenic potential is an astrocytic property or if it could be influenced by specific neuronal responsiveness, cerebral cortex and midbrain neuronal cultures were treated with DMEM/F12 (control) (Fig. 2a, g) or ACM from cortex, hippocampus, midbrain, and cerebellum (Fig. 2b–e, h–k). We observed that the ACMs from the four regions significantly increased the number of synapse between cortical and midbrain neurons compared to control conditions (Fig. 2f, l). There was no significant difference between the effects of the ACMs.

Therefore, these results indicate that astrocytes from different brain regions are strong regulators of synapse formation

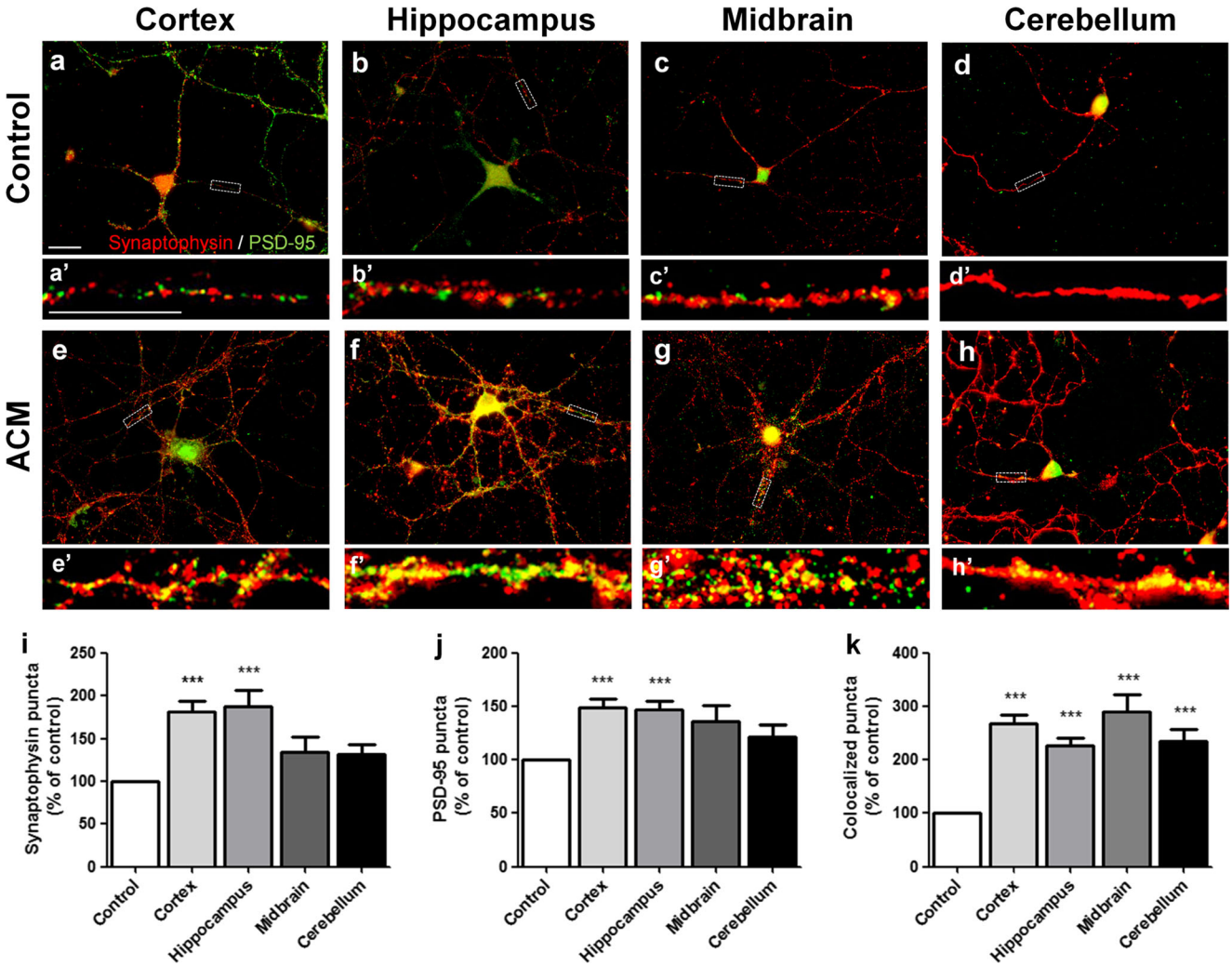

Fig. 1 Astrocytes induce synapse formation in different brain region. **a–d, a′–d′** Neuronal cultures with 12DIV from cerebral cortex, hippocampus, midbrain, and cerebellum were treated with DMEM/F12 (control) and with astrocyte-conditioned medium (ACM) from cerebral cortex (**e**, **e′**), hippocampus (**f, f′**), midbrain (**g**, **g′**), and cerebellum (**h, h′**), respectively, for 3 h. **i–k** Excitatory synapse formation was analyzed by imunocytochemistry for the synaptic proteins, synaptophysin (**i**), and PSD-95 (**j**), followed by quantification of puncta numbers (**k**). (n = 3). *Scale bars* 20 μm (**a**) and 5 μm (**a′**). ***$P < 0.001$

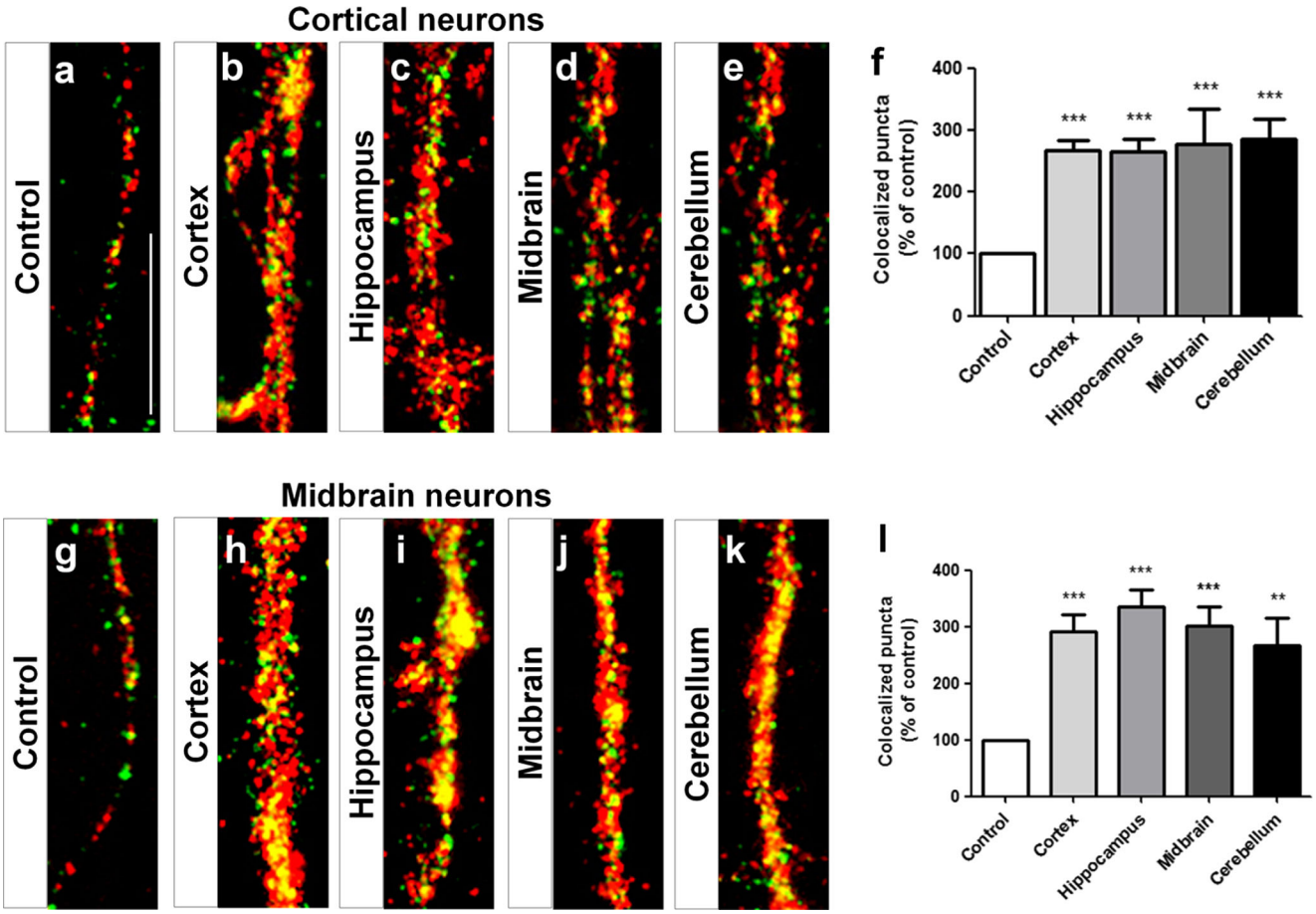

Fig. 2 Astrocytes from different brain regions induce synapse formation between cerebral cortex neurons. **a, g** Neuronal cultures with 12DIV from cerebral cortex and midbrain were treated with DMEM/F12 (control) and with ACM from cerebral cortex (**b, h**), hippocampus (**c, i**), midbrain (**d, j**), and cerebellum (**e, k**), for 3 h. **f, l** Excitatory synapse formation was analyzed by quantification of synaptic proteins puncta numbers**.** ($n = 3$). *Scale bar* 5 μm. $^{**}P < 0.01$; $^{***}P < 0.001$

and, as well as neurons, capable to modulate synaptic responsiveness.

Astrocytes from Different Brain Regions Have Heterogeneous Synaptogenic Potential

It is well known that astrocytes regulate synapse formation and maturation through the release of soluble factors. In general, these factors are secreted in very low concentrations and have saturation points for their effects. Thus, we asked if the ACMs used previously contained saturated concentrations of synaptogenic factors, which could avoid us from discerning small differences between them. For this, we performed synapse formation analysis with diluted ACMs. The ACMs from the four regions were used in full (100 %) and diluted forms (two times; 50 %; five times; 20 %) to treat neuronal cultures from the cerebral cortex, for 3 h. Then, the cells were fixed and immunostained for synaptic proteins and the number of synapses formed was quantified (Fig. 3).

We observed that increasing dilution of ACM clearly emphasized their differences in synaptogenic potentials. When two times diluted (50 %), ACMs from cerebral cortex and hippocampus were less synaptogenic than the ACMs from midbrain and cerebellum. Five times dilution (20 %) completely impaired cortex ACM effect and significantly diminished hippocampus and midbrain ACMs; while having no effect on cerebellar ACM, which maintained highly synaptogenic. Altogether, these data show that different populations of astrocytes have heterogeneous synaptogenic potential due to the secretion of different concentration of synaptogenic proteins.

Astrocytes from Different Brain Regions Express Different Profile of Synaptogenic Molecules

Although the identity of several synaptogenic molecules secreted by astrocytes is already known, there is little knowledge about differences in the expression of these molecules from distinct astrocytic populations and their impact on synaptogenesis. Firstly, to confirm the proteic nature of the synaptogenic factors in the ACMs, we tested whether their biological activity was sensitive to high temperatures. As

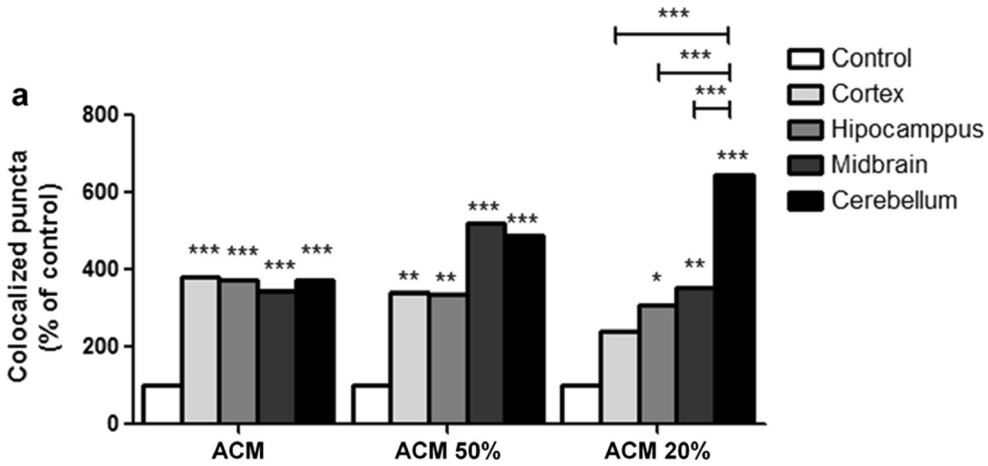

Fig. 3 ACM from distinct brain region have different amount of synaptogenic factors. **a** Cortical neuronal cultures with 12DIV were treated with DMEM/F12 (control) and with different concentrations (100, 50, and 20 %) of ACM from cerebral cortex, hippocampus, midbrain, and cerebellum, for 3 h, and synapse formation was analyzed. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

expected, ACM from cerebral cortex significantly increased the number of synapses between cortical neurons compared to control. In contrast, boiling of ACM completely inhibited its synaptogenic activity, indicating the protein nature of the synaptogenic molecule (Fig. S1). To identify some of these molecules, we analyzed by quantitative PCR, the gene expression of eight known synaptogenic molecules in cortical, hippocampal, mesencephalic, and cerebellar astrocytes in vitro.

We observed that astrocytes from distinct regions have different levels of expression of genes encoding TSP-1, glypicans 4 and 6, hevin, SPARC, TNF-α, BDNF, and TGF-β1 (Fig. 4a–d). In general, among the investigated molecules, SPARC mRNA was the most abundant in all regions, followed by hevin and glypican 4, while the mRNAs of glypican 6, TNF-α, BDNF, and TGF-β1 were transcribed in minor amounts.

In order to better analyze the variances on astrocytic expression of synaptogenic factors, the expression of each gene was also represented in separated graphs. We found major variances within regions, in the transcription of hevin, SPARC, and the glypicans 4 and 6 (Fig. 4e–l). While hevin is more expressed by cerebellar astrocytes compared to the other regions, SPARC is more expressed by cortical astrocytes. We also observed that glypican 6 is more expressed in the hippocampus and glypican 4 in the cerebellum. Although there was no significant difference on the expression of the other factors, we observed that TGF-β1 and TSP-1 tend to be more expressed by hippocampal astrocytes, and BDNF is apparently more expressed in the cerebral cortex.

In order to complement these data, we analyzed the distribution of Hevin, in different astrocytes in vivo and in vitro. Hevin is an extracellular matrix protein secreted by astrocytes in the synaptic environment and it is known to induce formation of excitatory synapses [18]. Cultured astrocytes from all different regions (cerebral cortex, hippocampus, midbrain, and cerebellum) produced hevin (Fig. 5a–h). It is interesting to note, however, that astrocytes from the same region present distinct levels of hevin production (Fig. 6a, e), suggesting that subpopulations within the same region might account for heterogeneity in the synaptogenic potential of astrocytes. Hevin staining was colocalized with GFAP+ astrocytes, showing that astrocytes from the four regions also produce significant amounts of hevin protein in vivo (Fig. 6a–l, i′–l′). In order to investigate whether qPCR expression profiling of cultured astrocytes matches in vivo expression patterns, we have quantified hevin levels across regional astrocytes in vivo. The pattern of hevin immunostaining was similar to those observed for mRNA expression. As shown in Fig. 6m, cerebellar astrocytes present increased hevin immunostaining compared to their counterparts in the other regions.

Together, our results report the genic and protein expression of synaptogenic factors in different subtypes of astrocytes. We suggest that variation in the profile of these factors might account for heterogeneity in the synaptogenic potential of distinct populations of astrocytes.

Discussion

In this study, we investigated the heterogeneity in the synaptogenic potential of astrocytes from different brain regions. We found that astrocytes from the cerebral cortex, hippocampus, midbrain and cerebellum regulate differently the formation of excitatory synapses. We also found that these cells exhibit different gene expression profile for some synaptogenic factors. Our data contribute to understand the role of glial cells to synapse formation in different brain regions, and shed light on the cell-autonomy of glia-induced synapse.

The structural and functional association between neurons and astrocytes at the synapse prompts the concept of the "tripartite synapse," which regards glial cells as integral elements of synaptic connections [32]. In spite of this well-accepted role for astrocytes, the position and requirement of perisynaptic astroglia might differ among mature brain regions [28]; whereas 90 % of cerebellar synapses have some perisynaptic astroglia, less than 50 % of cortical and

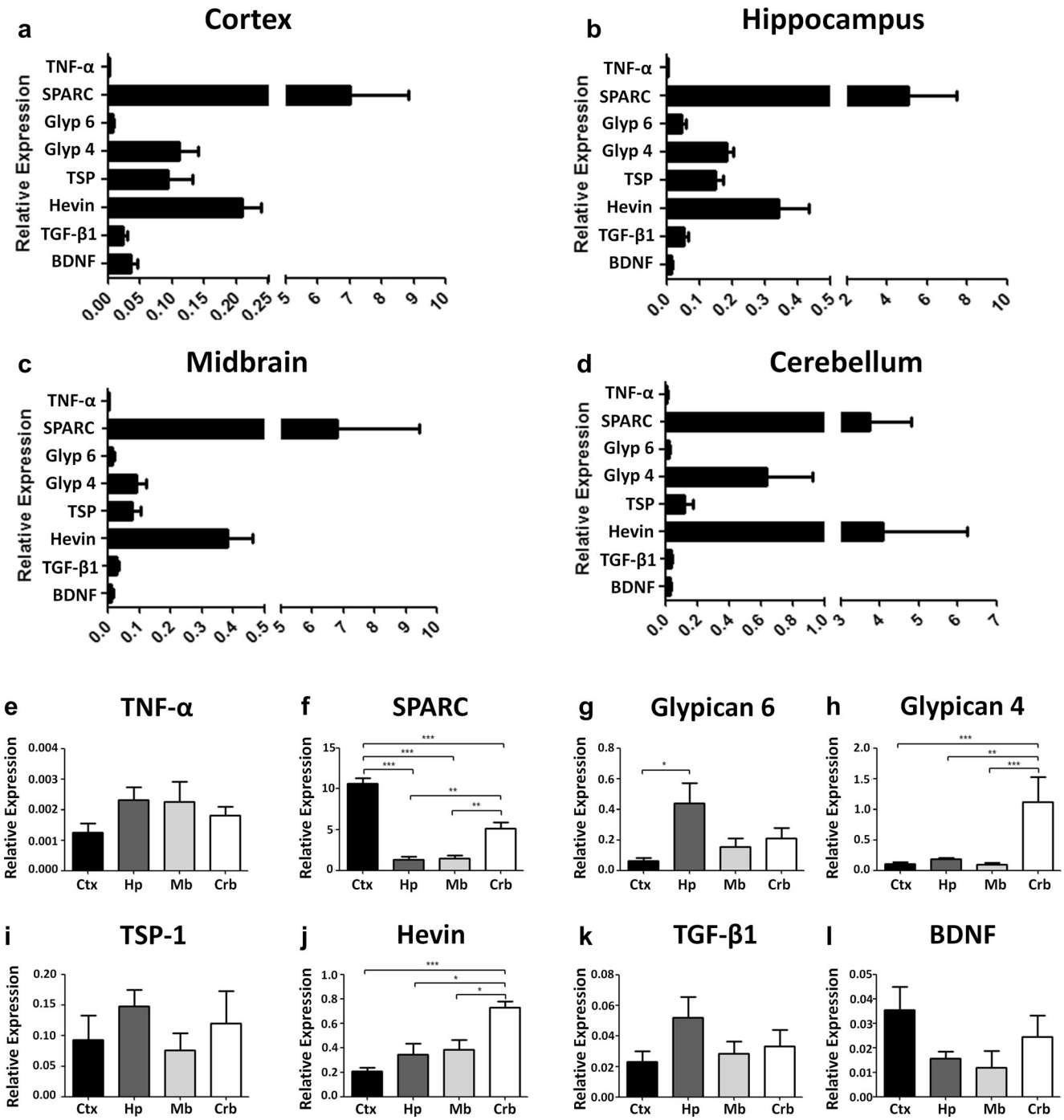

Fig. 4 mRNA levels of synaptogenic factors vary in astrocytes from different brain regions. The expression level of the genes for the synaptogenic factors was evaluated by real-time qPCR of cultured astrocytes from cerebral cortex (**a**), hippocampus (**b**), midbrain (**c**), and cerebellum (**d**). Each gene was represented separately: TNF-α (**e**), SPARC (**f**), glypican 6 (**g**), glypican 4 (**h**), TSP-1 (**i**), hevin (**j**), TGF-β1 (**k**), and BDNF (**l**). Values were normalized to that of beta actin transcript. One-way analysis of variance (ANOVA) followed by Tukey's test was used to evaluate differences among regions. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

hippocampal synapses have any [28, 29]. These observations raised some issues: Is glia-driven synapse a general principle that applies to all neurons and astrocytes in the brain? Which features confer the requirement of synapses to be ensheathed by astrocytes? What are the structural and functional differences between glia-modulated and glia-free synapses? Is glia-driven synapse astrocytic or neuronal-autonomous? So far, there is no clear evidence of the heterogeneity in the synaptogenic potential of different subtypes of astrocytes.

In the present study, we evaluated and compared the synaptogenic activity of astrocyte-conditioned medium (ACM) from cerebral cortex, hippocampus, midbrain, and

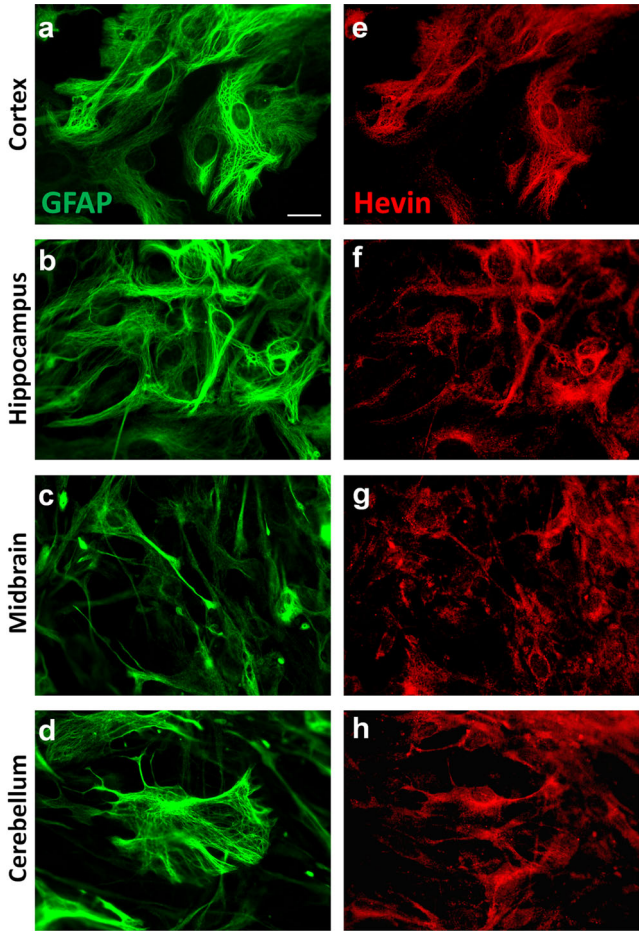

Fig. 5 Characterization of the distribution of hevin protein in astrocytes from different regions in vitro. Astrocyte cultures from cerebral cortex, hippocampus, midbrain, and cerebellum were immunostained for GFAP (*green* **a-d**) and hevin (*red* **e-h**). *Scale bars* 20 μm

cerebellum in neuronal cultures from the respective region. As expected, we observed an increase in synapse number in all four regions. Similar effect was observed when the ACMs were used to treat cultured neurons from heterotypical regions (different origin from the astrocytes), such as the cerebral cortex and midbrain. These data indicate that the synaptogenic potential we observed is an astrocytic property that is not restricted by neuron response capacity.

Although different neuron-astrocyte partners might be efficient in providing a synaptic niche as demonstrated here, in vivo evidences suggest that specificity of tripartite synapse might depend on the astrocyte domain [33]. Domain-specific depletion of astrocytes in ventral spinal cord resulted in abnormal motor neuron synaptogenesis [33]. Astrocytes from neighboring progenitor domains were unable to invade and rescue the depleted area, indicating essential region-specific neuron-astrocyte interactions. These results suggest that astrocytes in the various spatial domains might become specialized for interactions with their own particular neuronal neighbors as result of common patterning mechanisms.

We also found that although the ACMs have similar synaptogenic potential, qualitative and/or quantitative differences may impact the ability of astrocytes to induce synapse formation. Our findings demonstrate that dilution of ACMs emphasizes their differences in synaptogenic potential. They further suggest that each population of astrocytes might exhibit different repertoire of synaptogenic and anti-synaptogenic factors. Among the synaptogenic molecules secreted by astrocytes that have already been identified are the extracellular matrix proteins, thrombospondin, SPARC and hevin [17, 18], the proteoglycans, glypicans 4 and 6 [18], and the growth factors, TNF-α, BDNF, and TGF-β1 [22, 34]. However, it is still unclear whether these factors are equally expressed in different populations of astrocytes; further, if neurons derived from distinct regions are equally responsive to the molecules.

It is interesting to note that five times dilution of ACMs significantly impaired cortex, hippocampus, and midbrain ACMs effects; while had no effect on cerebellar ACM, which maintained highly synaptogenic. This response might be attributed to a misbalance in the ratio between synaptogenic/anti-synaptogenic factors in these media.

Several studies have shown that astrocytes from different regions are molecularly distinct. In this regard, cultured astrocytes from the brainstem, neocortex, and cerebellum are more closely related to each other compared to astrocytes from the optic nerve [35]. This molecular heterogeneity is also found in vivo in directly isolated astrocytes from different brain regions, with cortical astrocytes having different gene expression patterns from cerebellum astrocytes [36]. Similarly, a transcriptional analysis combining both in vitro and in vivo samples from several regions and developmental stages found out that astrocytes from each brain region have unique molecular profiles [37]. Altogether, these studies indicate the molecular and functional heterogeneity between different populations of astrocytes, but they do not provide an obvious connection with the astrocytic role in synapse formation.

Our data show that different populations of astrocytes have different gene expression profile of some of the known synaptogenic factors. While the mRNA levels of TSP-2, TNF-α, BDNF, and TGF-β1 are more similarly expressed between different populations of astrocytes, we found that the expression of hevin, SPARC and glypicans 4 and 6 varied significantly between distinct regions.

The glypicans 4 and 6 are heparin sulfate proteoglycans secreted by astrocytes and capable to induce functional glutamatergic synapses between ganglionar retinal neurons through recruitment of AMPA glutamate receptors [19]. Interestingly, besides both glypicans having the same synaptogenic potential; glypican 4 was more abundant than isoform 6 in all subtypes of astrocytes analyzed, and was enriched in the cerebellum in comparison to the other regions. On the other side, glypican 6 was more abundant in the

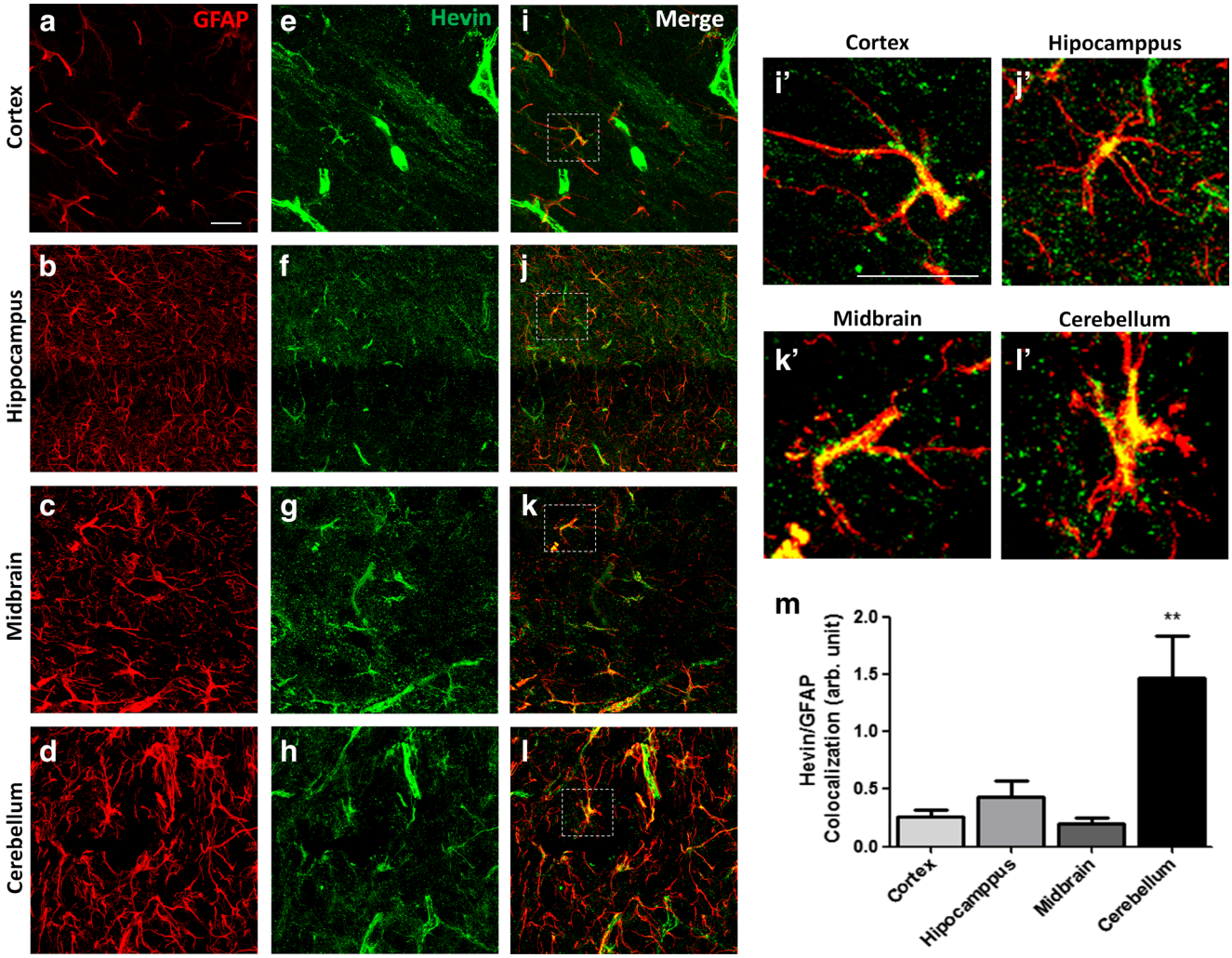

Fig. 6 Characterization of the distribution of hevin protein in astrocytes from different regions in vivo. Sagittal brain sections from P15 mice were stained with antibodies against GFAP (*red* **a**–**d**) and hevin (*green* **e**–**h**). *Merged images* indicate that hevin is distributed mostly on the cell body of GFAP+ astrocytes (**i**–**l**). High magnitude of selected squares (**i′**–**l′**). Individual cells were selected to quantify the levels of hevin in astrocytes as measured by hevin/GFAP colocalization (**m**). $^{**}P < 0.01$. *Scale bars* 20 μm (**a**–**l**) and 5 μm (**i′**–**l′**)

hippocampus in comparison with the other regions. The study of the role of glypicans 4 and 6 in synaptic formation and maturation [19] revealed that the expression of these proteoglycans overlaps in some regions during development. Our data are in contrast to previous work that showed that while glypican 4 is more expressed in the hippocampus, the expression of glypican 6 is higher in the cerebellum in P12 mice. Some possibilities may explain this discrepancy: (1) our data are derived from astrocytes from newborn animals and not P12 mice as in the previous work. This might be relevant since cerebellar development takes place mainly in the post natal period; (2) we used real-time PCR, a quantitative technique which is much more sensitive than in situ hybridization, the one previously used; (3) we analyzed the expression of these molecules in isolated cultured astrocytes, without interference from interactions with other cell types, which certainly have an impact in vivo.

Hevin and SPARC are extracellular matrix proteins, secreted by astrocytes to regulate the formation of synapses [18]. Hevin increases the number of synapses in retinal neurons and is required for proper formation and maturation of these synapses in their postsynaptic targets in the superior culicullum [18]. However, hevin expression is not restricted to retinoculicular system; it is regulated during cerebral cortex development [38], with higher levels between P15 and P25, which coincides with the period of intense formation, maturation, and elimination of synapses in this region. Hevin expression is restricted to astrocytes in this region and SPARC, a protein of the same family as hevin, acts as a negative regulator of synapse formation [18]. The structural homology of hevin and SPARC (53 % identity between amino acids) has led to speculation of possible genetic compensation between the two proteins [39]. Recently, a novel mechanism underlying hevin function has been described; astrocytes modify the interaction between the trans-synaptic

adhesion molecules, neuroligins and neurexins, through the secretion of hevin, thus modulating the formation and plasticity of excitatory synapses [40].

Here, we showed that hevin is significantly more expressed by astrocytes in the cerebellum compared to other regions. In contrast, SPARC expression showed the opposite pattern, with higher levels in astrocytes from the cerebral cortex. These findings corroborate the antagonistic functions of these proteins.

In order to reduce the number of animals used in this work, accordingly to animal ethical procedures, all astrocyte cultures were prepared from newborn mice. This procedure might have conceptual issues, since astrocytes develop and maturate differently across the brain [41]. While in the midbrain, astrocytes are generated during embryogenesis [5], in the cerebral cortex, hippocampus, and cerebellum they appear mostly after birth [3, 30]. Although the events that underlie the development and arrangement of these cells in different brain regions have been extensively studied, they are still not well understood. The major constraint is that there is no ideal marker to study the heterogeneity of astrocytes at any region and time course [3, 42]. Moreover, even in a specific region, subtypes of astrocytes are generated at different stages of development [43]. In order to investigate if expression profiling of cultured astrocytes matches in vivo patterns, we have quantified hevin levels across regional astrocytes in vivo. The pattern of hevin immunostaining was similar to those observed for mRNA expression, with increased hevin immunostaining in cerebellar astrocytes compared to their counterparts in the other regions.

The distribution of the synaptogenic protein hevin in astrocytes has been demonstrated in vivo [18, 38]. In the visual cortex of P25 mice, higher concentrations of hevin were found in the perinuclear region, while the processes and especially the end-feet of the cells did not reveal the presence of the protein. Our data corroborate this work and show that the distribution of the protein in P15 mice follows the same pattern on different populations of astrocytes, with higher concentrations around the nuclei and poorly distributed along the processes of some, but not all, astrocytes. Our results show that cultured astrocytes from the four regions also display hevin protein; however, in vitro, the protein is not concentrated only on the cell body; instead it is broadly distributed along the cells.

Accumulating evidences support the concept that heterogeneity in astrocyte-synapse interactions might impact several essential events for neural connection, including regulation of the levels of neurotransmitters, neuronal support for survival and maturation, control of the distribution of synaptic adhesion molecules, and receptors assembly, among others. Glutamate receptors and transporters are differentially expressed by astrocytes from different regions; whereas functional NMDA receptors are found in astrocytes from cerebral cortex and spinal cord [44, 45]; they have not been identified in hippocampal astrocytes [46]. Glutamate transporters GLT-1 and GLAST are also region-specific, with GLT-1 being expressed in the adult cortex, hippocampal gray matter, and spinal cord, and GLAST in the cerebellum, hippocampal dentate gyrus, and spinal cord [6]. Recently, Molofsky et al. showed that postnatal spinal cord astrocytes express several region-specific genes, mainly Semaphorin3a (Sema3a), required for proper motor and sensory neural circuit organization [47]. These works, together with ours, suggest that distinct region-restricted subpopulations of astrocytes provide a molecular signature that contributes to coordinate neural circuit refinement. Taken together, our data provide new information on the basis underlying the heterogeneity of astrocytes, especially on the formation of excitatory synapse in different regions of the CNS.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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