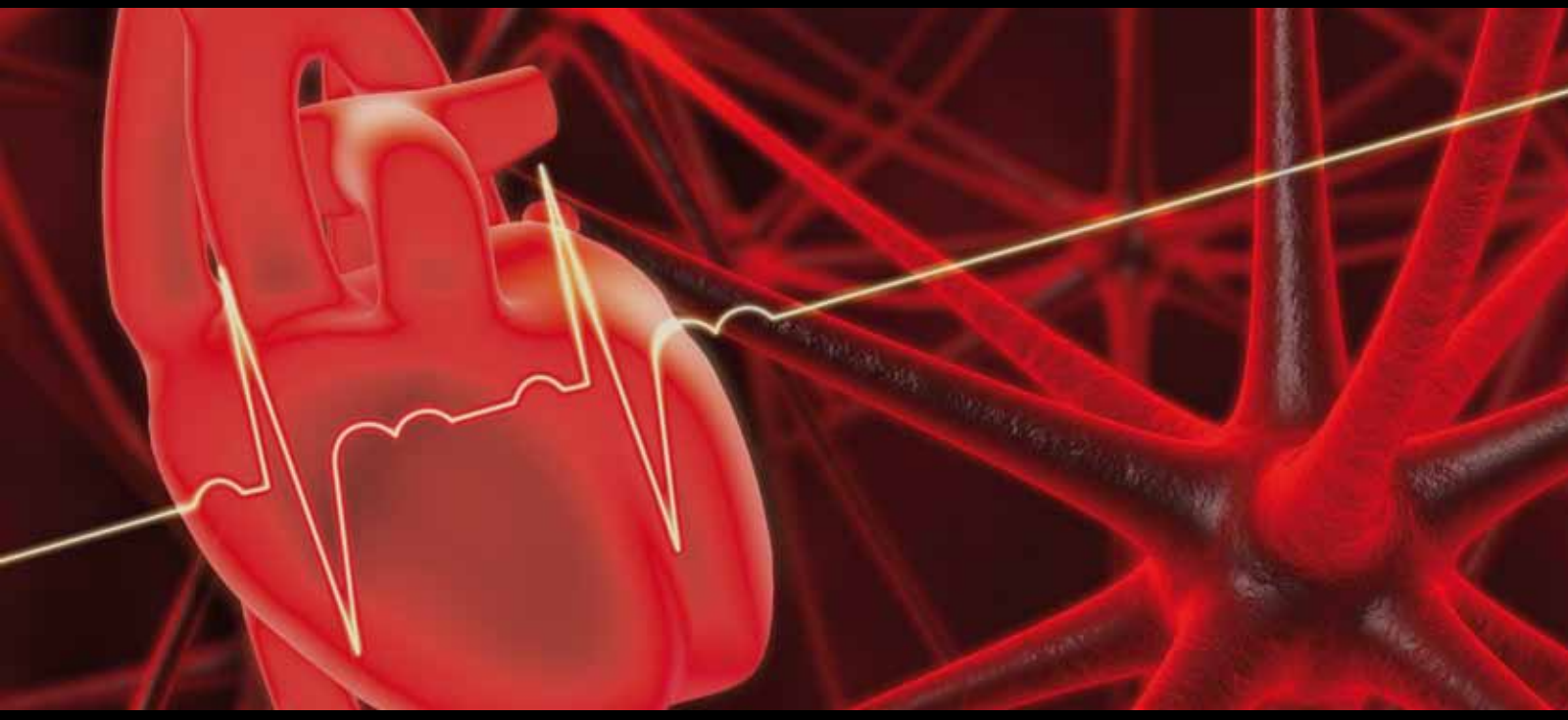
Cardiovascular Psychiatry and Neurology

S100B Protein in the Nervous System and Cardiovascular Apparatus in Normal and Pathological Conditions

Guest Editors: Rosario Donato and Claus W. Heizmann



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Editorial

S100B Protein in the Nervous System and Cardiovascular Apparatus in Normal and Pathological Conditions

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Accumulating evidence suggests that S100B, a Ca^{2+}-binding protein of the EF-hand type, functions as a regulator of intracellular activities and as an extracellular signal. Within cells, S100B interacts with a relatively large number of target proteins thereby regulating their functions (Figure 1). While most of these interactions are Ca^{2+} dependent, in some instances S100B/target protein interactions are not. S100B is also secreted by certain cell types and released by activated/damaged/necrotic cells. Secreted/released S100B can affect cellular functions with varying effects depending on its local concentration.

S100B is not a ubiquitous protein, its expression being restricted to astrocytes, Schwann cells, ependymocytes, certain neuronal populations, adipocytes, chondrocytes, melanocytes, dendritic cells, muscle satellite cells, skeletal myofibers, arterial smooth muscle cells, and bronchial epithelium, in normal physiological conditions. However, the S100B cell expression pattern during prenatal and postnatal development might be different (there is limited information about this issue); S100B expression levels in certain cell types may be varied in response to extracellular factors; levels of S100B are high in several cancer cells; S100B expression may be induced in cardiomyocytes and arterial endothelium in response to norepinephrine. Serum levels of S100B in normal prepubescent and postpubescent human subjects are relatively high and low, respectively, and increases in S100B serum levels are found in physiological conditions (such as intense physical exercise) and in several pathological conditions (mostly, brain diseases, certain psychiatric disorders, melanoma, and heart infarction and insufficiency).

The present special issue of "*Cardiovascular Psychiatry and Neurology*" focuses on the brain-heart role of S100B. In the adult brain, S100B amounts to ~0.5% of cytoplasmic protein content and is most abundant in astrocytes (with an estimated concentration of ~10 μM), where the protein is found diffusely in the cytoplasm and associated with microtubules, GFAP intermediate filaments, and intracellular membranes. Such a high concentration and its diffuse localization in the cytoplasm explain S100B's ability to interact with enzymes, enzyme substrates, scaffold/adaptor proteins, transcription factors, and cytoskeleton constituents thereby regulating energy metabolism, Ca^{2+} homeostasis, transcription, and cell shape, proliferation, differentiation, and motility (Figure 1). Besides, ~5% of S100B is being constitutively secreted by astrocytes; S100B secretion can be enhanced or reduced by a number of factors and/or conditions; in case of brain damage, large amounts of S100B are being passively released, with a fraction of the protein diffusing into the cerebrospinal fluid (CSF) and blood.

Whereas increases in the CFS and/or serum S100B content are taken as an indication of brain damage (yet increases in serum S100B content might point to nonbrain damage as well), a large body of evidence indicates that brain extracellular S100B behaves as a neurotrophin in normal physiological conditions and as a damage-associated molecular pattern (DAMP) factor upon massive release consequent to astrocyte activation or necrosis. In this latter case, S100B

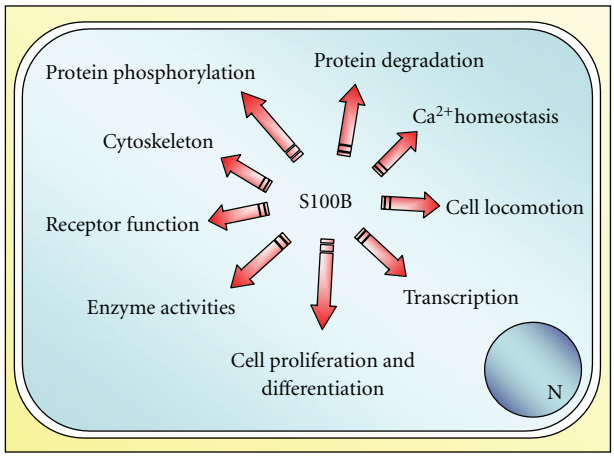

Figure 1: Schematic representation of intracellular regulatory effects of S100B.

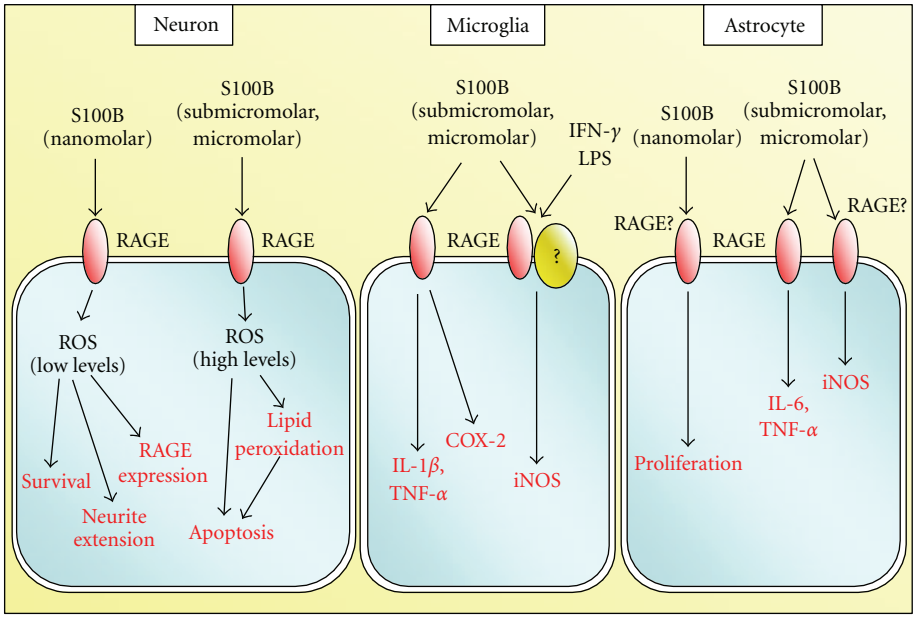

Figure 2: Schematic representation of extracellular regulatory effects of S100B on neurons, microglia, and astrocytes.

participates in the amplification of the brain inflammatory response by activating microglia and astrocytes and exerting toxic effects on neurons. RAGE (receptor for advanced glycation end products) has been identified as the receptor transducing both trophic and toxic effects of S100B in the brain and might act as a coreceptor supporting certain S100B effects on microglia (Figure 2).

As an intracellular regulator, S100B also participates in myocardium remodeling post infarction inhibiting the hypertrophic response in cardiomyocytes surviving the insult, and once released from necrotic cardiomyocytes, it acts as a DAMP factor causing cell death again via RAGE engagement. Adipocytes also release S100B in response to catecholamines with unidentified effects, though. Lastly, as a DAMP factor, S100B also participates in the pathophysiology of atherosclerosis stimulating vascular smooth cell proliferation and activating monocytes/macrophages.

The present issue of "*Cardiovascular Psychiatry and Neurology*" attempts to delineate the functional roles of S100B in the brain and the cardiovascular apparatus and to update the information about this multifaceted protein. We are confident that both cell biologists and clinicians will benefit by the reading of the papers presented therein.

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Review Article

S100B Protein, a Damage-Associated Molecular Pattern Protein in the Brain and Heart, and Beyond

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S100B belongs to a multigenic family of Ca^{2+}-binding proteins of the EF-hand type and is expressed in high abundance in the brain. S100B interacts with target proteins within cells thereby altering their functions once secreted/released with the multiligand receptor RAGE. As an intracellular regulator, S100B affects protein phosphorylation, energy metabolism, the dynamics of cytoskeleton constituents (and hence, of cell shape and migration), Ca^{2+} homeostasis, and cell proliferation and differentiation. As an extracellular signal, at low, physiological concentrations, S100B protects neurons against apoptosis, stimulates neurite outgrowth and astrocyte proliferation, and negatively regulates astrocytic and microglial responses to neurotoxic agents, while at high doses S100B causes neuronal death and exhibits properties of a damage-associated molecular pattern protein. S100B also exerts effects outside the brain; as an intracellular regulator, S100B inhibits the postinfarction hypertrophic response in cardiomyocytes, while as an extracellular signal, (high) S100B causes cardiomyocyte death, activates endothelial cells, and stimulates vascular smooth muscle cell proliferation.

1. Introduction

S100 is a multigenic family of small (~10 kDa) Ca^{2+}-binding proteins of the EF-hand type comprising 25 members exclusively expressed in vertebrates [1, 2]. In humans, the genes encoding S100A1-S100A16, S100A7L2, S100A7P1, and S100A7P2 map to chromosome 1q21, and the genes encoding S100A11P, S100B, S100G, S100P, and S100Z map to chromosomes 7q22-q31, 21q22, Xp22, 4p16, and 5q13, respectively [3]. With the exception of S100G which is a Ca^{2+}-modulator protein involved in the buffering of cytosolic Ca^{2+}, the members of this protein family are Ca^{2+} sensor proteins which once activated by Ca^{2+} interact with intracellular target proteins thereby regulating their activities. However, S100A10 is a constitutively activated protein involved in regulatory functions irrespective of the cytosolic Ca^{2+} level. It should be pointed out that: (1) the Ca^{2+}-binding affinity of S100 proteins (as measured in vitro) is considerably lower than that of the universal, intracellular Ca^{2+} sensor protein, calmodulin [4]; (2) however, S100's Ca^{2+}-binding affinity increases in the presence of target proteins (5) and/or, for some S100 members, Zn^{2+} [5, 6]; and (3) Ca^{2+}-independent interactions have been described for certain S100 proteins [7]. Importantly, differently from the ubiquitous calmodulin, individual S100 proteins exhibit a cell-specific expression, and some S100 proteins exert both intracellular and extracellular regulatory activities [1, 8]. Moreover, with the exception of S100G which is monomeric, all other S100 proteins exist within cells as dimers (mostly homodimers, and in certain cases heterodimers), and some of the secreted/released S100 proteins form oligomers [1, 9, 10].

S100B was the first member of the S100 protein family to be identified. This protein is highly abundant in the brain where it localizes to astrocytes (which represent the most abundant source of S100B in absolute), although certain neuronal populations also appear to express it [1, 10]. S100B is also expressed in cells outside the brain, such as melanocytes, adipocytes, chondrocytes, Schwann cells, glial cells of the gastrointestinal apparatus, supporting cells of the

adrenal medulla, dendritic cells, mature skeletal myofibers, skeletal muscle satellite cells, and arterial smooth muscle cells [1, 10–12]. Cardiomyocytes do not express S100B, but S100B becomes expressed in the cardiomyocytes surviving an infarction under the action of catecholamines [13–15].

S100B is constitutively secreted by astrocytes and its secretion can be regulated by a number of factors [10]. It is also secreted by adipocytes along with free fatty acids under the action of catecholamines [16]. Moreover, S100B is passively released from damaged and/or necrotic cells. The presence of S100B in the cerebrospinal fluid, serum, and amniotic fluid above threshold levels is used for diagnostic/prognostic purposes [17].

S100B exerts regulatory activities within cells and, once secreted/released, it acts as an extracellular signal. However, accumulating evidence suggests that intracellular regulatory activities of S100B differ substantially from its extracellular effects; that is, no unitary theory of intracellular and extracellular S100B's effects can be envisaged at present.

2. Intracellular S100B

As an intracellular regulator, S100B has been implicated in the regulation of protein phosphorylation, energy metabolism, the dynamics of cytoskeleton constituents (and hence, of cell shape and migration), Ca^{2+} homeostasis, and cell proliferation and differentiation [10]. The large variety of cell activities regulated by S100B can be explained by the high abundance of the protein in S100B-expressing cells and its cytoplasmic localization. Thus, the enhanced expression of S100B in melanoma cells has been suggested to be causally related to tumor progression given that S100B not only interacts with the tumor suppressor, p53, blocking its phosphorylation [18] but also downregulates p53 expression (with p53 in turn downregulating S100B expression) [19], and pharmacological blockade of S100B activity with pentamidine, a drug that disrupts S100B-p53 interactions [20], results in a significant tumor growth inhibition [21]. However, interaction with and/or downregulation of p53 might not be the sole mechanism whereby intracellular S100B stimulates cell proliferation. Indeed, intracellular S100B has been shown to stimulate proliferation and modulate cell differentiation via activation of PI3K and its downstream signaling pathways in neuronal and astrocytic cell lines [22, 23] and to modulate cell differentiation via activation of IKKβ/NF-κB in myoblast cell lines [12], and its induction by the so-called SOX trio in chondroblasts has been causally related to inhibition of chondrocyte differentiation [14]. Moreover, S100B positively regulates migration in astrocytes via activation of a Src/PI3K/RhoA/ROCK module [23]. Thus, intracellular S100B might serve the function of negatively regulating cell differentiation and stimulating proliferation and migration in cell lines. Whether S100B serves these functions during development and tissue regeneration remains to be established. A tight regulation of S100B expression appears to take place during neurogenesis, with relatively high S100B expression levels in neural progenitor cells as long as they are proliferating and migrating, followed by repression of S100B expression in coincidence with glial precursor cell differentiation and resumption of S100B expression in differentiated astrocytes [23, 24]. Interestingly, S100B appears to be required for MIO-M1 cells, a human Müller glia cell line, to form neurospheres (i.e., spherical aggregates of highly proliferating, round cells characterized by low adhesiveness to the substrate and the expression of transcription factors characteristic of neural stem cells [25]) [23]. Indeed, S100B knockdown in these cells has been shown to result in reduced neurosphere formation and proliferation and acquisition of an astroglial phenotype (unpublished results). Also, rat primary astrocytes transiently downregulate S100B expression when exposed to the differentiating agent, db-cAMP, and reexpress S100B at later stages of db-cAMP-induced differentiation [23]. In this case, as well as in the case of glioma cell lines induced to acquire a differentiated phenotype by serum starvation, reexpressed S100B firstly appears to be located at the origin of cell extensions in proximity of F-actin bundles [23]. These results are compatible with the possibility that: (1) S100B is required for neural progenitor cells to maintain stemness and migratory capacity; (2) transient repression of S100B expression is functionally associated with early steps of astrocytic differentiation; (3) persistence of S100B expression in neural progenitor cells might result in disturbances in neural cell differentiation [26, 27]; and (4) reexpression of S100B in differentiated astrocytes might be functionally linked to the maintenance of astrocytic processes as well as other cell activities (see above). The molecular mechanism regulating S100B expression in astrocytes depending on the developmental stage remains to be identified; preliminary evidence suggests that EGF signaling might cause downregulation of S100B expression in differentiating astrocytes [24]. Characteristically, S100B expression is enhanced in astrogliosis, a process consisting of proliferation and activation of astrocytes followed by their hypertrophy as observed after a brain insult that compromises brain tissue integrity or during chronic brain inflammatory states [28]. This raises the possibility that S100B might contribute to astrocyte reactivity following brain damage by favoring both the migration of activated astrocytes to the site(s) of damage and the formation and/or stabilization of F-actin cytoskeleton in astrocytic processes, likely via a Src/PI3K/RhoA/ROCK pathway and a Src/PI3K/Akt/GSK3β/Rac1 pathway [23], and possibly by regulating as yet unidentified intracellular activities via the Src/PI3K module. PI3K is known to play a regulatory role in inflammatory cells [29, 30], and astrocytes are active players in innate immunity in the brain [31]. Indeed, there is evidence that PI3K signaling might play an important role in both astrogliogenesis [32] and astrocytic activation in neuroinflammation [33–36]. Experimental evidence suggests that administration of arundic acid (ONO-2506), an agent suggested to inhibit S100B synthesis [37], in a rodent ischemia model results in inhibition of overexpression of S100B in astrocytes and the subsequent activation of signaling pathways in the peri-infarct area, in a reduction of delayed infarct expansion and in amelioration of neurologic deficits [38]. Conversely, after permanent middle cerebral artery occlusion in S100B

transgenic (TG) mice, infarct volumes are significantly increased during the first postinfarct days and astrogliosis is enhanced compared with controls [39]. Moreover, S100B TG mice show increased susceptibility to perinatal hypoxia-ischemia [40], and overexpression of S100B has been shown to accelerate Alzheimer disease-like pathology with enhanced astrogliosis and microgliosis [41]. In this regard, association between elevated brain levels of S100B and several brain pathologies including Alzheimer disease is a well-established notion [10, 42]. Although in the aforementioned cases [38–41] it is difficult to distinguish between intracellular and extracellular effects of S100B, it is tempting to speculate that elevated levels of intracellular S100B might contribute to astrocyte activation during the course of brain damage and to astrogliosis. Yet, these putative effects of intracellular S100B appear to be counterbalanced by S100B extracellular effects in part (see below).

As mentioned earlier, cardiomyocytes do not express S100B in normal physiological conditions; however, S100B becomes expressed in cardiomyocytes surviving an infarction under the action of catecholamines and acts to inhibit the cardiomyocyte hypertrophic response with a mechanism that remains to be elucidated [13–15].

Genetic evidence has been presented that S100B exerts inhibitory effects on caffeine-induced rises in the free Ca^{2+} concentration in astrocytes, suggesting that S100B might act to reduce cytosolic Ca^{2+} concentration [43]. However, the molecular mechanism underlying this S100B effect has not been identified. On the other hand, acute infusion of S100B knockout (KO) mice with norepinephrine (NE) after a 28-day treatment with NE results in a significantly smaller increase in mean arterial pressure (MAP) compared to wild-type (WT) and S100B TG mice, with a tendency of S100B TG mice to respond with higher MAP values compared with WT mice [11]. Arterial smooth muscle cells (ASMCs) from S100B KO mice are less responsive to NE treatment compared with WT ASMCs due to either reduced Ca^{2+} mobilization from internal Ca^{2+} stores or reduced extracellular Ca^{2+} influx [11], pointing to a requirement of S100B for appropriate Ca^{2+} responses to NE. However, this does not apply to cardiomyocytes from S100B KO mice pointing to a dissociation between effects of S100B in cardiomyocytes and those in ASMCs in the same experimental setting (i.e., stimulation with NE) [11]. The molecular mechanism underlying S100B's ability to enhance cytosolic Ca^{2+} concentration in ASMCs also remains to be identified. The giant phosphoprotein AHNAK, which modulates L-type Ca^{2+} channels in response to β-adrenergic stimulation [44], interacts with phospholipase C$\gamma$and PKC-αincreasing intracellular Ca^{2+} mobilization [45] and is expressed in smooth muscle cells and cardiomyocytes [46], is an S100B target protein [47]. Thus, AHNAK is a potential intermediate linking S100B to elevation of cytosolic Ca^{2+} levels [11]. However, additional intermediates appear to come into play because S100B-AHNAK interactions also occur in cardiomyocytes which do not require S100B for increasing cytosolic Ca^{2+} levels in response to NE, as mentioned earlier. Together, these results point to a differential ability of S100B to intervene in the regulation of the cytosolic Ca^{2+} concentration in activated cells depending on the cell type, suggesting that cell-specific intermediates might link S100B to regulators of cytosolic Ca^{2+} levels.

3. Extracellular S100B

3.1. S100B Acts as an Extracellular Signal in Brain, Vasculature and Heart. The first evidence for the presence of S100B outside neural cells was provided by Michetti et al. [48] who detected measurable amounts of S100B in the cerebrospinal fluid of patients with multiple sclerosis. S100B was ever since taken as a marker of brain disease [17]. Subsequently, Shashoua et al. detected S100B in the brain extracellular fluid [49], and later on, Van Eldik and Zimmer [50] demonstrated that an astrocyte cell line secreted S100B under conventional culture conditions. Secretion of S100B from astrocytes, which occurs via a noncanonical secretion route, was subsequently shown to be regulated by a number of factors/conditions, of which some enhance secretion (e.g., serotonin, lysophosphatidic acid, low levels of glutamate, forskolin, low extracellular Ca^{2+} and/or K^+ levels, TNF-α, IL-1β, metabolic stress, serum deprivation, kainic acid, the neurotoxin 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine, natural antioxidants, and antipsychotic drugs) while some other reduce secretion (e.g., high levels of glutamate, glucose and K^+, inhibition of Src kinase activity, cell confluence, Ca^{2+} channel blockers and gap junction inhibitors) [10, 51–53]. However, a rather small fraction of intracellular S100B is being secreted constitutively by astrocytes, and S100B secretagogues cause a 2-4-fold increase in secretion at most [10]. Given that the total brain S100B concentration amounts to 10–20 μM; that the brain intercellular space is relatively narrow; and that a fraction of secreted S100B diffuses into the cerebrospinal fluid, the brain extracellular S100B concentration should amount to a few nM under normal physiological conditions. Yet, the brain S100B concentration outside cells might be several orders higher in case of astrocyte damage and/or necrosis due to a combination of passive release of the intracellular protein, defective clearance of the extracellular protein in consequence of inflammation, and/or Ca^{2+}-induced formation of S100B oligomers [54, 55] and adhesion of S100B oligomers to the extracellular matrix.

The existence of an extracellular fraction of S100B was soon put in relation to effects of the protein on brain cells inasmuch as a neurite extension factor from bovine brain was identified as a disulfide-linked dimer of S100B [56]. Whereas early reports on extracellular S100B supported the possibility that the protein might function as a trophic factor towards neurons and astrocytes [10], the observation that elevated levels of S100B were present in the temporal lobe of patients with Alzheimer disease in conjunction with the presence of highly reactive S100B-positive astrocytes surrounding the neuritic plaques [57] raised the possibility that S100B might contribute to Alzheimer disease neuropathology. Thus, research on extracellular S100B proceeded ever since along two main directions, on the basis of the protein's dual role as a neurotrophic factor and as a neurotoxic factor. These apparently contradictory effects of S100B in the brain were

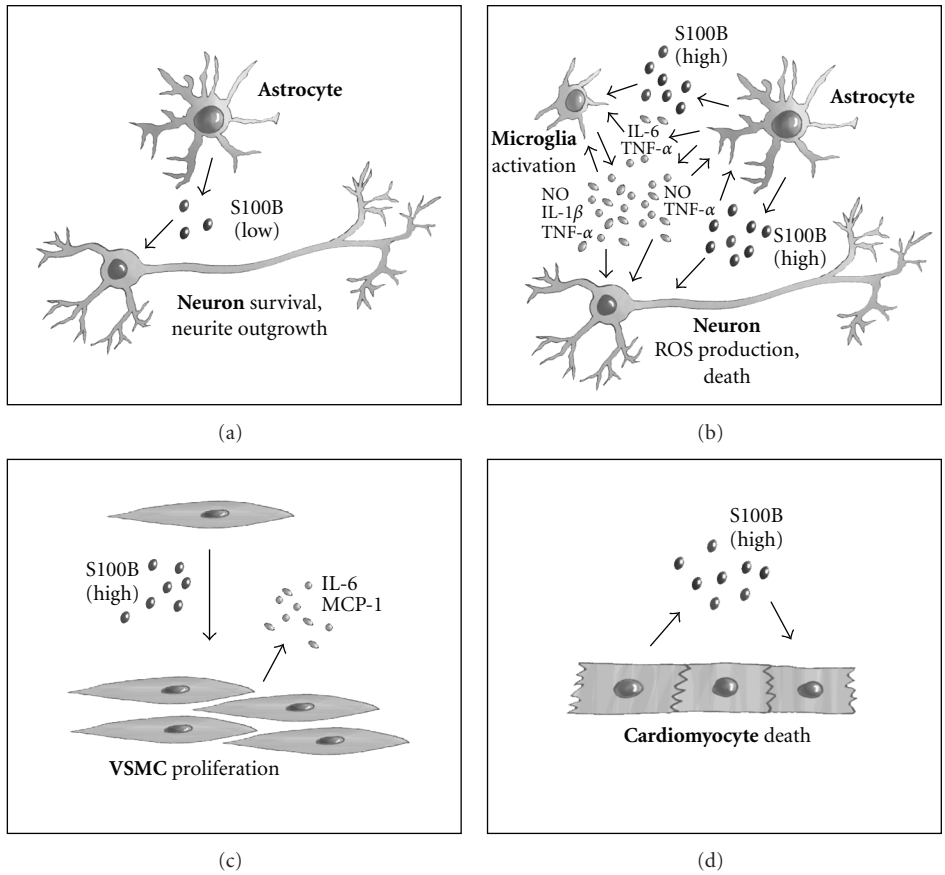

Figure 1: Schematic representation of extracellular effects of S100B in brain, heart, and vasculature. (a) At low concentrations, S100B promotes neuronal survival and stimulates neurite outgrowth via stimulation of RAGE signaling. (b) At high concentrations, S100B causes neuronal death both directly via excessive stimulation of RAGE signaling in neurons and indirectly via RAGE-dependent activation of microglia and astrocytes. (c) At high concentrations, S100B stimulates VSMC proliferation via RAGE activation. See text for details. (d) S100B released from necrotic cardiomyocytes kills nearby, surviving cardiomyocytes via RAGE activation.

shown to be dependent on the protein's concentration, with doses of S100B up to a few hundred nM being neurotrophic and higher doses being neurotoxic [42]. Indeed, S100B at high doses causes neuronal apoptosis both via a direct action on neurons [58] and via stimulation of nitric oxide (NO) release by astrocytes [59] (Figure 1(a)). Moreover, at high doses, and in the presence of cofactors (either bacterial endotoxin or interferon-γ [IFN-γ]), S100B enhances NO release by microglia [60, 61], the brain resident macrophages (Figure 1(b)). The finding that S100B can activate microglia, albeit at high doses, suggests that the protein might have a role in neuroinflammation, a possibility substantiated by other studies of effects of S100B on microglia and astrocytes [62–66] (Figure 1(b)).

A strong impulse to research on extracellular effects of S100B in the brain came from the observation that RAGE (receptor for advanced glycation end products), a multiligand receptor of the immunoglobulin superfamily expressed in several cell types including neurons during development as well as in activated inflammatory cells, transduces S100B's effects on endothelial cells and microglia [67]. Indeed, RAGE ligation by S100B on neurons has been shown to be responsible for both the protein's neurotrophic effects (at low S100B doses) via activation of a Ras/MEK/ERK/NF-κB/Bcl-2 pathway and a Ras/Cdc42-Rac1 pathway, and pro-apoptotic effects (at high S100B doses) via hyperactivation of the Ras/MEK/ERK pathway and consequent overproduction of reactive oxygen species (ROS) [68] (Figure 1(b)). These latter results anticipated that: (1) extracellular S100B might affect any RAGE expressing cell; (2) the outcome of S100B action might be dependent on RAGE functionality and/or concentration; (3) the different outcome of S100B effects depending on the protein's concentration might be dependent on the number of RAGE molecules engaged on the cell surface and/or the physical state of S100B; and (4) the relatively high doses of S100B required for RAGE-dependent activation of inflammatory cells, compared with absence of effects at low doses, might reflect a differential ability of RAGE to recruit different intermediates linking the receptor to intracellular signaling pathways depending on the cell type, RAGE's physical state and/or the intensity/duration of RAGE stimulation.

As to point (1), S100B has been indeed used ever since as a generic RAGE agonist [8, 10]. However, RAGE-independent effects of low and high doses of S100B have been documented in the case of cultured myoblasts (which express RAGE) in differentiation medium [69–71], which raises the possibility that extracellular factors might regulate S100B/RAGE interactions. Indeed, recent evidence suggests that in high-density myoblast cultures S100B (up to 1 nM) induces the formation of a multimeric RAGE/S100B/bFGF/FGFR1 transcomplex in which bFGF/FGFR1 antimyogenic signaling is enhanced while RAGE promyogenic signaling is inhibited, as opposed to RAGE-dependent regulatory effects of S100B in low-density myoblast cultures (a condition in which S100B does not bind to bFGF/FGFR1 and thus fully activates RAGE) (F. Riuzzi, G. Sorci and R. Donato, submitted for publication).

As to point (2), in general no effects of S100B could be detected in neurons, astrocytes, and inflammatory cells that had been transfected with a dominant negative RAGE mutant (i.e., RAGE lacking the cytoplasmic and transducing domain) or in which RAGE expression had been knocked down (11). However, S100B has been shown to stimulate NO release from IFN-γ-treated microglia to the same extent in microglia that had been stably transfected with either full-length RAGE or dominant negative RAGE, but to a significantly larger extent in these cases compared with mock-transfected microglia [72]. This suggests that at least in the case of S100B-induced NO release by microglia, the RAGE extracellular domain might serve to concentrate S100B on the microglial cell surface thereby allowing S100B to potentiate IFN-γ effect. Whether S100B interacts with IFN-γ thereby potentiating IFN-γ effects remains to be established. Yet, at high doses S100B causes overproduction of ROS via activation of the NADPH oxidase complex in monocytes/macrophages in the absence of co-factors, with ROS in turn, activating Src tyrosine kinase which recruits a Ras/MEK/ERK1/2/NF-κB pathway and a Rac1/Cdc42/MKK6/p38 MAPK/NF-κB pathway with ensuing upregulation of IL-1β, TNF-α, COX-2 and iNOS expression [73, 74]. In these latter works, RAGE-signaling has been proposed, but not directly demonstrated to have a role in S100B effects. By contrast, at high doses S100B causes myoblast death via ROS overproduction, via a RAGE-independent mechanism that remains to be elucidated [70]. On the other hand, S100B/RAGE signaling-dependent RAGE induction and activation in neuronal cells might contribute to the protective effect of low S100B doses towards β-amyloid neurotoxicity and to amplification of β-amyloid neurotoxicity by high S100B doses [75].

As to point (3), preliminary evidence suggests that at high, but not low doses S100B chemoattracts microglia in a RAGE-dependent manner; however at low doses the protein does chemoattract RAGE-overexpressing microglia (R. Bianchi, Eirini Kastrisianaki, I. Giambanco, and R. Donato, submitted for publication). This suggests the possibility that at the levels found in normal physiological conditions brain extracellular S100B cannot affect microglia migration due to the very low, if any expression levels of RAGE in "resting" microglia, but the protein might contribute to chemoattract microglia at the beginning of a brain insult as a result of activation of microglia, a condition which is accompanied by induction of RAGE in these cells [76]. Thus, increasing the density of RAGE molecules on the microglial cell surface might cause S100B to switch from a neurotrophic factor to a proinflammatory factor. In this regard, it is known that RAGE is induced in a variety of cell types by RAGE-activating ligands [77, 78]. Moreover, recent evidence suggests that extracellular S100B exists in the form of octamers and higher-order multimers in the nonreducing, high Ca^{2+} conditions found in the extracellular fluid; that RAGE exists in the form of oligomers on the cell surface; and that ligand-induced oligomerization is required for RAGE to signal adequately [54, 55, 79]. Thus, the neurotoxic and proinflammatory effects of S100B might require the occurrence of high levels of S100B oligomers/multimers interacting with RAGE oligomers (and/or causing/enhancing RAGE oligomerization). Importantly, doses of S100B causing neuronal apoptosis do not cause microglial apoptosis, a difference which is likely to depend on the different scavenging ability of neurons and microglia towards oxidants. Microglia activation and chemotaxis are being usually considered in the context of neuroinflammation. However, it should be pointed out that microglia chemoattraction is not necessarily a dangerous and/or inflammation-related event; "resting" microglia are not exactly resting, microglia continuously patrolling the territory, exerting a protective action by virtue of their ability to keep the neuronal and astrocytic extracellular milieu clean, and likely resolving mild degree brain insults [80, 81]. According to these views microglia are active players in brain tissue homeostasis under normal physiological conditions, and thus (low) S100B's ability to chemoattract microglia might be beneficial in case of mild degree brain insults. However, whereas there is information about the increased susceptibility to perinatal hypoxia-ischemia and accelerated Alzheimer disease-like pathology with enhanced astrogliosis and microgliosis in a background of overexpressed S100B [82, 83], no information is available in a background of deletion of the S100B gene. Thus, whether or not is there any role of S100B in microglia-mediated brain tissue homeostasis in normal physiological conditions remains to be established.

As to point (4), RAGE engagement has been shown to result in the activation of several downstream signaling pathways [77, 78]. However, there appears to be no univocal set of signaling pathways that are being activated by RAGE in different cell types. For example, whereas the Ras/MEK/ERK1/2/NF-κB pathway plays a major role in (low) S100B/RAGE-induced neuronal survival (via upregulation of the antiapoptotic factor, Bcl-2) and (high) S100B/RAGE-induced neuronal death (via overproduction of ROS) [68], its activation is not critical for (high) S100B to upregulate the expression of the proinflammatory enzyme, COX-2, in microglia, S100B/RAGE activating a Cdc42/Rac1/JNK/AP-1 pathway and a Ras/Rac1/NF-κB pathway in this latter case [64, 66]. Yet, the MEK/ERK1/2 pathway does mediate the S100B/RAGE-induced upregulation of IL-1β and TNF-α expression and secretion by microglia [84]. Incidentally, at low doses S100B does not

activate microglia [60, 61], albeit synergizing with IL-1β and TNF-α to upregulate COX-2 expression [66]; instead, low S100B blocks trimethyltin-induced increase in TNF-α expression in microglia (via a mechanism that remains to be identified) [85]. Also, whereas high S100B has been shown to activate a RAGE/ROS/PI3K/Akt/NADPH oxidase/ROS pathway leading to lipid peroxidation and caspase-3 activation that cause dorsal root ganglia neuron apoptosis [86], the PI3K/Akt module does not appear to have any role in S100B/RAGE-induced upregulation of COX-2 expression in microglia [66]. Moreover, high levels of S100B cause GSK3β-dependent hyperphosphorylation of τ protein (a hallmark of Alzheimer disease) via RAGE-dependent activation of JNK and upregulation of Dickopff-1, a stimulator of GSK3β activity, in human neural stem cells [87]. So far, intermediates linking S100B/RAGE to signaling pathways include Src [88, 89] and diaphanous-1 [90]. It is tempting to speculate that the amount of RAGE expressed on the cell surface (which should condition the extent of RAGE oligomerization) and the relative fractions of octameric and multimeric S100B outside the cell might play a role in the choice between the intermediates recruited to RAGE, not to mention the potential recruitment to RAGE of other, cell-specific intermediates, possible crosstalks among intracellular signaling pathways, and autocrine/paracrine effects of factors released by the affected cells in consequence of the S100B/RAGE interaction.

Interestingly, levels of brain S100B in epileptic patients are increased compared with controls [91], and S100B release is increased in a mouse model of epilepsy [92]. In this latter experimental setting, the amplitude of hippocampal kainic acid-induced gamma oscillations is significantly reduced compared with WT mice, and released S100B enhances hippocampal kainic acid-induced gamma oscillations, an event that is abrogated by the local infusion of either an S100B neutralizing or a RAGE-neutralizing antibody [92]. Thus, S100B-activated RAGE signaling appears to make neurons more sensitive to the epileptogenic activity of kainic acid. Although no information is available about the cellular localization of RAGE in these studies, it is possible that S100B hyperpolarizes inhibitory interneurons in the hippocampus via RAGE engagement thereby causing dysinhibition of pyramidal neurons and enhancing their sensitivity to kainic acid. Indeed, some evidence suggests that S100B affects neuronal electrical discharge activity by modulation of potassium currents at low doses [93].

A role for S100B has been suggested in the pathogenesis and/or pathophysiology of schizophrenia based on the observation that serum levels of the protein are increased in this psychiatric disorder [94–96]. However, whereas in early studies the increased levels of S100B in patients with schizophrenia have been reported to occur without an indication for significant glial or neuronal damage, a finding that has been interpreted as an indirect evidence for increased active secretion of S100B by astrocytes during acute psychosis [97], other studies have shown that astrocyte and/or oligodendrocyte activation occurs in schizophrenic patients [98, 99]. Whereas alterations in glial and/or serum S100B levels may be indicative of participation of glial cells in the pathophysiology of schizophrenia, it is not known whether the increased serum S100B levels are indicative of the participation of the protein in the pathogenesis/pathophysiology of schizophrenia and what the role of (intracellular and/or extracellular) S100B in this psychiatric disorder might be. Recent work has shown that serum S100B levels normalize while levels of sRAGE (i.e., a product of digestion of RAGE acting as a scavenger of RAGE ligands) increase under antipsychotic treatment [100], suggesting that antipsychotic drugs might enhance the secretion/activity of matrix-metalloproteinases responsible for sRAGE production. Given the established role of RAGE in inflammation and of sRAGE as a protective factor against a number of inflammatory diseases [77, 78], and since a neuroinflammatory component characterizes psychotic states [101–103], one may hypothesize that during the course of acute schizophrenia activated astrocytes release more S100B either to aid in protecting neurons or to amplify neuroinflammation; the concomitant liberation of sRAGE from inflammatory cells (e.g., activated astrocytes and microglia) might then act to reduce the activity of RAGE ligands such as S100B, so as to extinguish/reduce the inflammatory response. However, this simplified model does not take into account the role of conventional cytokines and chemokines coming into play in the context of neuroinflammation and schizophrenia. In addition, enhancement of S100B release from astrocytes might not be causative of psychotic states per se because schizophrenia does not necessarily occur in Down syndrome (which is characterized by chronically elevated S100B levels (see [104, 105])), in aged people (who also show elevated S100B levels (see [106])), or in a variety of neuroinflammatory states characterized by elevated S100B levels [17]. Moreover, recent work raises the possibility that release of S100B from adipocytes contributes significantly to the elevated serum S100B levels found in schizophrenia [107, 108]. Although there is suggestion that variants within the S100B gene predispose to a psychotic subtype of bipolar affective disorder, possibly via alteration of gene expression [109–111], conclusions about the role of S100B in the pathogenesis/pathophysiology of schizophrenia should await more detailed analyses. The recent identification of *S100B* as a novel dyslexia candidate gene along with three other genes (i.e., *PCNT*, *DIP2A*, and *PRMT2*) mapping to chromosome region 21q22.3 suggests that decreases in S100B expression might contribute to certain dyslexia phenotypes [112]. This preliminary observation, however, lends support to the notion that alterations in S100B expression may have profound effects on brain functions and represents a further stimulus towards the elucidation of the functional role(s) of S100B at the cellular and molecular level.

Regulatory effects of extracellular S100B are not restricted to the brain. Aside from effects on monocytes/macrophages, neutrophils, myoblasts, and lens epithelial cells, S100B also exerts effects on vascular endothelial cells, vascular smooth muscle cells (VSMCs) (for review see [10]), and cardiomyocytes (see below). In fact, S100B engages RAGE in endothelial cells thereby activating NF-κB

transcriptional activity, increasing expression of vascular cell adhesion molecule-1, inducing monocyte chemoattractant protein-1 and RAGE transcripts and abrogating sodium nitroprusside-potentiated vasodilatation in response to ACh in endothelial dysfunction in type II diabetic ($Lepr^{db}$) mice. Also, S100B enhances the interaction of RAGE with the leukocyte β2-integrin Mac-1, thus increasing leukocyte adhesion to endothelial cells. RAGE engagement by S100B causes VSMC proliferation (thus impacting on the pathogenesis of atherosclerosis) (Figure 1(c)) via stimulation of NADPH oxidase, increased ROS generation, and activation of phospholipase D2 and janus kinase (JAK) 2 tyrosine phosphorylation, these effects being enhanced in the presence of high glucose concentrations or angiotensin II. Moreover, S100B/RAGE interactions in VSMCs result in recruitment of the nonreceptor Src tyrosine kinase and PKC and phosphorylation of caveolin-1, a component of caveolae, which are stable membrane domains that are kept in place by the actin cytoskeleton and act as multifunctional organelles. Effects of S100B/RAGE on VSMCs and stimulation of VSMC migration and release of IL-6 (Figure 1(c)), require p38 MAPK, ERK1/2, NF-κB and STAT3 activities, and ROS production. Recent evidence suggests that at concentrations >50 nM S100B induces cardiomyocyte apoptosis (Figure 1(d)) via RAGE-dependent phosphorylation of ERK1/2 and p53, increased expression and activity of proapoptotic caspase-3, and decreased expression of antiapoptotic Bcl-2 [113], another example of dissociation between intracellular and extracellular S100B effects.

3.2. Extracellular S100B: Just a DAMP Protein? Most of the results commented on thus far point to S100B as to a damage-associated molecular pattern (DAMP) protein that is, a factor released from damaged/necrotic cells and endowed with the ability to activate cells of the innate immune response, alter the function of cell types (such as astrocytes, endothelial cells, and VSMCs) that participate in the inflammatory response, and/or cause cell death. In this regard, serum and cerebrospinal fluid levels of S100B are of diagnostic and/or prognostic value [17]. Compelling evidence suggests that extracellular S100B can be considered as a DAMP factor in the context of accumulation of the protein in the extracellular space: in this case, S100B might contribute twice to the inflammatory response, as a RAGE-activating ligand and as a factor capable of upregulating RAGE expression in reactive cells. Thus, S100B would contribute significantly to propagation of inflammation, and S100B-blocking agents might thus prove beneficial, attenuating inflammation and consequent cell damage.

However, differently from S100A8, S100A9, and S100A12, which are secreted by activated macrophages/neutrophils in response to inflammatory stimuli [114, 115], S100B is constitutively secreted by astrocytes in normal physiological conditions [49–53], and serum levels of S100B are relatively high at birth and in otherwise normal infants decreasing to picomolar levels around puberty [116]. Also, astrocytes might not be the sole source of serum S100B in normal and pathological conditions [49, 107, 108]. These observations suggest that the S100B's function outside the cell might go beyond its role as a DAMP protein and/or that DAMP proteins may also play a role in tissue development and/or regeneration. As mentioned earlier, at low doses, S100B protects neurons against apoptotic stimuli [68, 75, 117–119], enhances neurite outgrowth [120–125], and stimulates astrocyte proliferation [126], and the intraventricular infusion of low doses of S100B induces neurogenesis within the hippocampus, which is associated with an enhancement of cognitive functions following experimental traumatic brain injury [127, 128]. Also, the protein is released by in vitro trauma and reduces delayed neuronal injury [129–131]. The S100B protective effect towards neurons may also be indirect, the protein-stimulating uptake of the neurotoxic glutamate by astrocytes [132], reducing neurotoxin-dependent activation of microglia and astrocyte [85], protecting neurons against β-amyloid neurotoxicity [75] and reducing neuronal and glial cytotoxicity under hypothermic conditions [133]. Moreover, it has been suggested that the proliferation of neuronal precursors in the adult brain reported to occur following chronic treatment with antidepressants might be dependent on upregulation of S100B expression in astrocytes and RAGE expression in proliferating neuroblasts [134] although a causal relationship between upregulation of astrocytic S100B and neuronal RAGE in consequence of treatment with antidepressants and proliferation of neuronal precursors has not been established. Furthermore, extracellular regulatory effects of S100B might not be restricted to the brain, vasculature and heart, nor might they be dependent on RAGE exclusively. For example, nanomolar S100B stimulates myoblast proliferation, thus potentially contributing to the expansion of the myoblast population [71], a critical event during muscle development and regeneration [135]. It is intriguing, however, that RAGE and certain RAGE ligands including S100B might play important roles in such diverse contexts as the innate immune response on one side (see above) and tissue development and regeneration on the other side [8, 10, 68, 77, 78, 136–139]. Comparative analyses of wild-type, S100B KO, S100B TG, and RAGE KO mice might shed light in this regard.

Learning and memory processes long represent another field of action of extracellular S100B (for review see Refs. [1, 10, 140]). Indeed, S100B KO mice exhibit enhanced spatial and fear memories and enhanced long-term potentiation (LTP) in the hippocampal CA1 region, and perfusion of hippocampal slices with S100B reverses the levels of LTP to those of the wild-type slices [141]. This suggests that at physiological levels, extracellular S100B might play a role as a regulator of synaptic plasticity, although the molecular mechanism underlying this activity remains to be elucidated. Recently, hyperactivity and increased sensitivity to auditory stimuli have been reported in RAGE KO mice, with no significant differences between KO and wild types in behavioral tests for spatial memory and anxiety, though [142]. This preliminary evidence suggests that S100B/RAGE interactions might not be important for normal motor activity or spatial and fear memories, although definite conclusions should await analyses of double S100B-RAGE KO mice.

4. Conclusions

During the last two decades, the interest in S100B protein function in the brain and the cardiovascular apparatus has increased remarkably, mostly in view of its extracellular effects. By a combination of structural and functional studies, the emerging picture is one in which extracellular S100B exerts trophic and toxic effects depending on the concentration attained locally and the density of RAGE molecules expressed on the surface of responsive cells. Specifically, at low (i.e., nanomolar) S100B concentrations and in the presence of a relatively low RAGE cellular density, the protein might exert trophic effects, whereas at high (i.e., submicromolar-micromolar) and/or in the presence of a relatively high RAGE cellular density, the protein might be toxic, participating in the inflammatory response and causing cell death. In general, S100B behaves like a DAMP factor in a background of chronically elevated extracellular concentrations, like those occurring in Down syndrome, Alzheimer-like dementia, chronic neuroinflammation, atherosclerosis, and probably schizophrenia as well as whenever RAGE is found on the cell surface above a certain threshold of density. Thus, extracellular S100B effects appear to be context-dependent. However, S100B's trophic/toxic effects might not be necessarily transduced by RAGE [69–72, 119].

The great deal of information presented on regulatory effects of extracellular S100B has somewhat obscured the protein's intracellular function(s). The changes in the expression levels of S100B during certain phases of neural cell development in vivo and in vitro [22, 24], the enhanced S100B expression in reactive astrocytes (astrogliosis) [57] and gliomas (as well as in several nonnervous tumor cells) [4–6] and the involvement of intracellular S100B in cell proliferation and differentiation [12, 21–23] call for a detailed analysis of the regulation of S100B expression at the transcriptional and posttranscriptional level and of physiologically relevant interactions of S100B within cells. The importance of this issue is highlighted by the fact that: (1) the amount of released S100B generally is a small fraction of the protein's intracellular content (thus, the greater the content the larger the released fraction especially in the case of S100B leakage from damaged/necrotic cells); (2) within certain limits, intracellular regulatory effects are proportional to the S100B concentration within cells (see for example Refs. [18, 21, 22]); (3) the intracellular and extracellular effects of S100B are not univocal (as an example, at micromolar concentrations extracellular S100B cause astrocyte death via overproduction of NO [62] and neuronal and myoblast death via overproduction of ROS [68, 70]; yet, at the submicromolar-micromolar concentrations found within astrocytes and myoblasts S100B stimulates migration and, to a lesser extent, proliferation [22], and reduces differentiation via inhibition of MyoD expression [12], respectively); and (4) synthetic compounds with ability to block S100B activity such as arundic acid [38] and pentamidine [19] might not discriminate between intracellular and extracellular S100B. While further work is required for having a complete picture of intracellular and extracellular regulatory effects of S100B, current studies of the protein in physiological and pathological conditions are shedding light on the variety of cellular functions in which S100B is involved.

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Review Article

The Calcium-Dependent Interaction of S100B with Its Protein Targets

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S100B is a calcium signaling protein that is a member of the S100 protein family. An important feature of S100B and most other S100 proteins (S100s) is that they often bind Ca^{2+} ions relatively weakly in the absence of a protein target; upon binding their target proteins, Ca^{2+}-binding then increases by as much as from 200- to 400-fold. This manuscript reviews the structural basis and physiological significance of increased Ca^{2+}-binding affinity in the presence of protein targets. New information regarding redundancy among family members and the structural domains that mediate the interaction of S100B, and other S100s, with their targets is also presented. It is the diversity among individual S100s, the protein targets that they interact with, and the Ca^{2+} dependency of these protein-protein interactions that allow S100s to transduce changes in $[Ca^{2+}]_{intracellular}$ levels into spatially and temporally unique biological responses.

1. Introduction

Ca^{2+} ions are important second messengers in all living cells [1]. Ca^{2+}-binding proteins, including members of the calmodulin/troponin/S100 superfamily, maintain the integrity of the Ca^{2+} signal and transmit it in a temporally and spatially coordinated manner [2]. S100s were discovered in 1965 [3], and as with other EF-hand containing proteins, S100s also transduce changes in $[Ca^{2+}]_{intracellular}$ levels (i.e., $[Ca^{2+}]_i$) into cellular responses by binding Ca^{2+} (Table 1), changing conformation, and then interacting with and modulating the activity of other proteins (target proteins) (Figure 1). The amino acid homology between the current family members ranges from approximately 20% to 55% [4]. Because of the extensive amino acid homology between S100B and S100A1, the first two family members identified, early models predicted that individual members were functionally redundant and essentially interchangeable. As the number of family members discovered has increased and differences in their cellular/subcellular localization, physical properties, and target proteins have expanded, additional models have arisen with specific S100 family members having unique biological functions [5–12]. This multigenic family now contains up to twenty-one members (humans) whose phylogenetic distribution is restricted to higher chordates. Oligomerization properties, affinities for divalent metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+}), and posttranslational modifications also contribute to diversity among S100 family members [7, 12]. Furthermore, each cell/tissue expresses a unique subset of family members [11, 12]. Collectively, these findings support the view that S100s often confer cell type specificity to Ca^{2+} signal transduction pathways in cells and tissues [11–16].

The 3-dimensional structure of numerous S100 proteins has been solved by NMR and X-ray crystallography techniques in the apo-, Ca^{2+}-bound, Zn^{2+}-bound, drug-bound, and target protein bound states, and with the exception of calbindinD_{9K}, S100s such as S100B are typically symmetric dimers with each subunit containing two EF-hand calcium-binding domains, although, some higher-order oligomeric

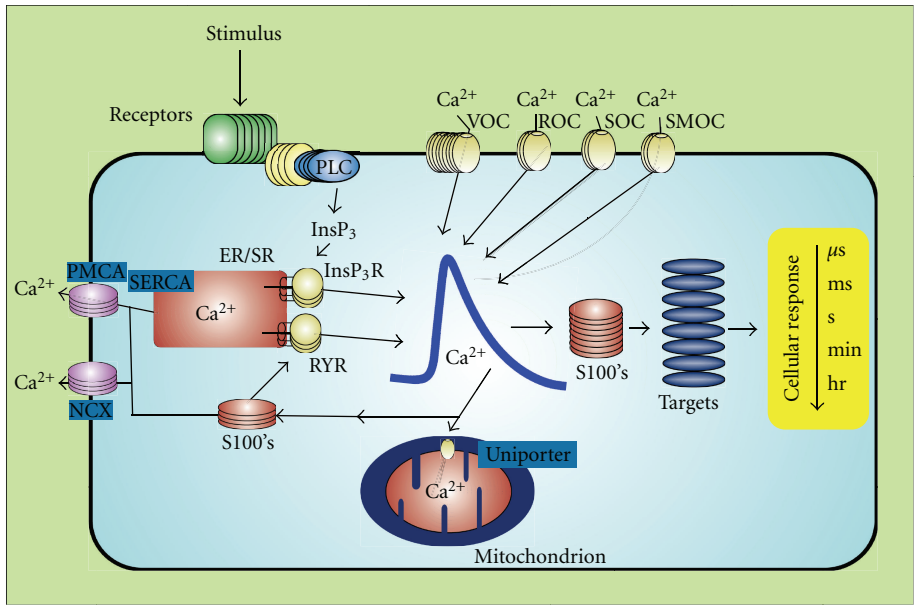


FIGURE 1: *S100s function as Ca^{2+}-signaling proteins.* S100s bind and regulate protein targets as well as other Ca^{2+}-signaling proteins in a Ca^{2+}-dependent manner. S100s are distributed in a cell-specific manner to generate cell-type specific activities [1, 2, 10].

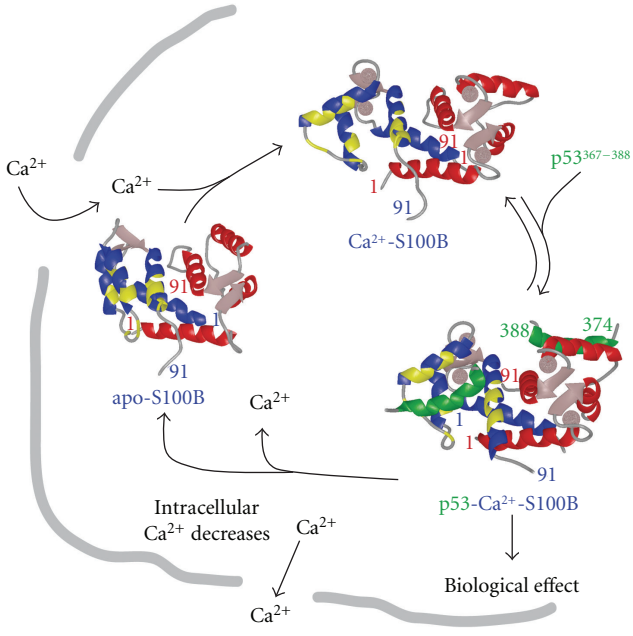


FIGURE 2: *The Ca^{2+}-dependent S100-target protein interactions.* In red/blue are subunits of S100B ($^{dimer}K_D$ < 500 pM [1, 17]) with regions shaded (yellow) for residues that bind targets such as $p53^{367-388}$ (green), p53 ($p53^{321-346}$, K_D = 24 ± 10 nM), or TRTK12 [10, 18].

states have also been detected and discussed [17–64]. The first EF-hand motif (EF1) in each subunit has fourteen rather than twelve residues and is termed the "pseudo-EF-hand" or "S100 EF-hand" [31, 65]. Another unique feature of the S100-hand (EF1) is that calcium-coordination is achieved via backbone carbonyl oxygen atoms rather than sidechain carboxylate oxygen atoms, and it typically binds Ca^{2+} rather weakly (K_D > 0.5 mM). The second EF-hand (EF2) is termed the "typical EF-hand" since it has the same number of residues as most EF-hand Ca^{2+}-binding proteins (i.e., twelve), and it exactly matches the consensus EF-hand in both sequence and three-dimensional structure [66]. As with the helix-loop-helix Ca^{2+} binding domains of other members of the EF-hand superfamily (i.e., calmodulin, troponin C, etc.), the second EF-hand of S100B coordinates Ca^{2+} via a backbone carbonyl oxygen at position 7, sidechain oxygen atoms from Asp/Glu at positions 1, 3, and 5, and via bidentate coordination from oxygen atoms from a Glu sidechain at position 12 [66]. Position 9 of the coordination scheme is occupied by a water molecule that is, in turn, hydrogen-bonded to a Glu sidechain oxygen atom from the pseudo-EF-hand. Another feature of S100B and several other S100s is that several of them bind Ca^{2+} ions relatively weakly, relative to $[Ca^{2+}]_i$, in the absence of a protein target (K_D ≥ 1 μM). However, upon binding their full-length target protein, Ca^{2+}-binding can then increase by as much as from 200- to 400-fold [60, 67]. This feature could allow for cells to contain high concentrations of some S100s, such as S100B (>1 μM), without depleting $[Ca^{2+}]_i$ levels and "short-circuiting" Ca^{2+} oscillations necessary for signaling.

The large number of protein targets that S100B and other S100 family members bind and regulate provides yet another level of diversity/complexity to S100 signaling. Examination of the extensive lists of S100 target proteins provides important insights regarding S100 signaling [15, 68, 69]. First, S100s regulate a wide-range of cellular processes that includes energy metabolism, cytoskeleton organization, gene expression, and signal transduction pathways. Second, the list of *in vitro* target proteins for individual family members can be extensive, 23–25 different protein

targets have been reported for S100B and S100A1 alone [5, 7, 13, 15, 68, 70]. Third, some target proteins interact with and have their activity modulated by multiple S100 family members suggesting functional redundancy among some family members. For example, both S100A1 and S100B interact with and activate aldolase A and aldolase C [71]. In contrast, S100A1 and S100B exhibit a Ca^{2+}-dependent interaction with phosphoglucomtutase but this interaction results in differential effects on target protein activity: S100A1 stimulates phosphoglucomutase activity and S100B inhibits phosphoglucomutase activity [72]. Like phosphoglucomutase, both S100A1 and S100B bind glycogen phosphorylase a [73]. However, S100A1 inhibits and S100B has no effect on glycogen phosphorylase activity. Interestingly, S100A4, S100A1, and S100A6 interact with methionine aminopeptidase 2, but with different affinities [74]. The S100A6 (calcyclin) binding protein CACYBP also interacts with S100A1, S100A12, S100P, and S100B but not S100A4 [75]. And several S100 proteins (i.e., S100A1, S100A2, S100A4, S100A6, and S100B) bind to p53, p63, p73 [29, 76–80], and affect their biological functions [81–84]. Fifth, S100 activation of some target proteins is Ca^{2+}-independent indicating that S100s sense changes in $[Ca^{2+}]_i$ levels and have important intracellular functions at both resting and stimulated $[Ca^{2+}]_i$ levels. Sixth, a subset of S100s have a conserved Zn^{2+}-binding site that contributes to altered Ca^{2+}-, target-, and in some cases, small molecule binding affinity *in vitro* [8, 20, 36, 40, 68, 85–99], which could potentially affect cellular functions. Seventh, the interaction of S100 family members with other family members as "target proteins" has been observed using yeast-two hybrid approaches [100–104]. Finally, S100 proteins may exert some of their effects via interaction with molecules other than proteins. For example, S100A8/S100A9 binds (poly) unsaturated fatty acids in a Ca^{2+}-dependent manner [105, 106] and S100A1 binds IP_3 (Baron, Coburn, and Zimmer, personal communication). New information regarding S100 family member specificity for individual target proteins and the structural motifs that mediate these interactions are detailed below. As discussed, the diversity among individual S100s, the protein targets that they interact with, and the Ca^{2+} dependency of these interactions are uniquely suited to conferring cell-type specificity to Ca^{2+}-signaling pathways.

1.1. The S100 Calcium-Switch. Biophysical and structural biology techniques are used to study molecular determinants involved in Ca^{2+}-dependent S100-target interactions. A comparison of apo- and Ca^{2+}-bound S100B (B-Ca^{2+}) indicates that the pseudo -EF-hand (EF1, $^{Ca}K_D > 350\,\mu M$, Table 1) has minor structural changes when Ca^{2+} binds, whereas, the typical EF-hand (EF2, $^{Ca}K_D = 56 \pm 9\,\mu M$, Table 1) has a large repositioning of several sidechain oxygen ligands during Ca^{2+} coordination (EF-2: D61, D63, D65, E67, D69, and E72) [23, 24, 26, 34, 78, 87, 107–109]. Much like S100A1, S100A4, and S100A5, Asp-61 and Asp-63, in positions 1 and 3 of the typical EF-hand, must rotate substantially to achieve a suitable Ca^{2+} binding orientation in S100B (Figures 2 and 4). That helix 4 is involved in the dimer interface explains why the entering helix 3 and not the exiting helix 4 moves significantly upon Ca^{2+} binding [23]. The conformational change, termed the "S100 Ca^{2+}-switch", is a feature unique to S100s and unlike other EF-hand proteins (i.e., calmodulin and troponin C) in which position 12 reorients the exiting helix upon Ca^{2+} binding [23, 26, 43, 44, 110, 111]. The conformational change of helix 3 involves breaking and forming hydrophobic contacts in several S100s with unique hydrophobic and/or hydrophilic residues becoming exposed for binding its protein target (Figures 2–4). However, S100A10 does not conform to other family members since it lacks a functional EF-hand Ca^{2+}-binding domain, so its target protein interactions are all independent of Ca^{2+} [11, 112]. In several S100-target structures, much has been learned about specific target- and inhibitor-S100 interactions involving the "hinge (loop 2)", the C-terminal loop, and the hydrophobic pocket involving helices 3/4 for several S100s (site 1), a second surface, nearby helix 4 and the Zn^{2+} site in S100B (i.e., site 2) was also discovered more recently in drug design studies aimed at inhibiting S100B [20, 21].

However, several important aspects pertaining to the Ca^{2+}- and target-binding properties of S100s are not yet answered. With S100A5 and S100Z as exceptions ($^{Ca}K_D \sim 0.2\,\mu M$, Table 1), dimeric S100s typically have a low affinity for binding Ca^{2+} *in vitro* ($^{Ca}K_D \geq 1\,\mu M$, EF2) [10, 124, 134](Table 1), leading some to classifying this family of proteins as "calcium-buffers" while others predicted that S100s only bound Ca^{2+} in locations where Ca^{2+} ion concentrations were relatively high (endoplasmic reticulum and the extracellular space). While low *in vitro* binding affinities were first thought to eliminate a role for S100 Ca^{2+}-binding in the cytosol, where $[Ca^{2+}]_i$ typically oscillates between 0.1 and $1\,\mu M$ [1, 2], we now know that for many S100s, their Ca^{2+}-binding affinity goes up significantly when its target protein is bound [34, 60, 109] (Figure 3). In fact, for S100A1 and the full-length ryanodine receptor (Figure 3(c)), the complex was detected at resting cytosolic $[Ca^{2+}]_i$ (i.e., at 100 nM) even though its Ca^{2+}-binding affinity *in vitro* in the absence of target is ~300-fold higher ($^{Ca}K_D = 27 \pm 2\,\mu M$) [60] (Table 1). One explanation for the tightening Ca^{2+}-binding affinity is that target binding induces a structural change to provide a more optimal Ca^{2+}-coordination geometry. Such an explanation was ruled out for S100B when the X-ray structures of Ca^{2+}-S100B (±TRTK12) and found that the Ca^{2+}-coordination in both EF-hands (EF1, EF2) was indistinguishable despite the fact that $^{Ca}k_{off}$ is ~20-fold slower when TRTK12 is bound [34]. Thus, the mechanism for "tightening" of Ca^{2+}-binding with target bound cannot be explained by structural data alone. Interestingly, B-factors from X-ray structures (Figure 4) and ^{15}N NMR relaxation rate studies of S100s have given us some indication that dynamic properties of S100 proteins are involved in the "tightening effect" (Figure 5). Thus, "induced-fit" versus "selected-fit" binding models for both the binding of Ca^{2+} and target to S100s have been considered. The underlying premise of the "selected-fit" model is that in the absence of target, the Ca^{2+}-S100 complex observed in the X-ray structure is in equilibrium with a dynamic state(s) that has

Table 1: Dissociation of Ca^{2+} and Mn^{2+} from the EF-hand calcium-binding domains in wild-type and mutant S100 proteins.

S100 protein	EF1	EF2
Ca^{2+} binding		
S100B (wt)	>350 μM[a,c]	56 ± 9 μM[b,c]
S100B (E31A)	>500 μM[a]	>500 μM[a]
S100B (E72A)	480 ± 130 μM[a]	>500 μM[a]
S100B (E31A + E72A)	>2 mM[a]	>2 mM[a]
S100B (+p53)	—	20 ± 3 μM[a]
S100B (E31A, +p53)	—	21 ± 7 μM[a]
S100B (E72A, +p53)	—	18 ± 4 μM[a]
S100B (E31A + E72A, +p53)	—	>300 μM[a]
S100B (wt, +TRTK12)	—	12 ± 7 μM[c]
S100A1 (wt)	—	27 ± 2 μM[d]
S100A1 (wt, +TRTK12)	—	8 ± 3 μM[e]
S100A2 (wt)	—	470 ± 50 μM[f]
S100A3 (wt)	—	~4 mM[g]
S100A4 (wt)	—	~2.6 μM[h]
S100A4 (wt, +p37)	—	~0.2 μM[h]
S100A5 (wt)	160 μM[i]	~0.2 μM[i]
S100A6 (wt)	—	~3.0 μM[j]
S100A7 (wt)	—	~1.0 μM[k,l]
S100A11 (wt)	—	~0.5 mM[m]
S100A12 (wt)	—	~50 μM[n]
S100A13 (wt)	~400 μM	~8 μM[o,p]
S100A16 (wt)	no binding	0.43 mM[q]
S100P (wt)	~800 μM	~2.0 μM[r]
S100Z (wt)	> 1 mM	~0.2 μM[s]
Mn^{2+} binding		
S100B (wt)	—	71 ± 12 μM[a,c]
S100B (wt, +p53)	—	27 ± 4 μM[a,c]
S100B (wt, +TRTK12)	—	6.0 ± 2.0 μM[a,c]

[a]The value listed is from previously published papers [109, 113], so direct comparisons of binding constants using similar methods/conditions could be made (+/− target, Figure 3). Several others report binding constants using different methods and varying conditions for EF1 (200 μM ≤ K_D ≤ 500 μM) and for EF2 (10 μM ≤ K_D ≤ 60 μM) [58, 78, 86, 87, 114–120].

[b]The dissociation rate constant for wild-type S100B was determined via stopped-flow methods and is k_{off} = 60 ± 22 s^{-1}. The off-rate together with the K_D enables the calculation of a macroscopic on-rate value of k_{on} = 1.1 × 10^6 M^{-1} s^{-1} that includes calcium-association plus a large conformational change. The K_D value for the mutants was also determined using competition studies of Ca^{2+} with the respective Tb^{3+}-bound S100B mutant in the absence and presence of p53 peptide. The dissociation constants together with the calcium off-rate values measured for the E31A and E72A mutants of 7.1 ± 3.7 s^{-1} and 6.8 ± 2.0 s^{-1}, respectively, were sufficient to calculate on-rate values of 3.4 ± 2.0 × 10^6 M^{-1} s^{-1} and 3.7 ± 1.3 × 10^6 M^{-1} s^{-1} for the mutants [109, 113].

[c]From Charpentier et al. (2010) [34].

[d]From Wright et al. (2005) [61].

[e]From Wright et al. (2009) [59]. S100A1 has also been shown to bind the full-length ryanodine receptor at 100 nM free Ca^{2+} [60, 67].

[f]From Franz et al. (1998) [89].

[g]From Fritz et al. (1998). A tenfold weaker affinity was reported when purified under aerobic conditions [90, 121].

[h]From Dukhanina et al. (1998). A weaker affinity was reported under different conditions in Pedrocchi et al. (1994) when S100A4 was originally discovered [122, 123].

[i]From Schäfer et al. (2000). For a direct comparison of Ca^{2+} and Zn^{2+} binding to S100A5 to those of other S100 proteins (i.e., S100B, S100A2, S100A3, S100A4, S1006, and S10011), under identical conditions and Methods, also see Schäfer et al., (2000) [124].

[j]From Kuznicki and Filipek (1987) and Mani and Kay (1990). Kordowska et al. also measured Ca^{2+}-binding for S100A6 under different conditions ($^{Ca}K_D$ ~18 μM) and found that binding to the target caldesmon (CaD) increased the affinity of S100A6 for Ca^{2+} by approximately 6-fold [96, 125, 126]. Other measurements under higher salt and other varying conditions are also reported with weaker affinities for S100A6 [68, 124].

[k]From Schäfer et al. (2000) [124]. Weaker binding to Ca^{2+} has also been reported for this protein in other conditions [127].

[l]No data is available for S100A8/A9, and S100A10 does not bind Ca^{2+}.

[m]From Allen et al. (1996) and Schäfer et al., (2000) [124, 128]. Note the affinity for Ca^{2+} increases by 10-fold upon the addition of a target molecule as found with other S100 proteins [128].

[n]From Dell'Angelica et al. (1994). Note that Zn^{2+}-binding to S100A12 significantly increases Ca^{2+}-binding affinity for this protein in the presence of Zn^{2+} (EF2: $^{Ca}K_D$ = 40 nM, EF1: $^{Ca}K_D$ = 15 μM) [129].

[o]From Ridinger et al. (2000). This protein is unique among S100 family members in that it does not bind to the hydrophobic binding dye, TNS, upon the addition of Ca^{2+} [130].

[p]No data is yet available for S100A14, and there is no S10015 [131].

[q]From Sturchler et al. (2006). The value in the table is for human S100A16, mouse S100A16 bound one calcium too, only weaker ($^{Ca}K_D$ = 0.75 mM) [132].

[r]From Becker et al. (1992) and Gribenko et al., (1998) [92, 133]. In Gribenko et al., (1998), the effects of Mg^{2+} binding on Ca^{2+} affinity are also rigorously addressed.

a lower Ca^{2+}-binding affinity; when target protein binds to an Ca^{2+}-S100 complex, the equilibrium shifts so "low affinity states" are eliminated when target binds to give less conformational exchange and an increase in measured Ca^{2+}-binding (i.e., less free Ca^{2+}, slower $^{Ca}k_{off}$, Figure 3). Important for drug design, we found that S100B inhibitors can mimic target binding to also cause higher affinity Ca^{2+} binding (i.e., $K_D < 1\,\mu M$). It is also now understood that weak Ca^{2+}-binding for S100s in the *absence* of target may also turn out to be biologically relevant (Figure 1). Since most target-free S100s have low affinity for Ca^{2+}, this allows numerous stable S100s to be at high concentrations in the cell ($>1\,\mu M$) without depleting $[Ca^{2+}]_i$ levels inside the cell and "short-circuiting" Ca^{2+} oscillations. Thus, several highly stable S100s can be "poised and ready" in any given cell for when their specific target(s) are expressed, as necessary for them to regulate numerous functions in mammalian cells (Figure 1) [109].

1.2. The S100 Model for Binding Calcium and Target. As a model system, Ca^{2+}-dependent S100-target interactions are attractive, since S100s, in essence, have only one "functional" EF-hand (EF2, Figure 2) per subunit, which for S100B does not cooperate with the EF1 or EF1′/EF2′ of the other subunit in Ca^{2+}-binding [10, 23, 31, 78, 87, 109]. This is very much unlike calmodulin (CaM) and troponin C (TnC), which have several functional and highly cooperative EF-hand Ca^{2+}-binding domains [66]. Thus, a typical dimeric S100-target interaction is Ca^{2+}-dependent and involves at least 11 possible states, 13 dissociation constants (K_1–K_{13}), four conformational changes (L_1–L_4), and the corresponding rate constants (k_1–k_{17}, k_{-1}–k_{-17}), per symmetric subunit [109] (Figure 5). As for Ca^{2+}-binding, the conformational changes and the binding events for a single target are also symmetric and occur independently of those on the other subunit (i.e., without cooperativity), so only one S100 subunit needs to be considered in the thermodynamic scheme [10, 23, 31, 78, 87, 109]. At low $[Ca^{2+}]$ (Figure 5), the predominant kinetic pathway for target binding to S100s can be simplified to K_I (k_{-1}/k_1, step 1), L_1 (k_{-14}/k_{14}), and K_X (k_{-10}/k_{10}, step 2) because an S100- or pseudo-EF-hand motif (EF1) binds Ca^{2+} about an order of magnitude more weakly than EF2 ($K_I \ll K_{II}$, i.e., $K_{II} \sim 0.5\,mM$) [109]. However, in addition to a two-step $K_I \cdot L_1 \cdot K_X$ pathway for target binding, we will also consider three additional equilibriums resulting from conformational exchange prior to Ca^{2+} binding (K_{XIV}), prior to target binding (K_{XV}), as well as for the S100-target bound state (K_{XIV}, Figure 5, Scheme 1). In step 1 (Scheme 1), structural data provides support for the "induced-fit" aspect of Ca^{2+} binding to S100B (i.e., A + Ca^{2+} ↔ [A − Ca^{2+}]‡ ↔ B − Ca^{2+}) since the apo-S100B (state A) has a patch of negatively charged residues comprising residues in the typical EF-hand (D65, E67, D69, and E72) sufficient for a fast bimolecular interaction with Ca^{2+} [23, 24]. Furthermore, no conformational exchange (R_{ex}) has yet been observed in ^{15}N-relaxation rate NMR studies of apo-S100B for any residues in helix 3 or in either of the Ca^{2+}-binding loops that subsequently undergo structural transitions when Ca^{2+} is added [136]. However, this needs to be examined more rigorously for apo-S100B and for other apo-S100s using ZZ-exchange and relaxation dispersion NMR methods (off-resonance $R_{1\rho}$, rcCPMG). Nonetheless, the lack of detectable R_{ex} in the apo-state currently provides an argument against the A ↔ B conversion (via K_{XIV}) (Figure 5, Scheme 1, in green). In step 2, for target binding to an S100 (i.e., B $-M_{II}+$ S ↔ B $-M_{II}-$ S, via K_X), NMR data does exhibit R_{ex} for both Ca^{2+}-S100B and Ca^{2+}-S100A1 [59, 60, 77, 136, 137]; these preliminary data are consistent with a model in which target binding selects a competent conformation(s) from an ensemble of dynamic states (via K_{XV}, i.e., "selected-fit" model [138]). The selected-fit hypothesis is also supported by the loss of exchange broadening upon binding of p53 and other targets to Ca^{2+}-S100B [77, 113]. Further, the rate of Ca^{2+} dissociation ($^{Ca}k_{off}$) from EF2 decreases from 60/s to 7/s when p53 binds as measured by stopped-flow methods [109]; similar results were found when other targets, including an S100B inhibitor (SBi1) bound to Ca^{2+}-S100B (Cannon and Weber, unpublished results). We now have X-ray crystal structures of S100B-Ca^{2+} and S100B-Ca^{2+}-TRTK12 that show Ca^{2+} coordination is indistinguishable in the two complexes [34], whereas, elevated B-factors were observed for S100B-Ca^{2+} for residues in EF2 in the absence of bound target (Figure 4). One interpretation of the elevated B-factors is that there is conformational dynamics affecting EF2 in the absence of target, however, other explanations cannot be ruled out such as the possibility that lattice contacts are different in the two structures giving rise to the changes in B-factors [34]. Thus, it is important to further examine the dynamics of Ca^{2+}-S100s, including EF2, directly by NMR to determine whether the decreased B-factors in EF2 (Figure 4) were due to a loss of conformational exchange as represented in Figure 5.

Additional evidence supporting Scheme 1 (Figure 5) is from stopped-flow experiments with S100-Ca^{2+} and S100B-Ca^{2+}-target complexes [109]. Here, a fast kinetic-step at the earliest time points in stopped-flow traces has been observed (i.e., biphasic, Cannon and Weber, unpublished results), indicative of K_{XV}. Also satisfying is that K_{XV} in Figure 5 provides a means for a single S100 protein to sample conformational space (i.e., at BM_{II}), as may be necessary to bind more than one target protein, an observation made for several S100 proteins [10]. Structurally similar S100 proteins may also bind the same protein target (i.e., TRTK12), although k_{off} (i.e., k_{-10}) in these cases usually varies due to specific differences in the binding site that give S100-target protein complexes unique conformations and hence varying "lifetimes" inside the cell (i.e., different $^{Ca}k_{off}$ values). These issues regarding specificity are important and require further examination. It is also necessary to remember that one assumption in this model (Figure 5) is that binding of Ca^{2+} to EF1 is not significant. While this assumption is valid based on existing K_D values (and verified for S100B) [87]), weak Ca^{2+} binding to this EF-hand (EF1) in other S100s may slightly populate additional states, which together with cooperative binding effects observed in some cases [15] could complicate interpretations with this simple model (Figure 5).

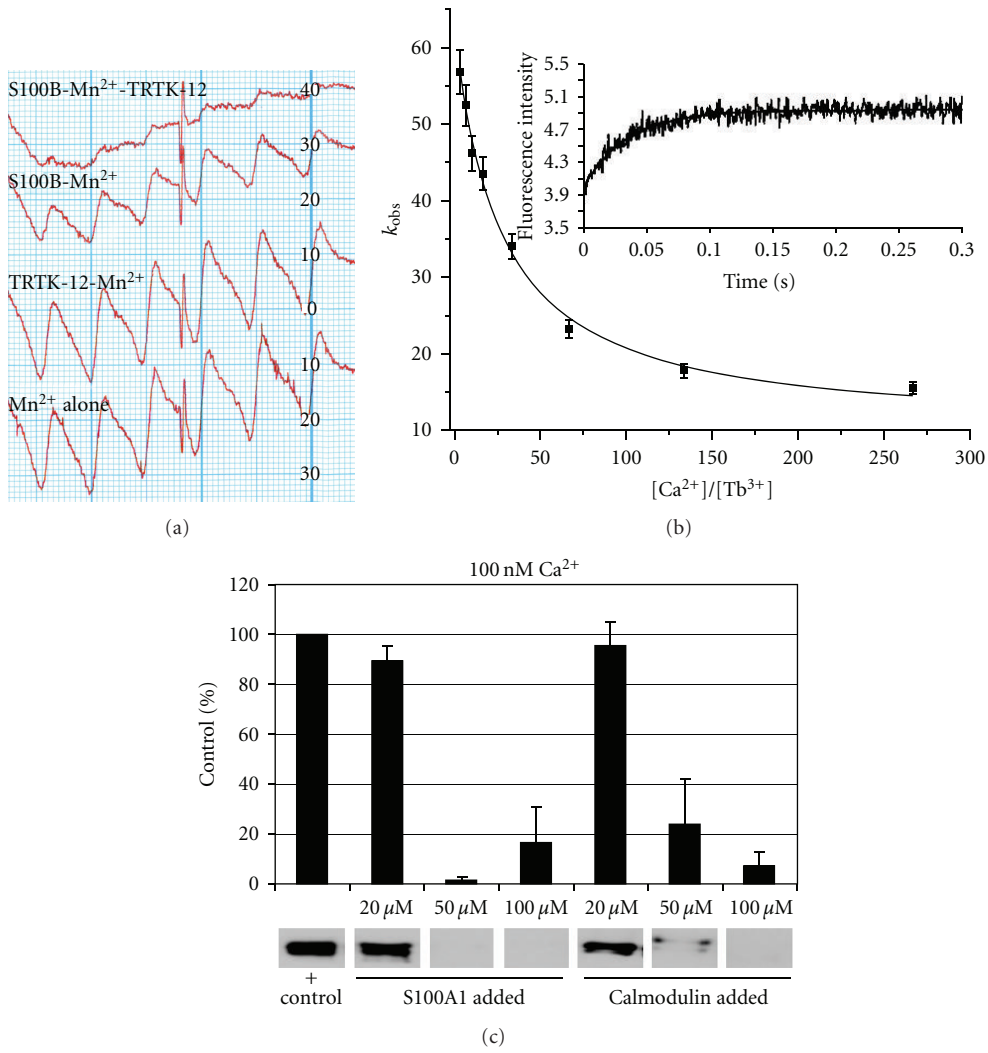


FIGURE 3: *Metal ion and target binding properties of S100 proteins.* (a) Binding studies with Mn^{2+} were completed since it is a good probe of the high affinity Ca^{2+} binding site on S100B (EF2) [113, 135]. Free Mn^{2+} was measured by electron paramagnetic resonance (EPR) in the absence and presence of S100B (+/− target peptide TRTK12) [34]. In all four traces, total $[Mn^{2+}]$ is identical (80 μM) with the bottom trace (4th trace) showing the signal for total $[Mn^{2+}]$. TRTK12 alone (1 mM) has no effect on the EPR signal (3rd trace), whereas, the addition of S100B (65 μM) binds Mn^{2+} and reduces free $[Mn^{2+}]$ (2nd trace). The addition of the same amount of S100B (65 μM) plus TRTK12 (1 mM, top trace) has the least free $[Mn^{2+}]$ and indicates that TRTK12 binding to S100B-Mn^{2+} enhances Mn^{2+} binding (compare traces 1 and 2). A similar effect was observed for SBi1 (unpublished) and for $p53^{367-388}$ [109]. As for $p53^{367-388}$ and SBi1, TRTK12 increased the affinity of S100B for Ca^{2+} in competition studies with Mn^{2+} and via stopped-flow kinetic measurements of $^{Ca}k_{off}$ as monitored in competition with Tb^{3+}. (b) Plot of the decrease in k_{obs} as a function of $[Ca^{2+}]/[Tb^{3+}]$ as used to determine the off rate of Ca^{2+} from the 2nd EF-hand (EF2, $^{Ca}k_{off}$). The k_{obs} values at each $[Ca^{2+}]/[Tb^{3+}]$ ratio were calculated from kinetic traces of stopped-flow experiments where Tb^{3+} (syringe C) is mixed with S100B at varying Ca^{2+} concentrations (syringe A) and $[Tb^{3+}]$ signal is monitored as a function of time (λ_{ex} = 230 nm, λ_{em} = 545 nm). A $^{Ca}k_{off}$ of 60 ± 8/sec was calculated from these experiments with S100B alone. When either TRTK12 or SBi1 is present, then the calculated $^{Ca}k_{off}$ value for S100B is reduced to 5 ± 3/sec similar to that found for $p53^{367-388}$ [109]. These studies demonstrated that TRTK12, $p53^{367-388}$, or SBi1 increased the affinity of S100B for Ca^{2+} at least in part by decreasing $^{Ca}k_{off}$. In (c), S100A1 was found to bind the full length ryanodine receptor (RyR) at 100 nM free calcium. Specifically, S100A1 competed full-length RyR1 away from agarose-linked CaM beads as judged by a decreased RyR1 band in an anti-RyR Western blot. Free CaM, a positive control, also competed the RyR away from CaM-linked beads [60, 67].

1.3. S100 Family Members Exhibit Overlapping but Distinct Target Protein Binding Profiles. While structural, biochemical, and biophysical approaches yield important structural and mechanistic details, they cannot be used to screen simultaneously the interaction of multiple S100 family members with an extensive array of full-length target proteins. Therefore, we developed a quantitative assay that monitors the interaction of fluorophore labeled S100s with membrane-immobilized target proteins that could be used to efficiently identify/prioritize S100-target protein interactions and S100

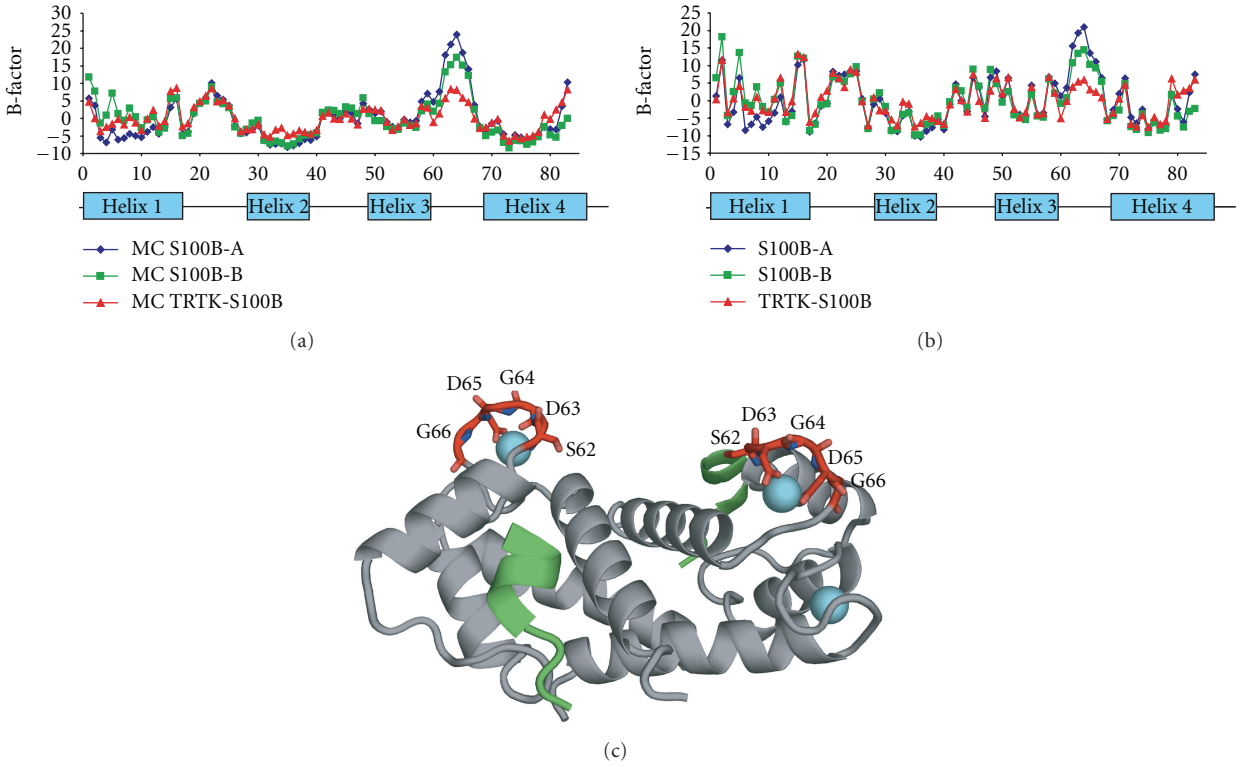


Figure 4: *B-factors for X-ray structures of TRTK12-Ca^{2+}-S100B (2.0 Å) and Ca^{2+}-S100B (1.5 Å) from the PI's laboratory.* (a) B-factors for backbone atoms for each subunit of Ca^{2+}-S100B (blue, green) and for TRTK-Ca^{2+}-S100B (red). (b) B-factors for sidechains with symbols as in (a). (c) Also shown is a ribbon diagram of the TRTK12-Ca^{2+}-S100B structure with residues colored red in EF2 (residues 61–72), which display lower B-factors in the TRTK12-bound state (in panels (a) and (b)). These data are all published in Charpentier et al., 2010 [139].

domains for structural/mechanistic analyses. First, commercially available Alexa-Fluor 488-conjugated calmodulin (CaM-488) and two well-characterized calmodulin target proteins, calmodulin-dependent kinase II (CaM kinase II) and phosphorylase kinase, were used to determine if this methodology would yield data representative of published K_Ds and K_As (Figure 6). The interaction of CaM-488 with both target proteins was Ca^{2+} dependent. Immobilized CaM kinase II bound 5.4 pmoles CaM-488 in the presence of Ca^{2+} and 0.1 pmoles CaM-488 in the absence of Ca^{2+}. Similarly, immobilized phosphorylase kinase bound 0.7 pmoles CaM-488 in the presence of Ca^{2+} and 0.3 pmoles CaM-488 in the absence of Ca^{2+}. Furthermore, the 8-fold difference in bound CaM-488 (5.4 versus 0.7 pmoles) is in qualitative agreement with the higher affinity reported for CaM kinase II when compared to phosphorylase kinase [140, 141]. These data demonstrate the feasibility of using a membrane binding assay and Alexa-Fluor conjugated Ca^{2+}-receptor proteins to monitor Ca^{2+} dependency of target protein interactions and to qualitatively compare the binding interaction.

To verify that this methodology could also be used to monitor S100-target protein interactions, the binding of S100A1-488 to a previously characterized target protein, glycogen phosphorylase a, was evaluated [73]. Consistent with results of previous gel overlay and affinity chromatography experiments, S100A1-488 exhibited Ca^{2+}-dependent binding to immobilized glycogen phosphorylase a at all points (Figure 7). Furthermore, S100A1-488 binding was saturable with a B_{max} of 30.0 pmoles and EC_{50} for binding value of 37.5 pmoles. While this type of assay does not provide a K_D, the B_{max} and EC_{50} can be used to compare S100-target protein interactions. Nonetheless, full-binding curves do not permit the simultaneous characterization of multiple S100s interacting with numerous target proteins. Therefore, two targets which exhibit differential interactions with S100A1 and S100B were used to determine if a single point assay like the one used in Figure 6 to characterize CaM target protein interactions would accurately reflect S100 target protein interactions. The quantity of target protein (50 pmoles) and probe concentration (100 nM S100A1-488 or S100B-488) were based on the EC_{50} (~40 pmoles) for glycogen phosphorylase a in 100 nM S100A1-488 (Figure 8). S100A1-488 and S100B-488 exhibited Ca^{2+}-dependent binding to phosphoglucomutase as well as glycogen phosphorylase, and the range of binding was similar to that observed for CaM target proteins. Glycogen phosphorylase a and b bound similar amounts of S100A1-488 (12–15 pmoles) and S100B-488 (15–20 pmoles). In contrast, phosphoglucomutase bound 5-fold more S100B-488 (14 pmoles) than S100A1-488 (2 pmoles). This differential binding was undetectable in previous gel overlay and affinity chromatography experiments despite the differential effects of S100A1 and S100B on phospohglucomutase activity [142]. The insensitivity/inability of the overlay and affinity chromatography

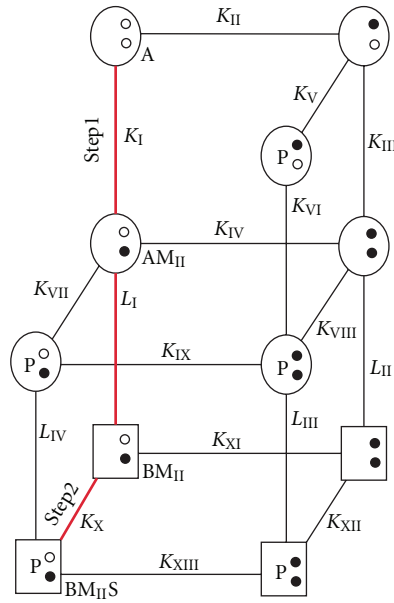
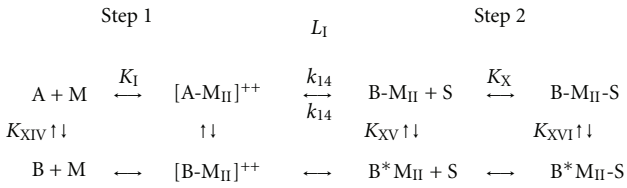


Figure 5: *Models for Ca^{2+}-binding and then target-binding to an S100 protein.* (Top) A model for the Ca^{2+}-dependent interaction of S100B with target proteins involves 13 equilibrium constants (K_I to K_{XIII}), 11 states, and 4 conformational changes (L_I–L_{IV}) [109]. The most highly populated states and the predominant pathway are colored red; this is due to weak Ca^{2+} binding in the pseudo-EF-hand (site I), which greatly simplifies this model (see Scheme 1). Specifically, the binding of Ca^{2+} to the pseudo- and typical EF-hand in each S100B subunit is described by six states (A, AM_I, AM_{II}, AM_IM_{II}, BM_I, and BM_{II}), five equilibrium constants ($K_I = [A][M]/[AM_I]$, $K_{II} = [A][M]/[AM_{II}]$, $K_{III} = [AM_I][M]/[AM_{I,II}]$, $K_{IV} = [AM_{II}][M]/[AM_{I,II}]$, and $K_{XI} = [BM_{II}][M]/[BM_{I,II}]$, two conformational changes (L_I: $AM_{II} \leftrightarrow BM_{II}$, L_{II}: $AM_{I,II} \leftrightarrow BM_{I,II}$) with corresponding rate constants, respectively, where A = S100B prior to the 90° reorientation of helix three of S100B, B = S100B after 90° reorientation of helix three, M_I = a Ca^{2+} ion bound to EF-hand I (pseudo-EF-hand), M_{II} = a Ca^{2+} ion bound to EF-hand II (typical EF-hand), $M_{I,II}$ = Ca^{2+} ions bound to EF-hand I and EF-hand II. Upon the addition of p53 or another target (S), the model expands to 11 possible states, 13 dissociation constants, and four possible conformational changes. Whether additional equilibriums occur (K_{XIV}, K_{XV}, and K_{XVI}) is considered in Scheme 1. (Bottom) In a second model (Scheme 1), state A is defined as the "closed" conformation observed in the apo-state (Figure 2), and state B is after a 90° reorientation of helix 3 termed the "open" conformation. In black, are states hypothesized to be populated. $[A\text{-}M_{II}]^{\ddagger}$ and $[B\text{-}M_{II}]^{\ddagger}$ represent short-lived intermediates, and L_1 is the Ca^{2+}-dependent conformational change involving helix 3 of S100B upon binding Ca^{2+} (Figure 2). Based on NMR relaxation rate data from the PI's lab [136], K_{XIV} highly favors state A. States are also considered via K_{XV} and K_{XVI} which result in B* states that represent an ensemble of dynamic structures, of which, only a subset fully coordinate Ca^{2+} as observed in X-ray structures [9]. It is hypothesized that K_{XV} favors the B^*M_{II} state(s), whereas, K_{XVI} favors B-M_{II}-S, explaining the apparent increase in Ca^{2+}-binding affinity using equilibrium binding measurements that monitor free [metal ion] (Figure 3).



Scheme 1

experiments to detect differential binding is most likely attributable to the high levels of receptor and/or ligand present. Altogether, these results demonstrate that a single point fluorometric assay can be used to quickly assess the relative affinity and Ca^{2+}-dependency of S100-target protein interactions.

Next, we compared the ability of different S100 family members (S100A1, S100A4, S100A5, and S100P) to interact with four previously reported S100B targets, glycogen phosphorylase a, glycogen phosphorylase b, tau, and phosphoglucomutase (Figure 8). As expected, all four target proteins bound S100B-488 (10–35 pmoles) in a Ca^{2+}-dependent manner. While none of the other family members exhibited the same target protein binding profile as S100B, there did appear to be two distinct groups: one group (S100A1 and S100P) with profiles that were similar to and another group (S100A4 and S1005) that clearly distinct from S100B. Glycogen phosphorylase a and b bound similar levels of S100A1-488/S100P-488. However, phosphoglucomutase did not bind S100P-488 and 6-fold less S100A1-488 when compared to S100B-488. In addition, tau bound >10 fold less S100P-488 than S100B-488 and binding to S100A1-488 was Ca^{2+} independent. In the case of S100A4 and S1005, binding to all target proteins was below 10 pmoles. Finally, labeled S100A1, S100B, S100A4, and CaM (100 nM) did not bind to immobilized S100 family members (S100A1, S100B, S100A2, S100A4, S100A5, S100A11, and S100A13), calmodulin, or

the negative control α-lactalbumin (75 pmoles) in the presence or absence of Ca^{2+} (data not shown). Collectively these data demonstrate that while there is extensive overlap among S100 family members with regard to the target proteins that they interact with, each S100 interacts with a unique compliment of target proteins. Nonetheless, there do appear to be family member-specific target proteins. For example, phospohglucomutase preferentially interacts with S100B. Furthermore, the relative affinity and Ca^{2+} dependency of the interaction can vary among family members. For example, tau is a target protein for multiple S100s including S100A1, S100A4, S100A5, and S100P, but only the interaction of tau with S100B is Ca^{2+}-dependent.

1.4. The Linker Region Mediates Family Member-Specific Binding. The linker region, the amino acid sequence that links the two EF-hands, exhibits the greatest sequence diversity among family members and has been postulated to regulate family member-specific binding to target proteins such as phosphoglucomutase. Consistent with this hypothesis is the accessibility of this region to solvent in both the apo- and Ca^{2+}-bound states of all family members for which 3D structures are available [10, 23, 57, 143]. To test this hypothesis, we checked the ability of a recombinant S100B-A1-B chimeric protein to bind to target proteins, in which the S100B linker region was replaced with comparable amino acid sequence from S100A1 (Figure 8). This chimeric protein was readily purified using similar procedures as used previously for S100B/S100A1, and the mutations to the hinge region did not exhibit any altered biochemical or biophysical properties tested. As anticipated, the interaction of the fluorophore-labeled chimeric protein with the S100A1/S100B targets glycogen phosphorylase a and b was indistinguishable from that of S100A1-488 and S100B-488, that is, Ca^{2+}-dependent binding in the 10–25 pmole range. In contrast, phosphoglucomutase, a target protein that preferentially interacts with S100B, bound 2–4-fold less S100B-A1-B-488 when compared to S100B-488. Interestingly, the presence of the S100A1 linker region did not lower and/or reverse the Ca^{2+}-dependent interaction with tau. Additional experiments will be needed to ascertain the linker region's contribution to other family-member specific target protein interactions as well as Ca^{2+}-independent target protein binding. Nonetheless, this is the first demonstration that the linker region does confer family-specific binding for some Ca^{2+}-dependent target proteins.

1.5. The Ca^{2+}-Dependent Target Protein Binding Domain. Carboxyl terminal aromatic residues (Phe88, Phe89, Trp90) of S100A1 have been previously shown to regulate Ca^{2+}-dependent interaction of S100A1 with the TRTK peptide, GFAP, and tubulin [72, 144, 145]. Analogous residues are found in several other members including S100B, S100A4, and S100A10 [78, 146–149]. To determine if these residues are obligatory for Ca^{2+}-dependent target protein interactions, we examined the interaction of the S100A1 (F88/89A-W90A)-488 with four additional S100A1 target proteins (Figure 9). As anticipated, mutant S100A1 binding to all four target proteins was decreased by ~4-fold in the presence of Ca^{2+}. Interestingly, there was a 4-fold increase in mutant S100A1 binding to tau in the absence of Ca^{2+}. These results confirm that carboxy-terminal aromatic residues contribute to the Ca^{2+}-dependent interaction of S100s with protein targets. However, it is not the only mechanism because not all family members that exhibit Ca^{2+}-dependent target protein

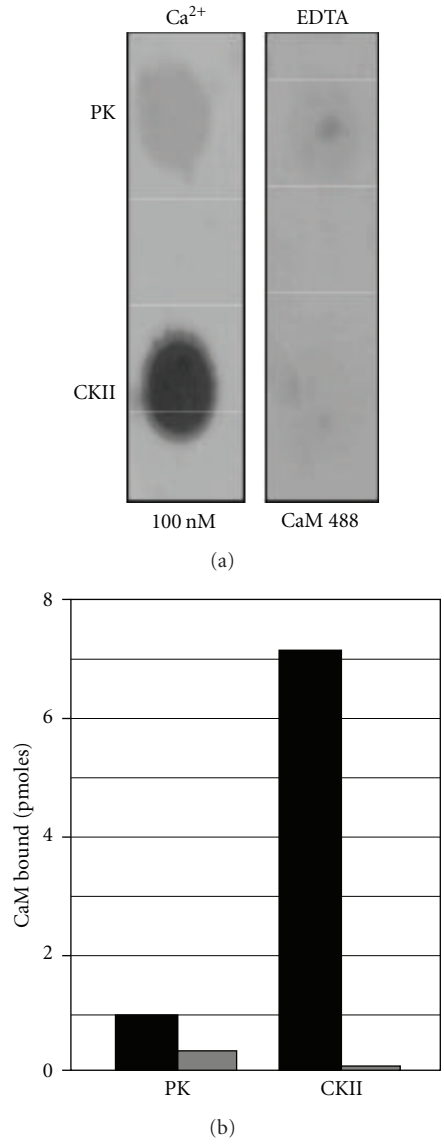


Figure 6: *Characterization of CaM-488 target protein binding.* Equimolar concentrations of CaM kinase II (CKII) phosphorylase kinase (PK) were immobilized on a PVDF membrane and incubated with 100 nM CaM-488 in the presence of Ca^{2+} or EDTA. Panel A contains a representative dot blot image. The histograms in Panel B represent the mean pmoles CaM-488 bound in the presence (black bars) and absence (gray bars) of Ca^{2+} assayed in triplicate in two independent experiments. Consistent with reported K_ds and K_as, both targets exhibited Ca^{2+}-dependent binding with the higher affinity target, CKII, binding more CaM-488 (7.0 pmoles) when compared to the lower affinity target PK (0.8 pmoles).

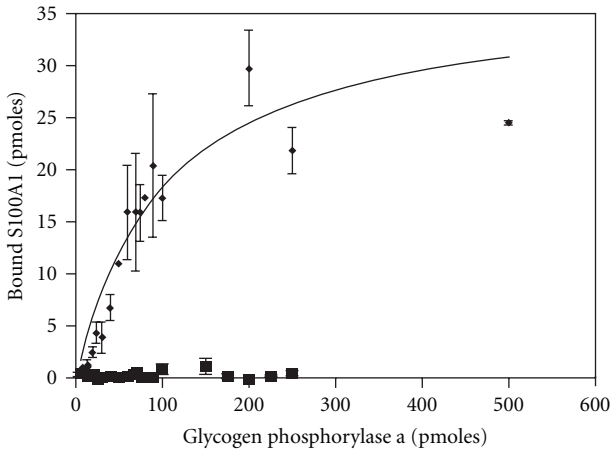


Figure 7: *S100A1-488 binding curves for glycogen phosphorylase a.* Membranes containing varying concentrations of glycogen phosphorylase were incubated in S100A1-488 in the presence (•) or absence (■) of Ca^{2+}. A standard curve of fluorescence intensity per mg of S100-488 was used to determine the experimental amount of labeled S100 per dot of target protein ($n = 17$).

interactions have hydrophobic residues in their C-terminal extension [150].

2. Summary

In summary, S100 family members have a distinct role in intracellular Ca^{2+} signaling. The complement of S100s and S100 target proteins expressed in an individual cell allows that cell to transduce a universal change in $[Ca^{2+}]_i$ into a unique biological response. Furthermore, their unique metal binding properties and diverse lists of target proteins provide mechanisms for conferring Ca^{2+}/metal sensitivity to cellular processes as well as integration and cross-talk among these processes. Thus, delineating S100-regulated processes in different cell types, ascertaining the relationship between intracellular and extracellular S100s, determining how S100-regulated processes are altered by Ca^{2+} dysregulation in disease states, and identifying that the molecular events involved are critical for understanding the function of this versatile protein family. The combination of structural, biochemical, molecular, cell biological, and *in vivo* techniques, which has been successful to date, will ultimately identify "inhibitors" of S100 function that can be used to normalize Ca^{2+} signaling in diseased cells.

3. Materials and Methods

3.1. Bacterial Expression Vectors for Wild-Type S100 Family Members. The pVex expression vector for human S100P was a generous gift of Dr. George Makhatadze [151]. Bacterial expression vectors for S100A1 and S100B have been previously described [152]. Plasmids encoding rat S100A4 (GenBank accession number AA997272) and S100A5 (GenBank accession number 1772854) were obtained through the IMAGE consortium library (Research Genetics, Huntsville, AL). The coding sequences were amplified using gene specific primers containing synthetic Nde I and Hind III sites at the 5′ and 3′ ends of the coding sequence, respectively. The nucleotide sequences for the primers were 5′-TTCCATATGGCGAGACCCTTGGAGGAG-3′ and 5′-CCCAAGCTTCACTTCTTCCGGGGCTCC-3′ (Lone Star Labs, Houston, TX) for S100A4, 5′-TTCCATATGGAGACTCCTCTTGAGAAG-3′ (Invitrogen, Carlsbad, CA) and 5′-CCCAAGCTTCACTTGTTGTCCTCTAAG-3′ (Lone Star Labs) for S100A5. Gene amplification was performed using an initial heat step (94°C, 5 minutes) followed by 30 cycles consisting of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 68°C, and a final extension step of 7 minutes at 68°C. The resulting PCR products were subcloned into the TA cloning vector PCR2.1 (Invitrogen, Carlsbad, CA). The coding sequences were isolated from Nde I-Hind III digests of plasmid DNA and subcloned into pET21a$^+$ (Novagen, San Diego, CA). The entire protein coding sequence was verified by DNA sequence analysis.

3.2. Bacterial Expression Vectors for Mutant S100 Proteins. The bacterial expression vector for the S100A1 triple point mutant (F88/89A-W90A) has been described previously [152]. A two-step PCR protocol followed by directional subcloning was used to generate the expression vector for the S100B-A1-B chimeric protein in which the amino acid sequence for the linker region in S100B (HFLEEIKEQ) was replaced with the S100A1 linker region (SFLDVQKDA). A sense oligonucleotide containing the amino terminal S100B sequence with an engineered Nde I restriction site (5′-CGCCATATGTCTGAACTCGAGAAAGCTG, Invitrogen) and a 3′ antisense oligonucleotide encoding the amino terminal half of the S100A1 linker region and an Xba I site (5′-CCTCTAGAAAGCTGCTAAGTTC, Invitrogen) were used to generate the 5′ half of the chimeric protein. The 3′ half of the chimeric protein was generated using the same template, a sense oligonucleotide encoding the carboxyl terminal half of the S100A1 linker region and an Xba I restriction enzyme site (5′-GCCATTTTCTAGACGTCCAGAAGGACGCGGAAGTTGTAGAC-3′, Integrated DNA Technologies, Coralville, IA) and an antisense oligonucleotide encoding the amino terminus of S100B with an engineered Hind III site (5′-CCCAAGCTTATTCATGTTCG, Intergrated DNA Technologies). The PCR program consisted of 30 cycles (1 minute denaturing at 94°C, 1 minute of annealing at 55°C, and 3 minutes of extension at 72°C). The resulting PCR products were ligated into the TA cloning vector PCR2.1. Purified insert DNA encoding the two halves of the chimeric protein was ligated together and subcloned into pET21a$^+$ using the 5′ Nde I and 3′ Hind III restriction sites. Restriction enzyme digest and sequence analysis confirmed that the insert sequence encoded a protein with the appropriate amino acid sequence changes.

3.3. Expression/Purification of Recombinant S100 Proteins. Recombinant S100B, S100A1, and S100A1 triple point mutant were purified as previously described [152]. Minor modifications in this procedure were made to optimize

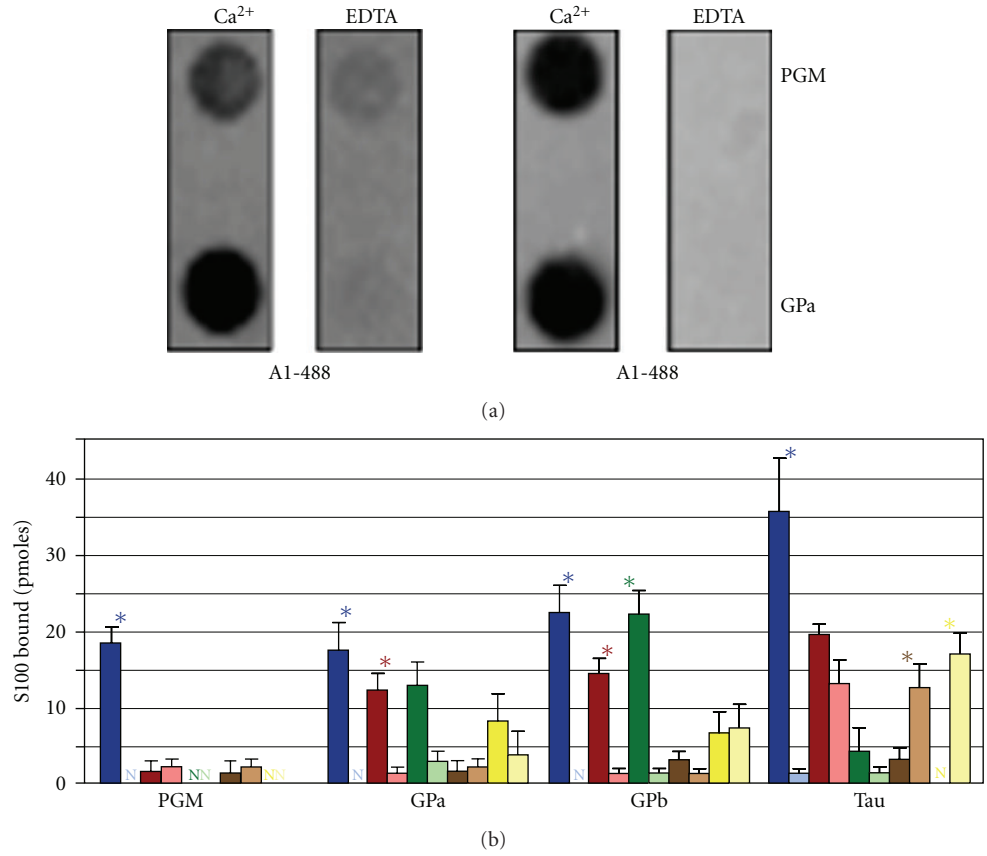


Figure 8: *Target protein binding profiles for S100 family members.* Membranes containing glycogen phosphorylase (a) (Gpa), glycogen phosphorylase (b) (Gpb), phosphoglucomutase (PGM), and tau (50 pmoles) were incubated in 100 nM Alexa Flour 488 labeled S100B (blue bars), S100A1 (red bars), S100P (green bars), S100A4 (brown bars), and S100A5 (yellow bars) in the presence (darker bars) or absence (lighter bars) of Ca^{2+}. The histograms depict that the mean pmoles S100 bound ± the SEM and N's denote no detectable binding. Asterisks denote $P \leq .05$ between the $\pm Ca^{2+}$ conditions.

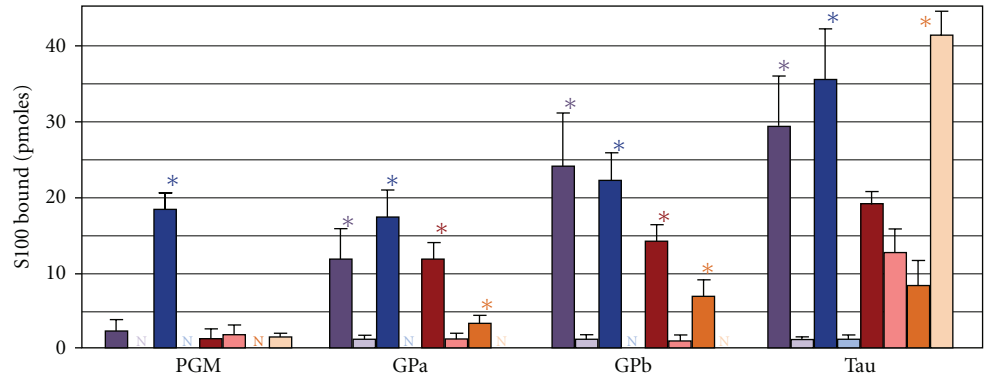


Figure 9: *Interaction of wild-type and mutant S100s with target proteins.* Membranes containing 50 pmoles glycogen phosphorylase a (Gpa), glycogen phosphorylase b (Gpb), phosphoglucomutase (PGM), and tau were incubated in 100 nM S100B-488 (blue bars), S100A1-488 (red bars), chimeric S100B-A1-B-488 (purple bars), or S100A1(F88/89A-W90A)-488 (orange bars) in the presence (darker bars) or absence (lighter bars) of Ca^{2+}. The histograms depict that the mean pmoles S100 bound ± the SEM and N's denote no detectable binding. The asterisks denote $P \leq .05$, and the N's denote no detectable binding between the $\pm Ca^{2+}$ conditions.

purification for S100A4, S100A5, S100P, and the S100B-A1-B chimera. Unboiled bacterial lysates for S100A4, S100A5, and S100P were fractionated by ammonium sulfate precipitation. Prior to phenyl-Sepharose chromatography, solid ammonium sulfate was added up to 30% (w/v) to S100A4 [153] and up to 60% for S100P and S100A5. The unboiled S100B-A1-B lysate and the S100A4, S100A5, and S100P ammonium sulfate fractions were chromatographed on

phenyl-Sepharose resin equilibrated with 50 mM Tris-Cl pH 7.4, 5 mM $CaCl_2$, and 1 mM β-mercaptoethanol. The resin was washed with buffer containing high salt (50 mM Tris-Cl pH 7.4, 5 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 500 mM NaCl). Protein was eluted in 50 mM Tris-Cl, pH 7.4, 5 mM EDTA, and 1 mM DTT. Fractions containing S100 protein were identified by SDS-PAGE and pooled. The purity of the protein preparations was assessed by SDS-PAGE. Protein samples were considered to be >95% pure when only a single band of ~10,000 kDa was visible on the Coomassie-blue stained gel.

3.4. Alexa Fluor Conjugation. All wild-type and mutant S100 proteins (5.0 mg/ml in PBS) were labeled with the photostable fluorophore, Alexa Fluor 488 (Molecular Probes, Eugene, OR), according to the manufacturer's recommendations. Prior to labeling, the pH was adjusted to ~8.3 with 1 M sodium bicarbonate and EDTA was added to a final concentration of 5.0 mM. Alexa Fluor 488 dye (1 mg) dissolved in DMSO was added to the protein sample. The reaction was incubated at room temperature for one hour in the dark with continuous stirring. Unconjugated dye was hydrolyzed by incubation of the solution overnight in the dark at 4°C. After dilution with 1-2 volumes of water, the pH of the solution was adjusted to 7.4 by the addition of 2 M Tris-Cl pH 7.4. Free dye was separated from protein conjugates by Ca^{2+}-dependent phenyl-Sepharose chromatography as described for protein purification. Next, we determined if this methodology could be used to quantify the binding of S100 proteins to their various target proteins. Alexa Fluor 488 labeled S100A1, S100B, S100A4, S100A5, and S100P exhibited physical characteristics that were indistinguishable from unlabeled proteins including Ca^{2+}-dependent binding to phenyl-Sepharose (data not shown).

3.5. S100-Target Protein Binding Assay. Glycogen phosphorylase a, glycogen phosphorylase b, phosphoglucomutase (PGM), and phosphorylase kinase were purchased from Sigma Chemical Company (St. Louis, MO). Bovine brain tau and calmodulin-dependent kinase II (CaM kinase II) were generous gifts of Gloria Lee (University of Iowa) and Tom Soderling (Vollum Institute, Portland, OR), respectively. Varying concentrations of the target proteins were immobilized on Immobilon-P PVDF membrane (Millipore) using a 96-well dot-blot apparatus (BioRad, Hercules, CA) per supplier's instructions. After a 30-minute incubation, wells were washed twice with three volumes of 20 mM Tris-Cl pH 7.4. The membrane was removed from the dot-blot apparatus, cut into strips, and incubated overnight at room temperature in 1 μM Alexa Flour 488-labeled protein in 50 mM Tris-Cl, pH 7.4 with 200 mM NaCl (buffer A) containing either 5 mM EDTA or 1 mM $CaCl_2$. The strips were rinsed three times in buffer A containing 5 mM EDTA or 1 mM $CaCl_2$ and fluorescence quantified on a Fuji FLA-5000 Image Analysis System (Stamford, CT). A standard curve of fluorescence intensity versus pmoles of PVDF-immobilized S100-488 (or Cam-488) was used to determine the pmoles of labeled protein bound to that concentration of target protein ($n \geq 2$). Data points for each target protein concentration were averaged together to yield the mean pmoles of bound S100-488 ± SEM which was calculated for each target protein. The student's t-test was used to determine the statistical significance ($P < .05$) of any measured differences between the means.

Acknowledgments

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Review Article

Perinatal S100B Protein Assessment in Human Unconventional Biological Fluids: A Minireview and New Perspectives

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Growing evidence is now available on the use of S100B protein as a valuable marker of brain damage and its role as a neurotrophic factor. Bearing in mind, among different S100B protein properties that are still being investigated, the possibility of measuring this protein in different biological fluids renders it suitable for use in several disciplines. This is the case with perinatal medicine where even more noninvasive techniques are particularly desirable in order to ensure the minimal handling diagnostic and therapeutic strategies. In this setting, the present minireview reports data on the presence and the usefulness of S100B protein as brain damage marker and as a neurotrophic factor in the so-called unconventional biological fluids such as saliva and human milk, respectively. Results offer new possibilities for the use of S100B in perinatal medicine as a key-protein for the investigations focusing on central nervous system development and damage.

1. Introduction

The term S100B refers to a member of a multigenic family of calcium-modulated proteins first identified in 1965 by Moore [1], each of which exhibits a unique pattern of tissue- or cell type-specific expression; the term S100 refers to their solubility in a 100%-saturated solution with ammonium sulfate. To date at least 25 proteins have been identified as belonging to the S100 protein family, characterized by the presence of a pair of so-called EF-hand (i.e., helix-loop-helix) calcium-binding motifs, first discovered in the crystal structure of parvalbumin, that induce conformational changes of the protein after binding to calcium. Most S100 proteins exist as dimers (frequently homodimers) within cells and are generally expressed and distributed in a cell-definite fashion, indicating a conserved biological role. In this setting, S100B is a homodimer of the beta subunit, mainly concentrated in the nervous system and in the adipose tissue. In the nervous system S100B appears to be most abundant in glial cells and its presence in specific neuronal subpopulations has also been described [2–6]. S100B is present intracellularly and extracellularly; it is believed to regulate several cellular functions (protein phosphorylation, protein degradation, calcium homeostasis, cell locomotion, transcription factors, cell proliferation and differentiation, enzymes, regulation of receptor function, cytoskeleton) while its biological intracellular role has not yet been completely elucidated in unifying terms. An extracellular biological role is also attributed to S100B, which is secreted by astrocytes as a cytokine exerting an autocrine or paracrine effect on glia, neurons and microglia; the protein may have a trophic effect during both development and nerve regeneration at physiologic (nanomolar) concentrations (the Jekyll side), but at high (micromolar) concentrations (the Hyde side) could be neurotoxic, participating in the pathophysiology of neurodegenerative disorders [7–10]. In this respect, transcriptional effects of micromolar S100B on neuroblastoma cells have been shown to result in perturbation of cholesterol homeostasis and interference in the cell cycle [11]. Both effects of S100B on target cells are believed to be mediated through RAGE engagement [5]. Apart from its still unknown function, the presence of S100B in biological fluids is

interesting, since at present it constitutes an established index of brain injury [10]. It should be noted in this respect that its half-life is approximately 1 hour and it is mainly eliminated by the kidney [12]. In particular, the assessment of S100B in biological fluids has been usefully employed in perinatal medicine [12, 13]. After its established use in conventional fluids (CSF, blood, urine, amniotic fluid), this minireview is focused on the assessment of S100B levels in unconventional fluids (saliva, milk), which may open new perspectives for studies on this protein.

1.1. S100B and Conventional Biological Fluids. S100B protein has been measured in several biological fluids (cerebrospinal fluid, blood, urine and amniotic fluid) by a series of immunoassays, which have been variously used in different fluids.

Cerebrospinal fluid (CSF)was the first of various biological fluids in which the role of S100B as a marker of active brain damage was demonstrated [14, 15]. In perinatal medicine, measurements of S100B protein in CSF have been used to monitor infants affected by perinatal asphyxia and post-hemorrhagic ventricular dilatation brain damage during cardiac surgery. S100B concentrations correlated with the extent of brain lesions, with long-term prognosis, and with neurological impairment at 1 year of age or death before that time [16–18].

The idea of measuring S100B into blood was based on the hypothesis that during active brain injury at least some of the S100B released from the damaged tissue could spread into the systemic circulation [19], also as a result of hemodynamic rearrangement of the blood brain barrier. Increased blood concentrations of S100B were indeed detected in cases of chronic hypoxia and/or intraventricular hemorrhage (IVH) in preterm infants, in full-term infants suffering by perinatal asphyxia and adverse neurological outcomes [20–23]. S100B was also measured in the blood of women whose pregnancies are complicated by intrauterine growth retard and whose newborns develop intraventricular haemorrhage [24].

In the urine S100B concentrations at birth were significantly higher in preterm newborns developing intraventricular haemorrhage and/or brain damage. In a cross-sectional study using urine obtained from preterm newborns, the protein has been found to be a valuable predictor of early neonatal death [25–28].

In the amniotic fluid S100B concentration has been shown to correlate with gestational age and with cerebral ultrasound scanning results in healthy fetuses [29]. In addition, amniotic S100B concentrations are higher in monoamniotic than in diamniotic and singleton pregnancies, hinting at the possibility that each foetus releases a physiologically defined amount of S100B during pregnancy [30]. Elevated S100B concentrations in the amniotic fluid have also been shown to constitute a reliable marker of foetus pathological conditions, including trisomy 21 [31–35].

1.2. S100B and Unconventional Biological Fluids. Among human biological fluids, in the perinatal period, the studies aimed at investigating the presence of S100B protein in biological fluids, namely "unconventional" biological fluids such as saliva and milk, are especially intriguing. In particular saliva, which is more easily collected than CSF, blood or urine offers the hope of simple non-invasive tests especially useful in perinatal medicine. This possibility could even represent the reaching of the so called "gold standard" for non-invasive longitudinal monitoring of occurring brain damage eagerly awaited in clinical practice. Future prospects also include the possibility of monitoring the true effectiveness of the even more risky therapeutic strategies such as mechanical ventilation, brain cooling and experimental medications. Even more interesting prospects are offered by the detection of S100B in breast milk, candidating the protein as a participant in the biochemical communication between mother and child. This might contribute to the identification of biochemical markers with a series of unknown functions that to date confirm that the role of maternal milk instead of artificial ones in brain maturation involvement is unique.

1.2.1. S100B and Saliva. The possibility that S100B could be assessed in different biological fluids has been substantiated in the last decade, thanks to continuous interactions among physicians of several disciplines. In this regard, the concept of minimal handling of patients derives from neonatologists and perinatologists who need, in the phase of critical intensive care medicine, non-invasive parameters able to detect cases at risk of brain damage at the earliest stage in order to prevent it. Among non-invasive biological fluids saliva meets the optimality requirements since it can be collected any time without newborn stress, its measurements are reproducible and can be compared with other "invasive" biological fluids.

Studies of human fetuses have shown that members of the S100 protein family are present in different tissues of the salivary glands during ontogenesis. Results showed that different S100 proteins are expressed during different phases of pregnancy up to adulthood, whilst S100B in particular has been found to be essentially absent from fetal salivary glands from 32 weeks of gestation onwards [36, 37]. Data on physiology of salivary glands reported that systemic blood and saliva are isotonic and contain the same concentrations of ions in the same ratios, thanks to a continuous exchange of ions and protein between blood and saliva. In addition, an increase or a decrease in the volume of saliva release is systemic circulation-dependent and mediated by ortho/parasympathetic activation [38]. These findings indicate that the salivary glands do not produce S100B in the third trimester of pregnancy and that S100B concentrations detected in saliva likely derive from systemic circulation. The first observation of the presence of S100B in saliva was supported by immunoluminometric assay confirmed by Western blot analysis and also offered a reference curve of the protein in this biological fluid in normal preterm and term newborns [39]. Results showed that saliva can be used in the clinical monitoring of S100B levels, which, as already established for other biological fluids, such as CSF, blood, urine or amniotic fluid, can constitute a reliable index to diagnose/monitor brain distress. Since saliva samples are more easily collected than other biological fluids, tests

performed on this biological fluid appear to be especially convenient in the clinical evaluation of newborns.

1.2.2. S100B and Milk. The hypothesis of assessing the protein in a so complex biological fluid was based on the following elements: (i) S100 proteins are highly conserved in amino acid composition among vertebrate species, suggesting a pivotal, although still unknown, biological role for the protein [40]; (ii) S100-like proteins have also been immunologically detected in spinach leaves suggesting a potential role in human food-chain [41], adipose tissue also constitutes a site of concentration for the protein [42]; (iii) breast milk is known to contain growth factors and cytokines [43–46]; (iv) milk is abundant in other calcium-binding proteins (e.g., alpha-lactalbumin, calmodulin, osteocalcin) [47–49]. Results showed S100B in human breast milk; in addition, S100B milk content was estimated to be 80/100 times higher than those detected in CSF, blood, urine [50]. Interestingly, significant differences have been found in S100B milk concentrations among different mammalian species such as human, cow, goat, donkey and sheeps. The S100B concentration is higher in human milk, thus raising possible speculations on a relationship between the milk concentration of this neurotrophic factor and species evolution [51]. Western blot analysis confirmed that the immunoreactivity observed using immunoluminometric assay refers to S100B protein and RT-PCR analysis also detected human S100B mRNA in the human milk [50]. This latter finding likely refers to the presence in the milk of cell types expressing S100B, including mammary epithelial cells and lymphocytes, which reasonably may be supposed to be the source of S100B in the milk. The presence of S100B at very high concentration in human breast milk may be related to its putative neurotrophic role, given that breast-feeding is believed to exert a stimulatory effect on brain maturation [52]. More detailed information will be needed to corroborate the possibility that S100B may participate in the nutritional aspects of milk, including possible effects on intestinal development and/or trophism of the enteric nervous system. In this respect it should be noted that human breast milk is known to contain different substances that may actively influence infant growth and development, including hormones, growth factors and cytokines [53]. Interestingly, S100B levels in human breast milk significantly increase during milk maturation, being very low in colostrum, intermediate in transition milk (7 and 14 days) and high in mature milk (30 days) [54]. Further studies investigated the impact of industrial preparation procedures (skimmed cow milk, protein sources supplementation, pasteurization and spray-drying procedures) on S100B content. Results showed that S100B has a sufficient thermo-stability to resist pasteurization but not spry-drying, suggesting that new feeding strategies in preterm and term infants are therefore warranted in order to preserve S100B and other potential brain constituents during industrial preparation [55]. It may be relevant in this respect that, thanks to the considerable technological improvement in dietetics preparations and progresses in laboratory biochemistry, evidence is growing that artificial milks could need to be enriched with recent discovered constituents: S100B can be included among these on account of its neurotrophic properties.

Finally, apart from the possible trophic role on the newborn exerted by the maternal breast milk, the examination of S100B level in this unconventional biological fluid could offer new prospects in the investigation of maternal physiopathological conditions with a potential direct impact on the newborn health.

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Review Article

Intracellular and Extracellular Effects of S100B in the Cardiovascular Response to Disease

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S100B, a calcium-binding protein of the EF-hand type, exerts both intracellular and extracellular functions. S100B is induced in the myocardium of human subjects and an experimental rat model following myocardial infarction. Forced expression of S100B in neonatal rat myocyte cultures and high level expression of S100B in transgenic mice hearts inhibit cardiac hypertrophy and the associated phenotype but augments myocyte apoptosis following myocardial infarction. By contrast, knocking out S100B, augments hypertrophy, decreases apoptosis and preserves cardiac function following myocardial infarction. Expression of S100B in aortic smooth muscle cells inhibits cell proliferation and the vascular response to adrenergic stimulation. S100B induces apoptosis by an extracellular mechanism via interaction with the receptor for advanced glycation end products and activating ERK1/2 and p53 signaling. The intracellular and extracellular roles of S100B are attractive therapeutic targets for the treatment of both cardiac and vascular diseases.

1. The Family of S100 Proteins

S100 proteins entail a multigenic family of calcium binding proteins of the EF-hand type (helix E-loop-helix F). These proteins are called S100 because of their solubility in a 100% -saturated solution with ammonium sulphate at neutral pH. They are small acidic proteins, 10–12 KDa, and contain two distinct EF-hands, 4 α-helical segments, a central hinge region of variable length, and the N- and C-terminal variable domains. To date, 25 members of this family have been identified [1]. Of these, 21 family members (S100A1-S100A18, trichohyalin, filagrin, and repetin) have genes clustered on a 1.6-Mbp segment of human chromosome 1 (1q21) while other members are found at chromosome loci 4q16 (S100P), 5q14 (S100Z), 21q22 (S100B), and Xp22 (S100G) [2]. S100 proteins are widely expressed in a variety of cell types and tissues. For example, S100A1 and S100A2 are found in the cytoplasm and nucleus, respectively, of smooth-muscle cells of skeletal muscle [3], S100P is located in the cytoplasm of placental tissue [4, 5], and S100B in cytoplasm of astrocytes of nervous system [6]. However, their expression might be repressed in other cell types by negative regulatory factors which are controlled by environmental conditions. For instance induction of S100B in rat myocardium postinfarction [7] implies that transcription regulation of these proteins is strongly controlled by negative and positive elements [8].

S100 proteins do not exhibit intrinsic catalytic activity. However, they are calcium sensor proteins and through interaction with several intracellular effector proteins they contribute to the regulation of a broad range of functions such as contraction, motility, cell growth and differentiation, cell cycle progression, organization of membrane-associated cytoskeleton elements, cell survival, apoptosis, protein phosphorylation, and secretion [1, 3, 9]. In order to modulate these types of activities, S100 proteins undergo conformational changes [10]. Upon calcium binding, the helices of S100 proteins rearrange, revealing a hydrophobic cleft, which forms the target protein binding site [11]. Although target binding of S100 proteins is calcium-dependent, calcium independent interactions have been reported [12]. Enzymes are the most common calcium independent target binding

for the S100 proteins. For instance, S100B and S100A1 bind with glycogen phosphorylase [13]. The most significant calcium-independent interactions of S100 proteins are their ability to bind to each other. Typically, they are homodimers, but heterodimerization adds to the complexity of this multiprotein family. Each subunit consists of two helix-loop-helix motifs connected by a central linker or so-called hinge region. The C-terminal canonical EF-hand motif is composed of 12 amino acids, whereas the N-terminal S100-specific EF-hand comprises 14 residues [3, 14].

Growing evidence indicates that in addition to intracellular activities, some S100 proteins (e.g., S100B, S100A1, S100A4, S100A8, and S100A9) exhibit extracellular functions [15]. However, secretion has been shown only for S100B, S100A8, and S100A9 [15]. The S100A8/A9 heterodimer is secreted by a novel secretion pathway that depends on an intact microtubule network and acts as a chemotactic molecule in inflammation [16, 17]. The extracellular effects of some S100 proteins require binding to the receptor for advanced glycosylation end products (RAGE) [18–21]. RAGE is a member of the immunoglobulin family of cell surface molecules recognizing multiple ligands including AGE, amphoterin, amyloid-β-peptide and β-fibrils, S100A12, S100A6, and S100B [22]. The 45-kDa receptor protein consists of 403 amino acids with an extracellular domain (1 variable and 2 constant Ig domains with disulfide bridges), a single transmembrane region, and a short cytosolic tail that triggers signal transduction [23]. RAGE ligands show selective binding to RAGE. S100B tetramer induces receptor dimerization by binding to RAGE [24]. S100B binds to domains V and CI whereas the RAGE ligand S100A6 binds to domains CI and CII [23].

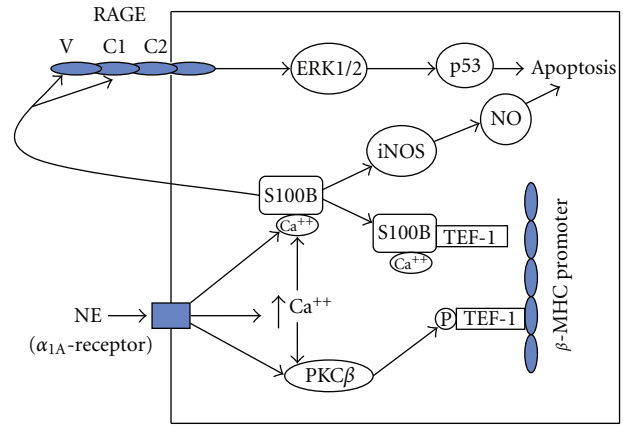

Figure 1: Schematic representation of proposed intracellular and extracellular effects of S100B in cardiac myocytes. Norepinephrine (NE) activation of the calcium-dependent protein kinase C (PKC)-β, mediated by the α_1-adrenergic receptor, phosphorylates (P) transcriptional enhancer factor (1) TEF-1, resulting in DNA binding and transactivation of the β-myosin heavy chain promoter. By contrast, S100B induction by NE and other hypertrophic signals (not shown) results in calcium-dependent block of PKC-β phosphorylation of TEF-1 and inhibition of β-MHC transcription. S100B can also induce apoptosis intracellularly via a inducible nitric oxide synthase (iNOS)-NO pathway or it can be secreted and via activation of the receptor for advanced glycation end products (RAGE) (extracellular components V and CI), and induce apoptosis via MEK-ERK1/2-p53 signaling.

2. Noncardiovascular Actions of S100B

S100B is predominantly expressed in astrocytes, oligodendrocytes, and schwann cells. S100B has intracellular and extracellular effects [1]. Intracellularly, S100B regulates the cytoskeletal dynamics through disassembly of tubulin filaments, type III intermediate filaments [1], and binding to fibrillary proteins such as CapZ [25] or inhibiting GFAP phosphorylation when stimulated by cAMP or calcium/calmodulin [26]. S100B interacts in a calcium-dependent manner with the cytoplasmic domain of myelin-associated glycoprotein and inhibits its phosphorylation by protein kinase [27]. It is implicated in the phosphorylation of tau protein [28], inhibition of Ndr kinase activity [29], inhibition of p53 phosphorylation [30], and regulation of the activity of the GTPase Rac1 and Cdc effector, IQGAP [31]. S100B can also be secreted by a number of cell types (e.g., astrocytes, glial cells) [32]. Astrocytes and glial cells secrete S100B, by a complex system involving alterations in intracellular calcium concentration [32]. S100B after secretion, or simply leakage from damaged cells, could accumulate in the extracellular space and/or enter the blood stream and cerebrospinal fluid [33, 34]. The action of S100B is strongly dependent on its extracellular concentration. At nanomolar quantities, it has trophic effects on neurite outgrowth; however, at micromolar concentrations it promotes apoptosis [35, 36]. Such high extracellular levels are detected after brain injury or in neurodegenerative disorders like Down's Syndrome, Alzheimer disease, or encephalitis [37, 38]. Both trophic and toxic effects of extracellular S100B are mediated in the brain by RAGE [36]. In addition to playing a major role in brain physiology [1], S100B has been implicated in cardiovascular development [39] and is considered a biochemical marker for brain injuries after bypass graft surgery [40] and dilated cardiomyopathy [41].

3. Cardiovascular Actions of S100B

3.1. Intracellular S100B and Myocyte Hypertrophic Gene Expression. The adult cardiac myocyte is terminally differentiated and has lost the ability to proliferate. The myocardium therefore adapts to increasing workloads through hypertrophy of individual cells in response to hormonal, paracrine, and mechanical signals [42, 43]. This process is initially compensatory but it can progress to irreversible enlargement and dilatation of the ventricle resulting in heart failure [44]. Myocyte hypertrophy is accompanied by the down-regulation of adult α-myosin heavy chain and a program of fetal gene reexpression including the embryonic β-myosin heavy chain (MHC), the skeletal isoform of α actin (skACT), and atrial natriuretic factor (ANF) [45, 46]. This response can be reproduced *in vitro* in cultured neonatal cardiac myocytes by treatment with a number of trophic factors including peptide growth factors and α_1-adrenergic agonists

[7]. Negative modulators of the hypertrophic response are essential to maintain a balance between compensatory hypertrophy and unchecked progression. Experimental evidence suggests that S100B acts as an intrinsic negative regulator of the myocardial hypertrophic response [47–49]. S100B not normally expressed in the myocardium, is induced in the peri-infarct region of the human heart after myocardial infarction [47] and in rat heart commencing at day 7 following myocardial infarction as a result of experimental coronary artery ligation [7]. In cultured neonatal rat cardiac myocytes, transfection of an expression vector encoding the human S100B protein inhibits the α_1-adrenergic induction of the fetal genes β- MHC and the skACT [7]. The inhibition of α_1-adrenergic induction is selective as S100B does not inhibit the capacity of thyroid hormone to induce α-myosin heavy chain. To establish that S100B blocked α_1-adrenergic induction of β -MHC and skACT by interrupting the PKC signaling pathway, the interaction between forced S100B expression and a constitutively activated mutant of PKCβ referred to as δPKCβ was tested [50]. δPKCβ transactivated the β-MHC and skACT genes supporting the notion that the α_1-adrenergic induction of these genes is mediated by activation of the class-I PKC isoform β-PKC [7, 50]. Forced S100B expression could only block δPKCβ-induced transaction of β-MHC and skACT amidst concomitant treatment with an α_1-adrenergic agonist or augmented extracellular calcium suggesting that the capacity of S100B to modulate the hypertrophic phenotype is calcium dependent [7]. The transcription factors TEF-1 (transcription factor-1) and related TEF-1 (RTEF-1) upon phosphorylation by PKCβ bind to MCAT elements on the skACT and β-MHC promoters and activate transcription [51]. In cotransfection experiments, forced expression of S100B inhibited the activation of the skACT and β-MHC promoters by overexpression of TEF-1 (unpublished observations). A direct interaction between S100B and TEF-1 was demonstrated using a coimmunoprecipitation strategy (unpublished observations). These data suggest that S100B modulates the activation of the fetal genes by direct binding to TEF-1. In addition to TEF-1, S100B interacts in a calcium-dependent manner with the giant phosphoprotein AHNAK/desmoyokin in cardiomyocytes and smooth muscle cells [49]. In cardiomyocytes, AHNAK plays a role in cardiac calcium signaling by modulating L-type calcium channels in response to β-adrenergic signaling [52, 53]. The S100B/AHNAK interaction may participate in the S100B-mediated regulation of cellular calcium homeostasis [53]. Whether there is any relationship between S100B-mediated effects on calcium fluxes and S100B-dependent inhibition of the α_1-induction of the hypertrophic phenotype remains to be elucidated. The function of the S100B/AHNAK interaction in smooth muscle cells is currently unknown. In the myocardium, S100B expression is transcriptionally controlled dependent on positive ($-782/-162$ and $-6{,}689/-4{,}463$) and negative ($4{,}463/-782$) elements upstream of the transcription initiation site, selectively activated by α_{1A}-adrenergic signaling via PKCβ and inhibitory and stimulatory DNA binding by transcription factors, TEF-1 and related RTEF-1, respectively [8] (Figure 1). This suggests that the same α_1-adrenergic pathway that initiates and sustains the hypertrophic response in cardiac myocytes by activating PKC signaling and which is subject to negative modulation by S100B also induces the S100B gene.

3.2. Intracellular S100B, Cardiovascular Hypertrophy and Apoptosis. To provide a physiologic model of S100B overexpression effects, transgenic mice were created that contained multiple copies of the human gene under the control of its own promoter. These animals demonstrate normal cardiac structure, and neuronal, but no basal cardiac expression of the transgene. In S100B transgenic mice, after chronic α_1-adrenergic agonist infusion, S100B is detected in the heart and increased in the vasculature [49]. In addition, the myocyte hypertrophy and arterial smooth muscle cell proliferation normally evoked in the heart and vasculature, respectively, in response to α_1-adrenergic stimulation in wild-type mice were abrogated in S100B transgenic mice [49]. In knockout mice, α_1-adrenergic agonist infusion provoked a potentiated myocyte hypertrophic response and augmented arterial smooth muscle cell proliferation. Furthermore, in knockout mice, both the acute and chronic increases in blood pressure in response to α_1-adrenergic agonist infusion were attenuated compared with wild-type mice [49]. To determine whether this inhibition is generalizable to other hypertrophic stimuli, transgenic and knockout animals were subjected to descending aortic-banding to produce pressure-overload. Aortic banding for 35 days increased left ventricular (LV)/body weight (BW) ratio in CD-1 controls (4.61 ± 0.06 g/kg versus 3.44 ± 0.16 g/kg in sham operated, $P < .05$, $n = 6$) and produced no hypertrophy in S100B transgenic mice (3.37 ± 0.12 g/kg versus 3.26 ± 0.11 g/kg in sham operated, $P < .05$, $n = 8$) and excessive hypertrophy in knock-out mice (5.12 ± 0.24 g/kg versus 3.19 ± 0.13 g/kg in sham operated, $P < .05$, $n = 6$). Similarly, thirty five days after experimental myocardial infarction, the S100B knockout mice mounted an augmented hypertrophic response compared to wild-type mice [48]. Fetal gene expression was induced to a greater magnitude in knockout mice compared to wild-type mice. The S100B transgenic mice did not develop the hypertrophic phenotype but demonstrated increased apoptosis in the peri-infarct region compared to wild-type and knockout mice. The postinfarct hypertrophic response in the myocardium is initiated by multiple trophic signals that include the state of local and systemic sympathetic hyperactivity through α_1-adrenergic stimulation [54]. These studies in S100B transgenic and knockout mice complement the culture data and support the hypothesis that S100B acts both as an intrinsic negative regulator of hypertrophy and an apoptotic agent. Intracellular S100B may modulate the apoptotic responses of postinfarct myocytes by activating a transcriptionally inducible form of nitric oxide synthase (iNOS) and production of nitric oxide (NO) [55] as has been described for astrocytes [35]. Forced expression of S100B may induce iNOS, NO production, and apoptosis. Thus NO could be an intermediate pathway in the induction of apoptosis by intracellular S100B (Figure 1). Similar to S100B, S100A6 is upregulated in post-infarct myocardium and is

selectively induced by TNF-α and serves to limit myocyte apoptosis [56]. S100B colocalizes with S100A6 in cardiac muscle [57], suggesting that heterodimerization may have distinct phenotypic consequences.

3.3. Extracellular S100B and Myocyte Apoptosis. Increasing evidence suggests that S100B plays a role in the regulation of apoptosis in post-MI myocardium by an extracellular mechanism after cellular release from damaged myocytes and interaction with RAGE [58]. Exogenously administered S100B to neonatal rat cultures induced apoptosis in a dose-dependent manner beginning at 0.05 μmol/L, a local or regional concentration that may be achieved in the peri-infarct myocardium [48]. Similarly, S100B at dose $\geq$0.05 μmol/L induced neuronal cell death [59]. Myocyte apoptosis is accompanied by cytochrome C release from mytochondria to cytoplasm, increased expression and activity of pro-apoptotic caspase-3, decreased expression of anti-apoptotic Bcl-2, and phosphorylation of ERK1/2 and p53 [58, 60, 61] (Figure 1). Transfection of a full-length cDNA of RAGE or a dominant-negative mutant of RAGE resulted in increased or attenuated S100B-induced myocyte apoptosis, respectively, implicating RAGE dependence. Inhibition of MEK signaling or overexpression of a dominant negative p53 inhibits S100B-induced myocyte apoptosis. This implies that RAGE activation by S100B increases MEK MAPK kinase signaling, p53 phosphorylation at serine 15, and p53-dependent myocyte apoptosis (Figure 1).

The effects of S100B on myocyte apoptosis stand in contrast to S100A1, the most abundant S100 protein expressed in cardiac muscle under basal conditions [62]. S100A1 exhibits increased expression in compensated hypertrophy, decreased expression in human cardiomyopathy, and down-regulation following experimental myocardial infarction [63, 64]. S100A1 knockout mice showed elevated systemic blood pressure, reduced endothelium-dependent vasorelaxation, and decreased survival after myocardial infarction [65, 66]. Like our proposed mechanism for S100B release, S100A1 is released into the extracellular space in the setting of myocardial injury and can bind RAGE [58]. Unlike S100B, extracellular S100A1 inhibits apoptosis independent of RAGE [67] or by RAGE signaling by interacting with a different extracellular domain of RAGE as has been shown with other RAGE ligands [23]. Thus, S100 proteins may differentially regulate myocardial structure and function. Given the capacity of S100A1 and S100B to heterodimerize, phenotypic consequences may depend on the availability and stoichiometry of S100A1 and S100B homodimers and heterodimers.

4. Concluding Remarks

In conclusion, the S100 family constitutes the largest subgroup of the EF-hand family of calcium-binding proteins with 25 members. S100 proteins have been implicated in pleiotropic calcium-dependent cellular events, with specific functions for each of the family members. S100B is induced in peri-infarct myocytes postmyocardial infarction in human subjects and experimental rodent models of myocardial infarction and in response to α_1-adrenergic stimulation. S100B plays an important role in negative intrinsic regulation of aortic smooth muscle cell proliferation, cardiac myocyte hypertrophy, and, via RAGE ligation, apoptosis. The intracellular and extracellular roles of S100B are attractive therapeutic targets for the treatment of both cardiac and vascular diseases.

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Review Article

Adipocytes as an Important Source of Serum S100B and Possible Roles of This Protein in Adipose Tissue

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Adipocytes contain high levels of S100B and *in vitro* assays indicate a modulated secretion of this protein by hormones that regulate lipolysis, such as glucagon, adrenaline, and insulin. A connection between lipolysis and S100B release has been proposed but definitive evidence is lacking. Although the biological significance of extracellular S100B from adipose tissue is still unclear, it is likely that this tissue might be an important source of serum S100B in situations related, or not, to brain damage. Current knowledge does not preclude the use of this protein in serum as a marker of brain injury or astroglial activation, but caution is recommended when discussing the significance of changes in serum levels where S100B may function as an adipokine, a neurotrophic cytokine, or an alarmin.

1. Introduction

Searching for peripheral molecular markers for brain damage and/or dysfunction, S100B protein appears to be a promising candidate [1–6]. In fact, studies in serum samples, after acute brain injury, show that S100B levels change; however the interpretation of results is complex, particularly because extracerebral sources contribute to the serum S100B content. Herein, we intend to discuss S100B from adipocytes as a source for the serum content of this protein and to compare the release of S100B from adipocytes with that of astrocytes, the major S100B-containing compartment in the central nervous system. We hope that this paper can contribute to the search for the biological role(s) of this protein in adipose tissue, as well as to help to understand how variations in the serum content of S100B affect physiological and pathological conditions.

2. Adipocytes

It is important to take into consideration some points about adipocytes, which are the target cells of this paper. Adipocytes are the main cell type in adipose tissue, which is distributed in three major anatomical areas: subcutaneous, dermal and intraperitoneal [7]. The population of adipocytes is heterogeneous in each area and subarea, based on their size and proliferative capacity, and also may be variable, depending on the region's blood flow and innervation density. In addition to adipocytes, adipose tissue contains stromal-vascular cells and immune cells and the traditional concept that tissue is a simple lipid store is no longer valid [8]. In fact, some adipocyte-derived proteins are messengers, acting on specific receptors found in endothelial cells, muscle cells, cardiomyocytes and neurons and disorders of communication between these cells are associated, for example, with diabetes and cardiovascular disorders [8, 9].

3. S100B in Adipocytes

S100B was initially described as a neuron-specific protein [10], but subsequent characterization revealed that this protein, in the central nervous system (CNS), is mainly localized in GFAP positive glial cells [11]. However, in the CNS, this protein was also abundantly detected in oligodendrocytes [12], in microglia [13] and even in cholinergic neurons of the

hindbrain [14]. Moreover, it was also found among various cells of non-neural tissues [15, 16], such as adipocytes [17], chondrocytes [18] and melanoma cells [19].

The first evidence of S100B in adipose tissue was independently obtained by Hidaka and coworkers, in Japan [20] and by Michetti and coworkers, in Italy [17]. Hidaka et al. found elevated levels of S100B in brain tissue, using a polyclonal anti-S100B and Michetti et al. found that S-100 protein in adipose tissue was comparable to that measured in the brain tissue, but that possibly $S100A_1$ was also present in the preparation. Regardless of this, these studies contribute to consolidate the view that S100B exists in non-nervous tissues [18, 19].

The mRNA expression of S100B was demonstrated later in adipose tissue [21]. It is important to mention that a direct correlation between the mRNA and protein levels was not observed. This absence of correlation also was observed in brain and adipose tissue in rats exposed to streptozotocin, a drug used to induce type 1 diabetes [22]. These data suggest a complex and cell-specific mechanism of S100B expression [23].

Preliminary results from Guaragna in our laboratory indicate that human adipose tissue also expresses high levels of S100B and that this amount varies depending on the anatomical area of adipose tissue. In rats, we found an elevated content of S100B in rat white adipose epididymal tissue (about 1.5 ng/μg of protein), comparable to that of the hippocampal tissue [24], but sample delipidation is a necessary experimental procedure to avoid an underestimation of S100B content in adipose tissue [25]. Moreover, both adipose and brain tissues increase S100B protein content when exposed to streptozotocin in diabetes or dementia models [22, 26].

4. Is S100B Released by Adipocytes?

There is clear evidence that adipocytes release S100B in different cell preparations, including epididymal fat pad and freshly-isolated adipocytes (Table 1). Based on cell integrity assay (lactate dehydrogenase release), the mechanism of S100B release from adipocytes should be appropriately referred to as S100B secretion, as occurs in astrocytes and differs, for example, from S100B release from melanoma cells [34]. A positive regulation of S100B secretion in adipocytes was observed in response to glucagon and catecholamines, likely triggered by a cAMP-mediated pathway, as occurs in astrocytes [35]. Insulin, which attenuates the cAMP pathway in adipocytes, caused a decrease in S100B release [31].

Nevertheless, it is important to evaluate whether this secretion could affect serum S100B levels. In other words, should variations in amount and activity of adipose tissue be considered in the interpretation of serum S100B levels? Human Brain tissue corresponds to 2% of weight body, while adipose tissue corresponds, in normal individuals, to 9–18% in men and 14–28% in women [7]. Thus, considering the amount and the *in vitro* secretion rate of S100B from the adipose tissue, it is reasonable to assume that this tissue is an important source of serum S100B that is even more important than brain tissue.

Some evidence to support this hypothesis arose during physical exercise conditioning studies, where serum S100B elevations, putatively, were not associated with brain damage; such exercise included swimming racing [36], marathon running [37] and playing basketball [38]. The suspicion that body weight could affect serum S100B levels was examined in a study in bipolar patients [39]. Two independent studies in anorexic patients indicate a relationship between serum S100B and body weight [40, 41]. However, an appropriate study about this issue was performed only more recently [42]. Steiner's group correlated mass body index (BMI), serum levels of S100B and two well-characterized adipose-derived proteins: leptin and adipocyte-type fatty acid-binding protein (A-FABP) in individuals without a prior history of neurological or psychiatric disorders. They observed that S100B levels were closely correlated with the body mass index, as well as levels of leptin and A-FABP.

In support of these findings, we investigated the levels of serum and CSF S100B in 48-h-fasting Wistar rats [33]. A significant (more than two-fold) increase in serum S100B levels was observed in these rats, without changes in cerebrospinal fluid S100B. These data are in agreement with *in vitro* hormonal changes induced in the adipose tissue, under stressing conditions, and suggest that S100B release from adipocytes might be linked to lipolysis, but definitive evidence is lacking.

5. Is There a Role for S100B in the Energy Metabolism of Adipocytes?

Adipocytes, like other cells, have an intense glycolytic metabolism (Figure 1). In the fed state, glucose intake goes to fatty acids and then to triacylglycerol (lipogenesis). It is important to mention that, in humans, fatty acid synthesis occurs mainly in hepatocytes. Then fatty acids, converted to triacylglycerols, are transferred and stored in adipose tissue. On the other hand, triacylglycerol stores are mobilized and brokendown to fatty acids and glycerol (lipolysis). In fact, a permanent cycle of lipogenesis and lipolysis occurs in adipose tissue, where about 70% of fatty acids released during lipolysis are reesterified. However, because adipocytes lack glycerol kinase, the glycolytic pathway provides glycerol-phosphate to lipogenesis; in addition and importantly, precursors of glucose, for example lactate (via pyruvate), are used as a source of glycerol-phosphate in a pathway denominated glyceroneogenesis (see [43] for a review).

Based on protein-binding assays, at least three putative targets of S100B have been implicated in energy metabolism: phosphoglucomutase [44], fructose-1,6-bisphosphate aldolase [45] and glyceraldeyde 3-phosphate dehydrogenase [23]. Apparently, S100B may inhibit phosphoglucomutase and stimulate aldolase. Together, these effects lead to an increase in the glycolytic pathway in adipocytes, which is coupled to lipogenesis and reesterification. However, the *in vitro* effects on the activity of these enzymes were preliminary characterized 20 years ago by Zimmer et al. and additional information is missing. Thus, until now a direct role of intracellular S100B on glucose metabolism in adipocytes (and astrocytes) remains a speculation.

Table 1: Evidence of modulated S100B release in adipocytes.

Modulatory Agent	Effect	Preparation	Reference
Catecholamines	↑ release	epididymal fat pads	[27]
Catecholamines	↓ intracellular content	*in vivo* (adipose tissue)	[28, 29]
Epinephrine, ACTH and cAMP	↑ release	epididymal fat pads isolated adipocytes	[30]
Insulin	↓ release	epididymal fat pads	[31]
Free fatty acids	↑ release	epididymal fat pads	[32]
Epinephrine	↑ release	isolated adipocytes	[33]
	↑ basal release	isolated adipocytes	[25]

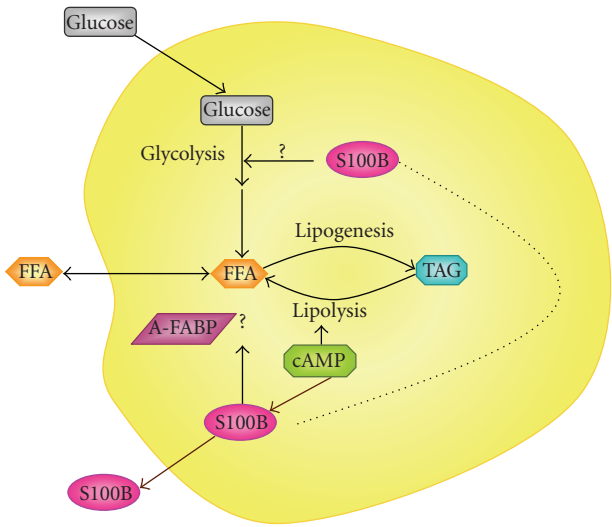

Figure 1: *Schematic representation of the putative roles of S100B and its secretion in adipocytes.* cAMP (e.g., induced by catecholamines—see Table 1) would trigger lipolysis and S100B secretion. The connection between these events remains to be established, as well as a role of S100B in the modulation of glycolysis (?) and in the transport of free fatty acids (?). A-FABP, adipocyte type—fatty acid binding protein; FFA, free fatty acids; TGA, triacylglycerol.

Moreover, the cAMP pathway, triggered by adrenaline and glucagon (and attenuated by insulin), which results in lipolysis (see Table 1), could, in parallel, be involved in S100B release (Figure 1). In fact, cAMP modulates S100B secretion in adipocytes [27, 31] and astrocytes [35, 46]. Twenty-five years ago, Suzuki and Kato suggested that S100B could serve as a carrier protein of fatty acids [31]. However, it is not clear whether S100B release is connected to the mechanism of lipolysis induced by cAMP. It is important to mention that some members of the S100 family, such as S100A7, A8 and A9, modulate fatty acid transport in keratinocytes and neutrophils due to the interaction with fatty acid-binding proteins (FABP) [47, 48]. Interestingly, Steiner et al. observed a strong correlation between serum circulating levels of S100B and adipocyte-type FABP (A-FABP) [42]. So far, however, no study has investigated a biochemical interaction between S100B and A-FABP.

6. What Is the Role of S100B Release by Adipocytes?

Regardless of whether S100B is released, or not, from adipocytes during lipolysis, it is also important to evaluate the extracellular role of this protein, that is, could have this protein an autocrine, a paracrine or an endocrine role? As yet, RAGE—the Receptor for Advanced Glycation End products (AGE)—is the only characterized receptor for S100B [49, 50] and, thus, we should discuss the possible extracellular effects of adipocyte-derived S100B based on RAGE activation. However, effects of S100B, independent of RAGE, cannot be rule out (e.g., [13]). Moreover, the effect of RAGE activation depends upon ligand, that is, RAGE activation by BSA-AGE or S100B does not necessarily induce the same response [51, 52]. The diversity of effects induced by RAGE activation are not only due to cell-specificity, but also depend on the oligomeric organization of the ligand (in the case of S100 proteins), as well as RAGE oligomerization [53, 54].

RAGE activation by AGE in cultures of adipocytes inhibited glucose uptake through the overgeneration of intracellular reactive oxygen species [55] and this could contribute to insulin resistance. Notice that *in vitro* S100B release by adipocytes is even higher than that of astrocytes [25]. Thus, beneficial or detrimental autocrine effects could be conceived. However, the direct effect of S100B on adipocytes has not been investigated until now.

Locally concentrated extracellular S100B in adipose tissue could exert an effect on neighbor cells. The innate immune response could be induced by S100B, recruiting monocytes [56] and activating macrophages [57], both in a manner that depends on RAGE. Moreover, also via RAGE, S100B is potentially able to increase endothelial adherence to leucocytes [58], to reduce vasodilatation induced by acetylcholine [59] and to increase neovascular proliferation [60]. Taken together, these effects could indicate a proinflammatory effect of RAGE activation by S100B, as has been proposed [61]. This is in agreement with the idea that adipocyte-derived proteins contribute to systemic inflammatory responses (e.g., [62]). However, a specific contribution of S100B to paracrine communication in adipose tissue demands further investigation, possibly providing a target for therapeutic intervention in obesity, diabetes and cardiovascular diseases.

With regard to any endocrine activity of the S100B released from adipocytes, two tissue targets could be proposed: the brain and heart, but weak biochemical bases of these connections can be established. S100B is not normally expressed in the myocardium, but it is induced in the peri-infarct region and potentially modulates myocyte apoptosis (see [63] for a review). Exogenous addition of S100B to cardiac myocyte cultures is able to cause apoptosis via RAGE, at a concentration of higher than 50 nM, a level that may be achieved in the peri-infarct local [64]. This concentration is 10,000 × higher than serum S100B. Thus, based on current evidence, it is quite difficult to conceive an endocrine effect of S100B, released from adipocytes, on cardiomyocytes. Furthermore, it is difficult to hypothesize an effect of S100B from adipocytes on neurons of the CNS, as the basal levels of extracellular S100B (from astrocytes and possibly from other neural cells) surpass serum levels. Therefore, the effect of S100B in the heart and CNS depends on local expression and secretion of this protein.

In conclusion, S100B released by adipocytes could work as an adipokine by modulating local microcirculation and immune response. In fact, due to local activation of the immune system, some S100 proteins (including S100B) have been considered a damage-associated molecular pattern (DAMP) or alarmin [65].

7. Contribution of Adipose Tissue to the Serum S100B Content

Generally, serum S100B is interpreted as a reflex of brain damage or astroglial activation. This is based on some properties: (1) brain tissue contains an elevated content of this protein, particularly in the astrocytes in gray matter; (2) astrocytes secrete S100B and, in fact, high levels can be measured in cerebrospinal fluid; (3) S100B is a small and very soluble protein. However, some points should be taken into consideration. Oligodendrocytes, choroid plexus epithelium and ependymal cells contains S100B [12] and potentially contribute to S100B cerebrospinal fluid (CSF) content. The size and solubility "per se" does not assure a free traffic from astrocytes or CSF to blood. In fact, there is some evidence that elevations of S100B in cerebrospinal fluid are not necessarily accompanied by elevation in serum S100B. The S100B traffic likely demands specific transporters, and it is possible that some brain diseases allow a higher S100B efflux.

Many extracerebral sources of S100B may contribute to the serum content of this protein. Here, we have emphasized adipocytes, but other sources include chondrocytes and cells of the marrow bone (in case of traumatism) [66, 67] and melanoma cells [68]. In these cases, S100B release appears to involve cell lysis, rather than actual S100B secretion. Other S100B-containing cells such as lymphocytes and cardiomyocytes are unlikely to contribute to serum S100B content.

Adipose tissue alterations, particularly insulin resistance, appear to be involved (whether preceding or associated) in many diseases, including type 2 diabetes, cardiovascular diseases and dementia [69, 70]. In addition to insulin resistance, adipose tissue alterations are also observed in bipolar affective disorders and schizophrenic patients [71], which are accompanied by elevations in serum S100B [39, 72]. Interestingly, insulin resistance in schizophrenic patients may be closely linked to serum S100B changes [73]. In support of this observation, rats fed on a ketogenic diet, which exhibits signals of insulin resistance [60], also demonstrate elevated levels of serum S100B (D Ziegler, unpublished observation).

Another important aspect to be considered is the ontogeny of the S100B protein. Serum S100B levels are negatively correlated with age [74]. This profile could be explained by the changes in S100B content in brain tissue (increase) and cerebrospinal fluid (decrease) observed during the postnatal development of rats [75]. Conversely, white adipose tissues increase with age [7]. This developmental characteristic apparently contradicts arguments regarding the contribution of adipocytes to serum S100B content. However, developmental evaluation of S100B content and secretion in white adipose tissue has, so far, not been investigated. Moreover, the contribution of brown adipose tissue, which contains S100B [76] and a decrease in an age-dependent manner [77], also remains to be characterized.

In conclusion, adipocytes contain high levels of S100B and *in vitro* assays indicate a modulated secretion of this protein by hormones that regulate lipolysis, such as glucagon, adrenaline and insulin. Although the biological significance of extracellular S100B from adipose tissue is still unclear, it is likely that this tissue might be an important source of serum S100B in situations related, or not, to brain damage. Current knowledge does not preclude the use of this protein in serum as a marker of brain injury or astroglial activation, but caution is recommended when discussing the significance of changes in its serum levels, where S100B could function as an adipokine, a neurotrophic cytokine or an alarmin.

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Review Article

Mood Disorders Are Glial Disorders: Evidence from *In Vivo* Studies

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It has recently been suggested that mood disorders can be characterized by glial pathology as indicated by histopathological *postmortem* findings. Here, we review studies investigating the glial marker S100B in serum of patients with mood disorders. This protein might act as a growth and differentiation factor. It is located in, and may actively be released by, astro- and oligodendrocytes. Studies consistently show that S100B is elevated in mood disorders; more strongly in major depressive than bipolar disorder. Successful antidepressive treatment reduces S100B in major depression whereas there is no evidence of treatment effects in mania. In contrast to the glial marker S100B, the neuronal marker protein neuron-specific enolase is unaltered. By indicating glial alterations without neuronal changes, serum S100B studies confirm specific glial pathology in mood disorders *in vivo*. S100B can be regarded as a potential diagnostic biomarker for mood disorders and as a biomarker for successful antidepressive treatment.

1. The Glial Hypothesis of Mood Disorders

Mood disorders—once considered "good prognosis diseases"—have, in fact, a less favorable outcome than previously thought [1, 2]. They are often very severe or even life-threatening illnesses. It has been suggested that impairment of neuroplasticity and cellular resilience may underlie their pathophysiology, and that optimum long-term treatment may only be achieved by the early use of agents with neurotrophic or neuroprotective effects. It has further been proposed that mood disorders are characterized by specific glial pathology [3]. Histopathological *post mortem* findings [1, 4–6] consistently showed reductions in glial cell density or glial cell numbers in prefrontal brain regions, such as the (subgenual) anterior cingulate cortex, the orbitofrontal cortex, and dorsolateral prefrontal cortex in association with reduced prefrontal gray matter in patients with mood disorders [3, 7–9]. Furthermore, alterations were described histopathologically for astrocytes [10–13] and oligodendrocytes [14–16] in these disorders. Specific reductions in oligodendrocytes have also been reported for the amygdala in major depressive disorder (MDD) [14], and microglial alterations in bipolar disorder (BD), also including manic episodes [1].

Rajkowska's hypothesis [3] of glial pathology in mood disorders has been supported by a recent study that specifically ablated astroglial cells in the prefrontal cortex of adult rats pharmacologically with L-alpha-aminoadipic acid (L-AAA) [17]. Indeed, rats treated with L-AAA showed depressive-like behavior in behavioral tests similar

to depression models based on chronic unpredictable stress. Conversely, the neurotoxic ibotenate did not show any effect. Remarkably, antidepressive treatment has been shown to successfully reverse reduction in astroglial density in animal models of depression [18]. Although density and size of cortical neurons are reduced in the orbitofrontal and dorsolateral prefrontal cortices in mood disorders, these neuronal reductions seem less pronounced than glial alterations and are detected only when specific morphological size-types of neurons are analyzed in individual cortical layers [1, 6].

2. The Glial Marker Protein S100B in Mood Disorders

Previous studies have shown that S100B, which is found in astro- and oligodendroglia, but not in microglia in the human brain [19], is altered in both serum [20, 21] and cerebrospinal fluid in mood disorders. Cerebrospinal fluid changes have been shown for drug-free depressive patients compared with euthymic patients [22] and in animal models of mania [23]. Interestingly, the levels of the glial marker protein S100B are specifically altered in the lateral prefrontal and parietal cortices in BD [24]. Roche et al. [25] demonstrated that S100B is a susceptibility gene for BD with psychosis. Although Yang et al. [26, 27] did not find an association between S100B gene polymorphisms and MDD in a Chinese population, they revealed an influence on age of onset and subgroups (first-episode versus recurrent episode depression) of MDD.

S100 proteins are a family of acidic proteins that can bind calcium and, thus, influence various cellular responses along the calcium-signal-transduction pathway [31–34]. S100B regulates cell shape, energy metabolism, contraction, cell-to-cell communication, intracellular signal transduction, cell growth [35], and can be actively released by astro- and oligodendrocytes [19, 36]. Interestingly, the effects of extracellular S100B depend on its concentration [33, 37]. In a nanomolar concentration S100B can act as growth and/or differentiation factor for neurons and astrocytes, whereas in a micromolar concentration it may induce apoptosis. Moreover, it has been suggested that S100 proteins, such as S100B, may play a crucial role in the pathogenesis of depression and its treatment [38–44].

To better evaluate the relevance of S100B in mood disorders, we recently conducted a systematic, quantitative meta-analysis using MedLine and Current Contents search engines (search strategy: [S100 OR S-100] AND [depression OR mania]) [20, 21]. The following inclusion criteria were applied: diagnosis according to internationally recognized diagnostic criteria (International Classification of Diseases, ICD-10; Diagnostic and Statistical Manual of Mental Disorders, DSM-IV [45, 46]), original and peer-reviewed studies, comparison with age-matched, healthy control subjects and no overlap with cohorts of other studies. Eight studies involving 193 patients suffering from mood disorders and 132 healthy control subjects were entered into the meta-analysis. Of the patients, 86 suffered from a major depressive episode, 63 from a manic episode, and 44 were

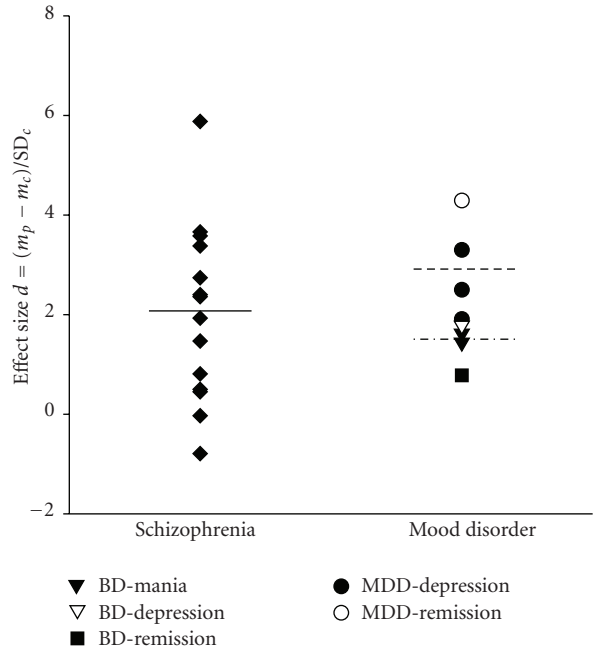

Figure 1: Effect sizes according to Cohen [28] of S100B serum concentration in schizophrenia, and mood disorders as identified by a systematic meta-analysis [21]. Median is shown for schizophrenia (solid line), major depressive disorder (MDD, dashed line) and bipolar disorder (BD, dashed & dotted line).

euthymic at the time of investigation. To adjust for systematic measurement effects, the effect size of each study (d) was calculated according to Cohen [28] as the difference of the means of the patient (m_p) and control group (m_c) divided by the standard deviation of the control group (SD_c). This measure represents normalized elevations of S100B in the patient groups. Effect sizes of the studies are shown in Figure 1. Cohen [28] defined values of ≥ 0.8 as large, > 0.5 as medium and > 0.2 as small. The mean effect size reached high values for all episodes of mood disorders [20], namely major depressive episode of MDD (2.57 ± 0.70), manic episode of BD (1.53 ± 0.13) and currently euthymic mood disorder (2.54 ± 2.48; mean $\pm$ SD). For major depressive and manic episodes, values were significantly higher than zero, confirming high serum S100B in acute episodes of mood disorder ($T = 6.4, = 17, df = 2, = 1, P = .024, = .037$; 2-tailed Student's t-test against 0), which was not the case for currently euthymic mood disorder ($T = 1.4, df = 1$, $P > .05$).

We set out to compare serum S100B in BD and MDD, because these types of mood disorder are classified as separate nosological entities and because we did not find significant differences between depressive/manic episodes and remitted mood disorder per se ($P > .05$; 2-tailed unpaired Student's t-test) [21]. As illustrated in Figure 1, serum S100B reached high effect sizes in both MDD (3.0 ± 1.03) and BD (1.4 ± 0.44; $T = 5.82, = 6.4, df = 3, = 3, P = .01, = .008$; 2-tailed one-sample Student's t-test against 0). Effect size was larger in MDD than BD ($T = 2.84, df = 6$, $P = .029$; 2-tailed unpaired Student's t-test). For mania in

BD and depression in MDD, only two studies with drug-free patients were available, each reporting high effect sizes (1.62, 3.3). Since the meta-analysis was conducted, a third study has been published with drug-free patients suffering from MDD (effect size 0,96 [47]).

Protein S100B has been detected in numerous other tissues in the human body besides glial cells, for example, in adipocytes, melanocytes, chondrocytes, myocardium, and Schwann cells [33, 35, 48]. Although changes elicited by adipocytes are at least theoretically possible in mood disorders [49, 50], no study has yet reported changes in S100B due to the aforementioned extracranial cell types.

Additionally, we compared results of the meta-analysis for mood disorders with another recent meta-analysis investigating serum S100B with the same method in 420 patients with schizophrenia [21, 51]. Although effects sizes also reached large values in schizophrenia (2.02 ± 1.78; $T = 4.25, df = 13, P = .001$; 2-tailed one-sample Student's t-test against 0), there were no significant differences in comparison with MDD or BD ($T = -1.03, = 0.68, df = 16, = 16, P = .317, = .509$; 2-tailed unpaired Student's t-test). In sum, results support the hypothesis that S100B is involved in the pathogenesis of mood disorders, particularly MDD.

3. Specificity of Elevations of Serum S100B in Mood Disorders

To validate the histopathologically generated hypothesis that mood disorders are characterized by specific glial pathology [3] *in vivo*, we recently measured S100B simultaneously with neuron-specific enolase (NSE) in the serum of patients with MDD and healthy age- and gender-matched control subjects [20]. NSE is located mainly in the cytoplasm of nerve cells and is not actively secreted [52, 53]. Hence, it has been regarded as a marker for neuronal injury or brain damage. If mood disorders are ultimately glial disorders as suggested by Rajkowska [3], one would expect elevated serum levels of S100B paralleled by unaltered neuronal marker protein NSE.

Figure 2 illustrates serum concentration of S100B and NSE in 10 control subjects, and in 10 patients with MDD at admission and discharge. As hypothesized, S100B concentrations were higher in depressive patients at admission and discharge compared to control subjects. NSE was not statistically different between patients (at admission or discharge) and control subjects. Moreover, antidepressive treatment had no significant effect on NSE serum levels. Three other studies have reported findings on serum NSE in major depression, but the choice of the according study samples was characterized by considerable limitations. Greffe et al. [54] investigated serum NSE in 6 subjects with refractory major depression in comparison to 274 psychiatric control patients that were not characterized in more detail. Similar to our study, they found no difference between groups. Another study [55] investigated treatment effects of clinically successful electroconvulsive therapy on serum NSE in 7 patients suffering from MDD. They did not find any significant changes during treatment and concluded that values were in the normal range across all measurements without showing control data. A comparable result was reported by Agelink et al. [56] although they did not distinguish between their patients with therapy-resistant major depression and subjects with schizodepressive psychosis. These data suggest that in MDD S100B is elevated while NSE remains unaltered, providing substantial support for Rajkowska's glial hypothesis for mood disorders [3]. However, only one study [57] has investigated NSE in mania so far, showing decreased values in 30 unmedicated and 15 patients undergoing lithium treatment in comparison with 30 healthy control subjects. These results make it difficult to generalize specific glial pathology in MDD to all mood disorders and have not yet been replicated.

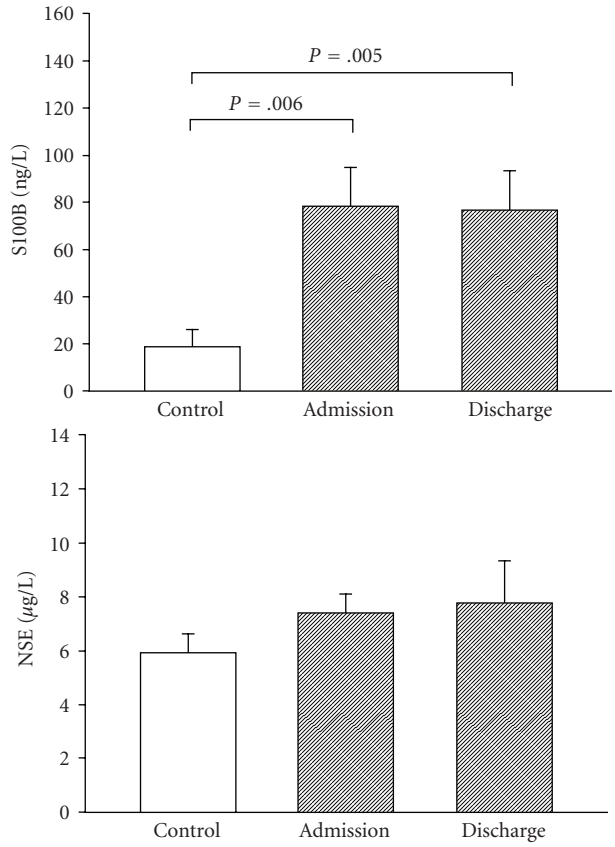

Figure 2: Serum concentrations of S100B and neuron-specific enolase (NSE) in patients with major depression immediately after admission and at discharge, compared with healthy age- and gender-matched control subjects [20]. P-values are reported for 2-tailed unpaired Student's t-test. Mean ± SEM.

Increased serum levels of S100B may indicate glial alterations in mood disorders either due to brain damage [58] or due to functional secretion of S100B by astrocytes and/or oligodendrocytes [19, 36]. Mathematical models suggest that levels of serum S100B exceeding approximately 350 ng/l indicate brain damage [59]. Mean serum levels of S100B as reported in our and other studies of mood disorders do not reach this threshold [20]. Likewise, our data together with earlier studies exclude possible neuronal

damage in MDD and mania as indicated by normal or even decreased serum NSE values [54, 55, 57]. One might therefore conclude that brain damage is not the primary cause of elevated S100B in mood disorders. Some authors regard serum S100B as a valid marker of blood-brain barrier integrity [59–63] and astrocytes might influence blood-brain barrier function [64–66]. Others argue that S100B might penetrate the blood-brain barrier easily, but this has not yet been proven experimentally [67, 68]. Accordingly, it remains to be clarified whether elevated serum S100B could indicate an impairment in the blood-brain barrier, as has been described for depression [69, 70].

4. Treatment Effects on Serum S100B

It has recently been suggested that a loss of neuroplasticity and cellular resilience may underlie the pathophysiology of mood disorders and that optimum long-term treatment can only be achieved by early neurotrophic and/or neuroprotective intervention [1, 2]. It is well-established that extracellular S100B can act as growth and/or differentiation factor for neurons and astrocytes via various intracellular signal cascades [1, 33, 71–73]. Antidepressive drugs influence the secretion of S100B by astrocytes via the serotonergic system [11, 35, 43, 44, 74–76]. S100B may even induce neurogenesis [77], which is required for the behavioral effects of antidepressants [78]. It has also been suggested that S100 proteins may play an essential role in the pathogenesis of depression and its treatment [40, 42], and that S100B-related mechanisms could be explored as potential targets for novel antidepressive therapeutics [38, 39]. Interestingly, levels of serum S100B might predict the response to antidepressive treatment in MDD [79].

To validate the impact of S100B as a marker for pharmacological treatment effects, we subsequently conducted a third systematic, quantitative meta-analysis (see above for search strategy and inclusion criteria) [20]. This meta-analysis identified three studies involving 46 patients with major depression and one study including 11 patients with mania. A fifth study examining changes in serum S100B immediately (1 and 3 hours) after electroconvulsive treatment did not include a control group [80] and because injury mechanisms following electroconvulsive treatment could represent a potential confounding factor, we did not include this study in the meta-analysis. A sixth study [56] did not differentiate between therapy-resistant major depression and schizodepressive psychosis when reporting their findings during electroconvulsive therapy, and was, accordingly, also excluded. The treatment effect size (d) for S100B and the severity of clinical symptoms was calculated for each study according to Cohen [28] as the difference of the means of the patient group at admission (m_{ad}) and discharge (m_{dis}) divided by the standard deviation at admission (SD_{ad}). Such treatment effect size reveals a measure for relative changes from baseline.

The mean treatment effect size derived from the three available studies could be calculated for serum S100B in major depression. As expected, it reached a large value for changes on the HAMD scale (3.47 ± 1.80), with a lower impact on serum S100B (0.43 ± 0.44; $T = 3.3, = 1.7, df = 2, P = .04, = .12$; 1-tailed Student's t-test against 0). As illustrated in Figure 3, effect sizes for clinical improvement during treatment (Hamilton Depression Rating Scale, HAMD) and respective changes of the serological marker S100B were significantly correlated with each other if the relationship for the three relevant studies involving major depression was examined ($r = 1.0, N = 3, P < .001$; correlation according to Spearman, 2-tailed p). This significant positive correlation between clinical treatment effects (HAMD) and serological treatment effects (S100B) indicates that serum S100B may be a reliable marker for treatment effects in major depression if clinical improvement is sufficient. For mania only one study examined changes of S100B during treatment, but without detecting any significant effects [29].

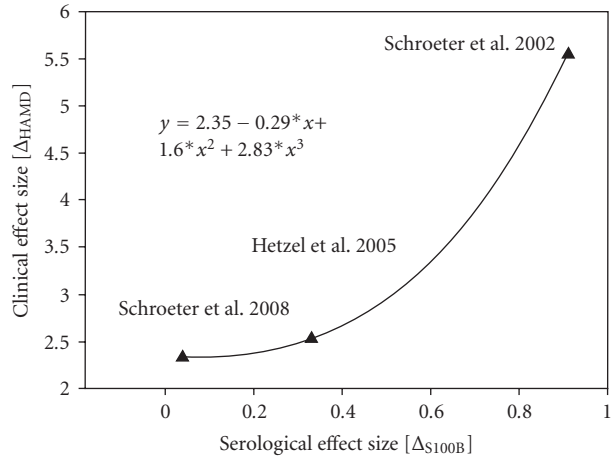

Figure 3: Effect sizes according to Cohen [28] for clinical (HAMD scores) and serological (serum S100B) treatment effects in major depression [20]. Effect sizes were calculated as changes between admission and discharge relative to standard deviation at admission for the three available studies [20, 29, 30]. Severity of depression was measured with the Hamilton Depression Rating Scale (HAMD).

However, one has to keep in mind the limitations of the meta-analysis for these treatment studies. All of the clinical studies on serum S100B in mood disorders involved several antidepressive/antimanic drugs or combinations with other psychotropic agents such as neuroleptics when psychotic symptoms were present [20]. Likewise, treatment studies to date have experienced significant limitations of sample size. Hence, future well-powered clinical studies are necessary to overcome this limitation. Furthermore, *in vitro* (cell culture) studies examining effects of different antidepressive treatment strategies on S100B with regard to the specific signaling pathways of the neurotransmitter system mainly targeted by the antidepressant would be of high interest.

5. Evidence from Studies with Serum Markers for the Glial Hypothesis of Mood Disorders

To summarize findings from the literature supporting the hypothesis of glial pathology in mood disorders [3], we list

the following key findings:

(i) Serum concentrations of the glial marker protein S100B are elevated in patients with mood disorder, major depression and mania, when compared with healthy control subjects.

(ii) Serum S100B is higher in major depressive disorder than bipolar disorder.

(iii) Successful antidepressive treatment reduces S100B in major depression. While only one study investigated treatment effects in mania, such an effect could not be found.

(iv) The neuronal marker protein NSE is unaltered in major depression and its treatment. NSE is not increased in mania; the only study in the literature reported mildly reduced serum levels.

(v) Data support the hypothesis that elevated serum S100B is related to active secretion by astrocytes and/or oligodendrocytes in acute episodes of mood disorders, particularly major depressive disorder, and that this secretion might decline with successful antidepressive treatment.

In conclusion, these findings strongly support the concept of serum S100B as a reliable and sensitive diagnostic biomarker for mood disorders and the clinical response to antidepressive treatment in unipolar major depressive disorder. Evidence of glial changes without neuronal alterations from *in vivo* studies is consistent with the histopathologically generated hypothesis that mood disorders are characterized by specific glial pathology.

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Review Article

Effects of S100B on Serotonergic Plasticity and Neuroinflammation in the Hippocampus in Down Syndrome and Alzheimer's Disease: Studies in an S100B Overexpressing Mouse Model

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S100B promotes development and maturation in the mammalian brain. However, prolonged or extensive exposure can lead to neurodegeneration. Two important functions of S100B in this regard, are its role in the development and plasticity of the serotonergic neurotransmitter system, and its role in the cascade of glial changes associated with neuroinflammation. Both of these processes are therefore accelerated towards degeneration in disease processes wherein S100B is increased, notably, Alzheimer's disease (AD) and Down syndrome (DS). In order to study the role of S100B in this context, we have examined S100B overexpressing transgenic mice. Similar to AD and DS, the transgenic animals show a profound change in serotonin innervation. By 28 weeks of age, there is a significant loss of terminals in the hippocampus. Similarly, the transgenic animals show neuroinflammatory changes analogous with AD and DS. These include decreased numbers of mature, stable astroglial cells, increased numbers of activated microglial cells and increased microglial expression of the cell surface receptor RAGE. Eventually, the S100B transgenic animals show neurodegeneration and the appearance of hyperphosphorylated tau structures, as seen in late stage DS and AD. The role of S100B in these conditions is discussed.

1. Introduction

S100B is a member of the EF-hand type of calcium binding S100 protein family which consists of approximately 20 different proteins. S100B is the only member found on chromosome 21, the remaining largely being found in a cluster on chromosome 1. S100B is the principle S100 found in brain, and makes up approximately 0.5% of all brain proteins. Under normal physiological states, S100B is expressed predominantly in astroglial cells of the central nervous system (CNS), and also to a lesser extent, in neurons, microglia, and oligodendrocytes [1–7]. However, in neuropathological conditions, including those induced by environmental stressors, infection, ischemia, trauma, psychiatric conditions such as depression [8–10], and schizophrenia [11, 12], the cellular and tissue distribution of S100B within the brain may change. As a brain-derived protein measurable in peripheral samples, S100B is often used as a biochemical marker for brain injury. In the periphery, S100B is expressed by Schwann cells, ependymocytes, adipocytes, chondrocytes, melanocytes, dendritic cells, skeletal muscles, and myocardium [13]. The fact that S100B is increased in a wide variety of pathological conditions is indicative of the diverse functions that this protein plays throughout the body and brain [14, 15].

Donato has suggested that S100B is important for the progression of cells through the cell cycle [16, 17]. We have used the term, "accelerated aging," to describe cell cycle changes in the CNS. Regardless of the term employed, the message is the same: S100B acts in the brain to promote development and aid in recovery, but also as an inflammatory protein with a role in aging and neuropathology.

Serum levels of S100B in humans are age-dependent [18] being highest in newborn, stable throughout adulthood and increasing again in aging. A similar profile is seen in rodents [19]. It has been suggested that the increased S100B in aging is related to a lifetime of proinflammatory events, including ischemia, trauma, and infections. The effects of S100B in brain are not only age-dependent, they are also concentration-dependent, such that it is protective and trophic at low concentrations [20], but toxic and proapoptotic at high concentrations [21, 22].

The neurobiological effects of S100B are known to occur intracellularly, in the cells which express the proteins, as well as extracellularly, as the protein can be released, notably in response to serotonin (5-HT) binding to the 5-HT1A receptor [16, 17, 23]. In addition to 5-HT, other factors known to stimulate S100B release include IL-6, adenosine, glutamate and cannabinoid receptors [24–26]. There is also a substantial amount of passive release into the neuropil.

Some of the detrimental extracellular effects of S100B may be mediated via the cell-surface receptor for advanced glycation end-products (RAGE). In the central nervous system, RAGE can be localized to neurons, microglial cells, and astrocytes. The RAGE receptor is a member of the immunoglobulin superfamily and leads to cellular dysfunction in a number of disorders. This receptor was originally identified and characterized for its binding of advanced glycation end-products (AGEs) which accumulate in diseases such as diabetes and renal failure [27]. More recently, RAGE was shown to be a multiligand, cell-surface receptor, responding to a number of ligands, including S100B. It is interesting to note that increased S100B results in increased RAGE [28]. Consistent with this notion is the fact that during development, or following an insult, both S100B and RAGE increase whereas in normal adult tissue, relatively low RAGE expression is found.

Elevated brain S100B expression occurs in various disease states, including Alzheimer's disease (AD) and Down syndrome (DS). The gene for S100B is found on chromosome 21 and is often part of the triplicated chromosome in DS. Interestingly, Down Syndrome almost inevitably leads to an extremely early development of AD and the increase in S100B is thought to contribute to the pathology of both. Although S100B has a variety of cellular effects throughout the body and brain, its role in neuroinflammation, and in the regulation and maintenance of the serotonergic nervous system is highlighted, with a particular focus on the hippocampus. Understanding neuroinflammation and serotonin neuronal plasticity in this brain region may help to explain many findings of changes in learning and memory, as well as the occurrence of depression symptoms. This is especially pertinent when considering co-morbidity in conditions wherein the levels of S100B are altered, such as AD and DS.

1.1. Alzheimer's Disease. Numerous human postmortem studies have shown increased S100B in the brain of subjects with AD [29–31]. This elevated S100B correlates with many of the neuropathological changes, including the presence of beta-amyloid plaques and neurofibrillary tangles. Elevated S100B has also been found in the cerebrospinal fluid of AD patients [32] and correlates with the degree of brain atrophy [33]. In a transgenic mouse model of amyloid pathology, increasing S100B has been shown to increase plaque formation [34]. Little work has been done on the role which S100B overexpression might directly play in the formation of neurofibrillary tangles. However, one recent study showed that S100B acts through the RAGE receptor to result in hyperphosphorylated tau, a contributor to neurofibrillary tangles [35]. In the current review, data have been included showing hyperphosphorylated tau in S100B overexpressing mice. Thus, chronic overexpression of S100B may lead to both plaque and tangle formation.

1.2. Down Syndrome. Serum levels of S100B are greatly increased in Down syndrome [36] and postmortem brain studies show lifelong overexpression of the protein [37, 38]. As in AD, many of the neuropathological changes in DS are thought to be related to the overexpression of S100B [39, 40]. Previous studies have shown behavioral, neuropathological and cardiovascular alterations in a transgenic mouse overexpressing human S100B protein which we use in our studies [40–46]. These mice were designed to model the elevated S100B that is often a part of the Down syndrome genotype [47]. Similar to DS patients who almost inevitably show premature signs of aging and AD, the S100B mice show signs of accelerated aging [48], neuropathology [44, 46, 49], and behavioral deficits [42, 43]. Thus, the S100B transgenic mouse is well-suited to study the influence of chronic S100B over-expression on brain and behavior in the context of DS, including the accelerated development of AD neuropathology.

2. S100B and Serotonin Neuroplasticity

The neurons which produce serotonin are amongst the earliest developing neurons in the mammalian brain and serotonin plays a role in the development and maturation of many brain regions [50]. Serotonin also becomes the most widely-distributed system throughout the cortex, touching on virtually every neuron in cortex [51]. This widespread distribution and early development allows for a role for serotonin in maintaining and promoting synaptic plasticity. Much of this effect of serotonin is mediated through the release of S100B. During development, S100B promotes process outgrowth from cortical cells [52, 53] as well as promoting dendritic development in the hippocampus [54]. A reciprocal relationship occurs, whereby serotonin (through 5-HT1A receptors) not only releases S100B [55, 56], but S100B also promotes development of serotonin terminals. Treatment with drugs and agents which increase serotonin, such as MDMA [57], or selective serotonin reuptake inhibitors [19, 58], increase the expression of S100B. Thus, S100B levels are important in regulating the terminal outgrowth and maintenance of serotonin terminals.

Interestingly, by twenty years of age, PET studies in normal populations have shown that the serotonin transporter

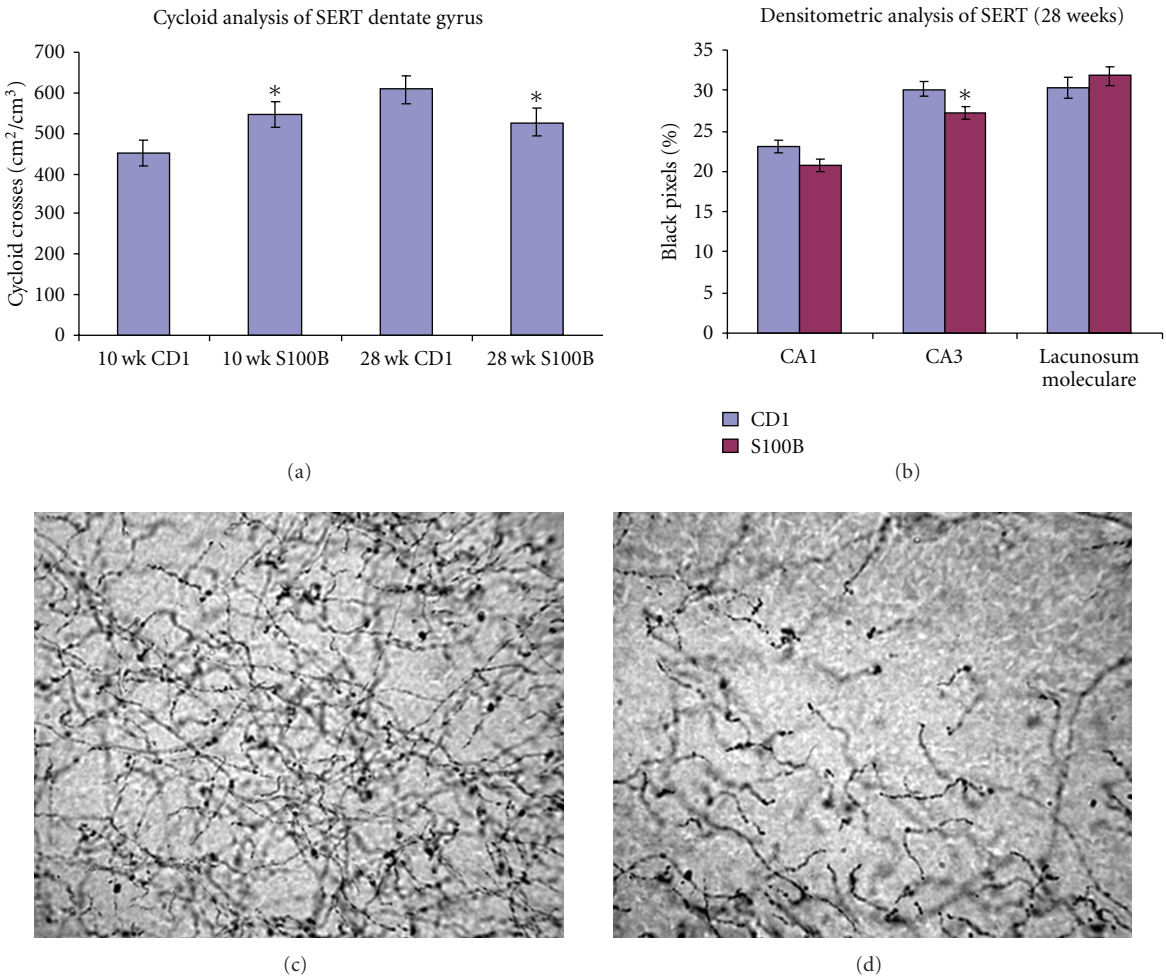


FIGURE 1: Serotonergic fiber analysis in the hippocampus of normal and S100B transgenic mice. In (a), a graph of the mean cycloid crosses (cm^2/cm^3) is shown to indicate the results from the stereological analysis showing a significant increase ($P < .05$) of SERT fibers in the dentate gyrus of 10-week-old S100B transgenic mice. Alternatively, at 28 weeks, significantly less ($P < .05$) SERT fibers were observed in the dentate gyrus of S100B transgenic mice. In (b), the results from the densitometric analysis are shown in areas CA1, CA3, and Lacunosum Moleculare of the hippocampus from 28-week-old mice. Note that there is a significant decrease ($^*P = .027$) in the density of serotonergic fibers in the S100B transgenic mice. It is pertinent to note that this densitometric method was verified in the infrapyramidal blade, where the results were consistent with the stereological analysis. There were no significant differences at the 10 week timepoint (data not shown) in CA1, CA3, or Lacunosum Moleculare. In (c) and (d), representative photomicrographs from the infrapyramidal blade of 28-week-old control (c) and S100B transgenic (d) mice. Note the apparent decreased density of serotonergic fibers in the S100B transgenic mice (d).

begins to decrease and continues to do so at the approximate rate of 10% per decade up until the 8th decade [59]. A further loss with aging may increase if S100B increases. For example, in neurodegenerative disease, such as Parkinson's disease and frontal lobe dementia, the forebrain serotonin fibers are decreased and become dystrophic, with enlarged and bulbous endings [60].

In midgestation DS fetuses, there is a significant (40%) loss of serotonin content in frontal cortex [61], although there is no loss of terminal development [62]. As subjects age, pronounced region-specific changes in serotonin terminal areas are seen. In the adult, loss of serotonin content is seen in caudate and temporal cortex, but increases are found in occipital cortex [63]. Studies of the serotonin terminal density, show that adult DS have increased terminals in frontal cortex [64]. Unfortunately, there are no reported studies on serotonin content or terminal density in hippocampus of DS, of which we are aware.

The increased levels of S100B in Alzheimer's disease may also be associated with a loss of serotonin. Neurofibrillary tangles have been shown to occur in the raphe nuclei, the site of serotonergic cell bodies [65, 66], and there is a loss of serotonin terminals in hippocampus and several other subcortical and cortical structures [67–71].

We have examined the development and maintenance of serotonin terminals in the S100B overexpressing transgenic mouse using an antibody raised against the serotonin transporter (SERT) which stains serotonergic fibers. Our results show increased serotonergic fibers in the dentate gyrus of the hippocampus of 10 week old S100B transgenic mice. Conversely, at 28 weeks, there is an accelerated loss of SERT-stained serotonin fibers as these animals age (Figure 1).

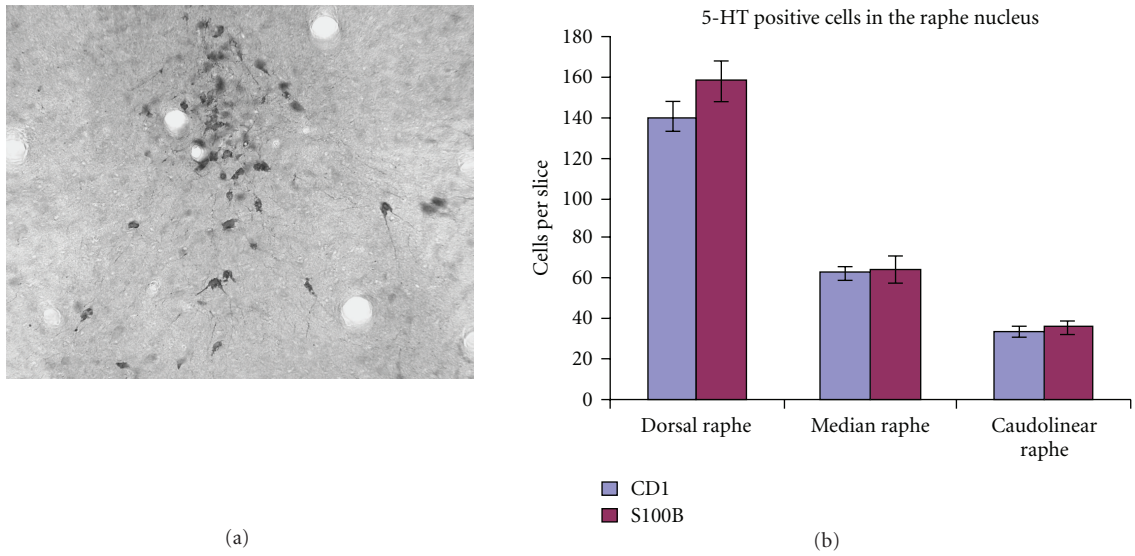


Figure 2: Serotonin cells in the raphe nucleus of 7-month-old control and S100B transgenic mice. In (a), a representative photomicrograph is shown to illustrate the 5-HT immunohistochemical labeling of serotonin cells in the raphe nuclei. In (b), a graph of the means showing no significant differences in the number of serotonin neurons between CD-1 control and S100B transgenic mice in the dorsal, median, and caudolinear segments of the raphe nucleus. It is pertinent to note that when all three regions were combined, the data were still not significantly different (data not shown).

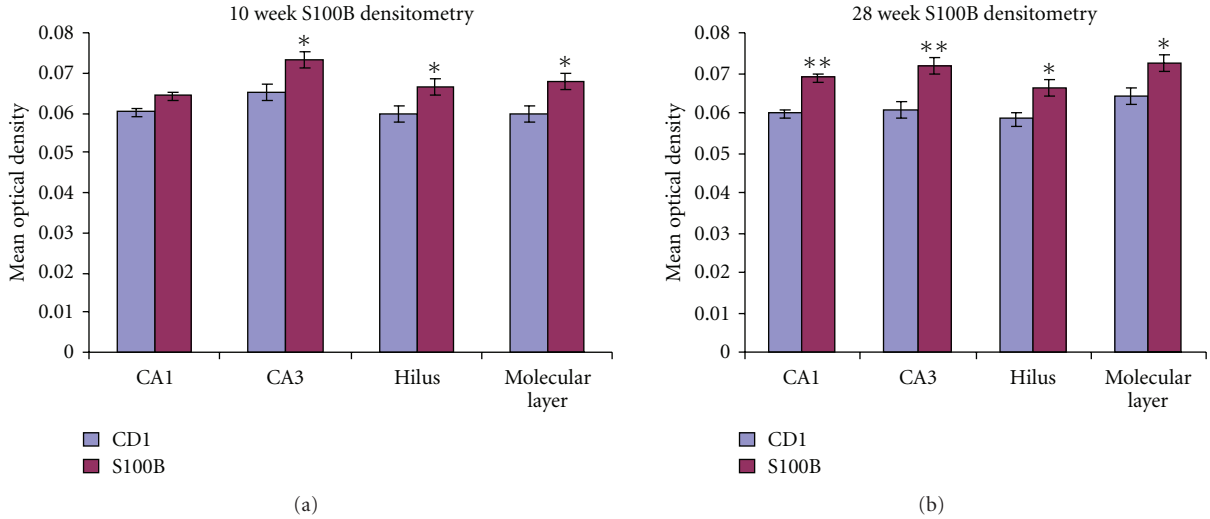


Figure 3: Immunodensitometry of S100B at 10-and 28-weeks- in CD1 control and S100B transgenic mice. In (a), the S100B mice shown significantly ($*P < .05$) elevated S100B in CA3 stratum radiatum, the hilus, and molecular layers of the dentate gyrus. There is also a trend towards significance in CA1 stratum radiatum. In (b), all of these regions are significantly elevated ($*P < .05$; $**P < .01$) at the 28 week time point.

Despite the decrease in serotonergic fibers, stereological analysis found no evidence for a decreased number of serotonin neurons in the raphe nucleus, as in the human postmortem studies of AD (Figure 2). A recent autoradiographic study found increased serotonin terminal density in the substantia nigra, but not the caudate of S100B overexpressing animals [72]. Therefore, changes to serotonin terminals in S100B overexpressing mice appear to depend on the age of the animals and on the structures examined, very much as has been observed in human DS tissue. In the two regions studied here, the findings suggest a loss of serotonin neuroplasticity in the memory center, the hippocampus, but no changes in the motor region of the caudate, which would correlate with the human cases.

These findings show that changes in S100B could lead to the changes in serotonin observed in DS and AD. Moreover,

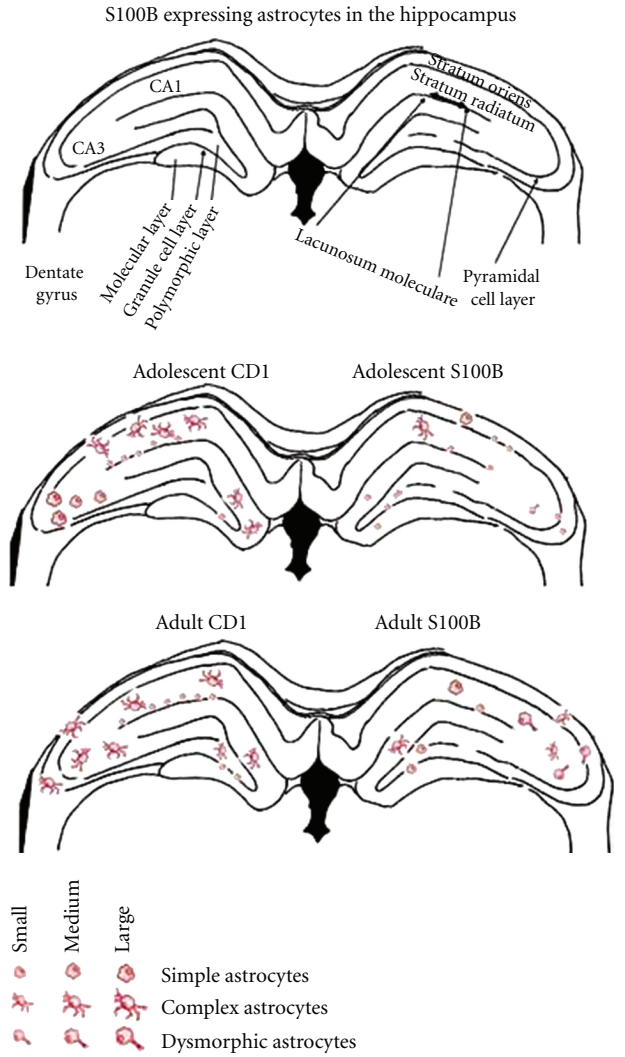


Figure 4: Schematic diagram summarizing the distribution analysis of S100B-labeled astrocytes in the hippocampus of 10 and 28-week-old CD-1 control and S100B transgenic mice. *10 weeks*: CD 1 animals showed a high density of large complex cells in CA1 SR, and a large number of small, simple cells in CA1 LM. Although similar cell types occurred in these regions of the S100B mice, their numbers were markedly reduced. In CA3, CD1 mice had a large number of medium to large simple cells, while the S100B mice again showed far fewer cells, which were smaller simple cells. In addition, there were a number of small atypical cells. Again, in the dentate gyrus, the adolescent CD-1 animals showed predominantly medium-sized complex cells, while the S100B animals showed small, simple cells. Occasionally, the S100 animals had small atypical cells within the polymorphic region of DG, which were virtually absent from the CD-1 mice. The cells of the S100B animals showed pronounced "haloes" of tissue S100B-IR around all cell types of all regions, suggesting large amounts of S-100B release. A representative photomicrograph of these findings is given in Figure 5. *28 weeks*: As the CD-1 animals matured, the number of complex cells in CA1 SR decline, but there continue to be a large number of small simple cells in LM. In CA3, the cells have become more complex. The dentate gyrus shows fewer complex cells, more simple cells, and now the occasional complex cell is seen within the polymorphic region. In the S100B animals, there continues to be an overall decrease in S100B-IR cells throughout the hippocampus, compared to adult CD 1 mice. The atypical cells first seen in adolescence are now larger and more numerous, particularly in CA3. These cells are virtually absent from the CD-1 mice at any age. CA1 cells are simple of mixed size, with an absence of complex cells. There are a small number of complex cells in CA3, more than in adolescent S100B animals, but smaller and fewer than in CD1 adults. Cells of the DG are predominantly medium sized simple and occasionally medium-sized complex. The atypical cells are not seen here. Again, large haloes (Figure 5) of S100B-IR are seen around the cells.

since serotonin has long been known to be related to depression, our findings may imply a role for S100B in depression. S100B levels are increased in CSF and serum of patients with depression [73, 74], and the best response to therapy is predicted by the highest levels of S100B [75]. Selective serotonin re-uptake inhibitors are a mainstay of treatment for depression and infants exposed prenatally to SSRI's have lower levels of S100B [76]. This may prove to be a serious teratogenic effect, given the role of S100B and serotonin in brain development.

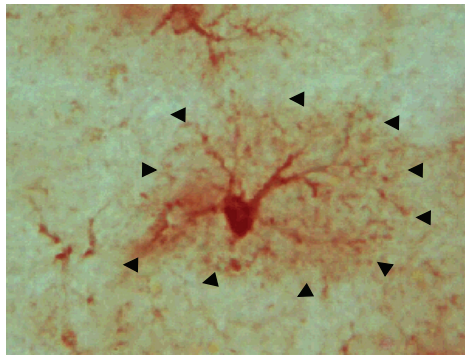


Figure 5: Photomicrograph of an S100B-positive astrocyte depicting a halo (outlined by arrowheads) of S100B-positive immunostaining around the astrocyte. Note that the majority of adult S100B+ astrocytes in the hippocampus of S100B transgenic mice display this type of appearance.

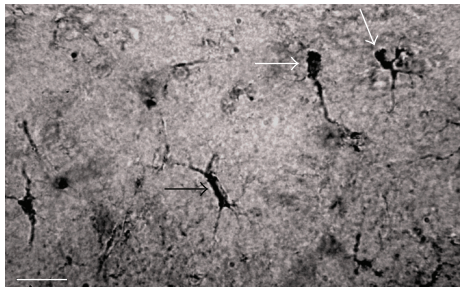


Figure 6: S100B-labeling of microglial cells in the hippocampus of 28-week-old S100B transgenic mice. In this micrograph, a rod-shaped cell (black arrow) is shown from an adult S-100B animal. This cell appears to be either an ependymal or microglial cell, based on descriptions by Hortega, (1920). Other cells (white arrows) in this photomicrograph appear to depict different stages of microglial differentiation. Scale bar = 20 μm.

3. Neuroinflammation

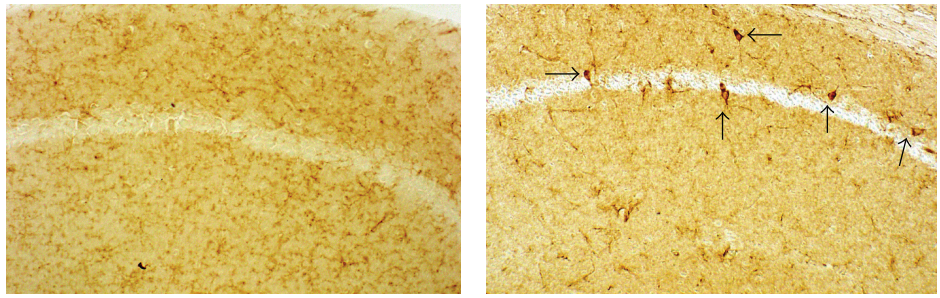
A combination of natural aging and numerous intermittent peaks in inflammatory processes, caused by environmental stressors such as infection, ischemia, or toxins, may ultimately lead to the pathological changes associated with aging. In the brain, these processes are termed neuroinflammation and generally refer to those processes known as "reactive gliosis." That is, the accumulation of enlarged or dystrophic microglial and astroglial cells [77]. Neuroinflammation can then lead to loss of neurons and loss of brain functions in a variety of neurodegenerative states [78, 79].

The role of S100B in neuroinflammation is becoming increasingly evident [19, 80]. S100B is predominantly expressed in astroglial cells of the mammalian nervous system, but during neuroinflammatory states, the protein can also be found in microglia [6], oligodendrocytes [1, 2], radial glia [7], and different classes of neurons [3, 5, 81]. In DS, activated astrocytes are already observed in the prenatal brain and are increasingly found with age. These increases correlate with the number of beta-amyloid plaques [37, 38]. Activated astrocytes are also found in AD [79] and S100B increases may play a role in their appearance. Activated microglial cells and elevated IL-6 are observed to correlate with elevated S100B in both disorders [82, 83]. In DS fetuses, microglial outnumber astroglia, which is not usually the case in normal fetuses [84]. Thus, chronic inflammation occurs in DS and may be involved in the inevitable, early development of AD.

In the S100B overexpressing mice, we have looked for effects of S100B on astroglial and microglial cells, in order to confirm the role of S100B in the neuroinflammatory changes seen in DS and AD. Finally, we have looked at markers of degeneration. The results of these studies are described in the following pages.

3.1. Astroglia. Astroglial appearance can generally be characterized by number, cell body size, and type of processes. Chronic overexpression of S100B results in changes to the number and morphology of S100B-labeled astrocytes at early (12 weeks) and later (28 weeks) timepoints. In the S100B overexpressing mice, astroglial cells are rarely observed to have the complex morphology with numerous processes, which are seen in control mice. Within astroglial cells, S100B regulates Ca^{2+} levels and once activated by Ca^{2+}, S100B interacts with intermediate filaments including GFAP and vimentin [85, 86]. This interaction leads to inhibition of filament polymerization, resulting in changes to the cytoskeleton and altered astroglial morphology [87–89]. Thus, in the S100B overexpressing animals, the excess S100B could lead to pronounced inhibition of intermediate filament polymerization and thus an instable cytoskeleton that lacks multiple processes. As the S100B transgenic mice age, the number of mature, multiprocessed S100B-labeled astrocytes is noticeably decreased (Figure 4). Moreover, as the transgenic animals mature, the atypical astrocytic morphology is more pronounced, with relatively larger cell bodies. This morphology is not observed in the control animals at either age (see Figure 4).

It is important to note previous studies showing that at low concentrations, S100B stimulates astroglial proliferation [90], and at high concentrations, the protein is toxic to astrocytes [21, 91]. Considering our findings showing that tissue levels of S100B are elevated throughout life in the transgenic animals (Figure 3), it is not surprising that the astroglial cells themselves are fewer in number and have an atypical morphology (Figure 4), changing with age. In addition to the changes in cell morphology, it is clear by the abundance of S100B in the parenchyma, that the astroglial cells are releasing relatively high amounts of S-100B into the surrounding neuropil. The released S100B is not evenly distributed throughout the neuropil, but rather is confined to characteristic "haloes" around the astroglial cell (Figure 5). Thus, chronic elevation of S100B in DS might be one mechanism whereby chronic astrocytic activation occurs. A second mechanism may be through the interaction between S100B and microglial cells.



(a) (b)

Figure 7: F4/80-labeling of macrophage/microglia in the CA1 pyramidal cell layer of 1-yr-old CD-1 and S100B transgenic mice. In CD-1 control animals (a), the labeling is found predominantly in resting microglial cells. Alternatively, in the S100B animals (b), several intensely labeled cells (arrows) are observed. These cells could be activated microglia, or another type of immune cell that may have infiltrated the hippocampus, such as macrophages or dendritic cells. 200X.

3.2. Microglia. Microglial cells are the primary component of the brain's immune system and are a key part of neuroinflammatory processes [78]. As with astrocytes, microglial cells exhibit various morphologies that correlate with a continuum of functional states of resting, reactive, chronically active and phagocytotic [92]. When the brain is damaged, whether from seizures [93], trauma, or diseases such as multiple sclerosis, Creutzfeldt-Jakob, DS, and AD [94–97], microglial cells become activated [98]. Activated microglial cells proliferate [99], migrate to the site of injury [100], alter their morphology, and begin two important functions: initiation of inflammatory processes by release of inflammatory proteins [101–103] and phagocytosis [104].

Microglial activation is mediated by numerous substances released by the injured tissue [105] one of which is S100B [106]. Moreover, in pathological states, microglial cells express S100B [6] suggesting that they may be involved in a feedback loop. As can be seen in Figure 6, microglial cells labeled with an antibody to S100B exhibit various activated morphologies in adult S100B overexpressing mice. This is consistent with in vitro studies showing that S100B is expressed by a class of microglial cells [6]. Considering that microglial cells are also prominent features of DS fetuses [85], the data support the idea that S100B overexpression influences microglial cells during development and in adulthood.

S100B has been shown to have at least two effects on microglial cells, one of which (production of nitric oxide) is RAGE-independent whereas the other (increases in the transcription factor NFkappaB) is dependent on binding to microglial RAGE [107]. When S100B binds to RAGE, the microglial cells become activated. Thus, chronic S100B elevation may influence chronic microglial activation via this mechanism. Support for this idea is derived from the fact that binding of ligands to RAGE leads to activation of signaling pathways which in turn can modulate gene expression [108]. One such pathway activated is that of the proinflammatory transcription factor, NFκB, which regulates cytokines, including interleukin IL-1, IL-6, and tumor necrosis factor [109, 110].

Both control and S100B-overexpressing animals show an increase in microglial cells and RAGE expression with age. However, this effect is more pronounced in the S100B animals, [46] (also see Figure 7). Thus, S100B binding to microglial RAGE may be a second mechanism whereby chronic S100B elevation in DS exacerbates chronic neuroinflammation.

3.3. Neuroinflammation and Neurodegeneration. Although a role linking S100B directly to cell death in DS and AD has not been established, there is evidence from animal data that this may indeed be the case [111, 112]. S100B transgenic animals show neuronal loss and increased expression of the proapoptotic protein clusterin [48] which is also increased in hippocampus and frontal cortex of AD [113]. In addition, previous animal studies have shown that using arundic acid to negatively regulate S100B will ameliorate beta-amyloid deposits, plaques, and glial (astrocyte and microglial) hypertrophy [114]. Interestingly, recent findings in the S100B transgenic mice show that S100B overexpression can lead to increases in the hyperphosphorylated tau protein found in neurofibrillary tangles (Figure 8). In a mouse model of AD, chronic over-expression of S100B has been shown to intensify gliosis and amyloidosis [115]. Moreover, chronic over-expression of human S100B increases brain damage and peri-infarct gliosis after focal ischemia. Together, these data support the idea that high levels of S100B can be detrimental in neuropathological conditions. Considering these findings, the S100B transgenic mice could be used to provide insight into possible prevention and treatments, for plaques, tangles, or other neuropathologies associated with S-100B overexpression.

For example, Vitamin E was examined in the S100B overexpressing mouse to determine if antioxidant treatment had the potential to attenuate neuroinflammatory damage associated with increased S100B. The results show that although Vitamin E decreased microglial activation in the control animals, it actually increased microglial activation and RAGE expression in S100B overexpressing mice [46]. We and others [53, 74] have hypothesized that in the state of

Figure 8: Tau-2-IR in the hippocampus of a one-year-old S100B transgenic animal. The antibody used in these studies recognizes a hyperphosphorylated form of tau usually associated with neurofibrillary tangles. In the developing brain, S100B promotes neurite outgrowth. However, in a brain chronically exposed to elevated levels of S100B, the neurites become dystrophic. (a) Cell body staining in pyramidal neurons of CA3, presumably to intracellular neurofibrillary tangles, 400X. (b) Neuropil threads in the lower blade of the dentate gyrus showing axonal accumulation of tau-2-IR, 600X. (c) Neuritic plaque in CA1 of stratum radiatum. This is a dense accumulation of neuritic fibers, similar to that described for this antibody in human AD subjects, associated with high levels of S100B (Sheng et al. [82]), 600X.

chronic overexpression of S100B, oxidation of S100B serves to preferentially induce the neurotrophic properties over the neuroinflammatory processes. Thus, treatment with an antioxidant to an animal already over-expressing S100B may interrupt this feedback, leading to further increases in RAGE upregulation and glial activation. Vitamin E may appear to be beneficial in a control animal, but animals already overexpressing S100B may show an increase in neuroinflammatory processes as evidenced by increased microglial activation [46]. This study makes the important point that studying potential treatments to prevent neurodegeneration should be done in animals which resemble the human form of neuropathology.

4. Conclusion

In conclusion, the data demonstrate that chronic S-100B overexpression results in serotonergic fiber alterations in the hippocampus, but no alterations to the number of serotonin neurons in the raphe nuclei. The data also show that chronic S100B over-expression results in activated astrocytes and microglia beginning during neonatal development and persisting through adulthood. Such changes are associated with an increase in RAGE expression. Considering the appearance of TAU-immunolabeling as a result of chronic S100B overexpression, future studies are needed to better understand the role of S-100B in mediating serotonergic and glial alterations, and their role in neuropathologies, such as, DS, aging, and AD.

Appendix

A. Methods

A.1. Animals. Male S-100B transgenic mice, aged 10 weeks ($N = 4$–6) and 28 weeks ($n = 4$–6) were compared to age matched, 10-week-old congenic CD-1 ($N = 4$–6) and 28 week CD-1 ($N = 4$–6) control mice. The S-100B mice and their congenic CD1 controls used in this study, were raised in the animal care facilities at SUNY Stony Brook, under identical conditions, of 12/12 light/dark cycle with food and water provided ad libitum.

A.2. Immunocytochemistry. All animals were perfused with 50 mL of .9% saline followed by 50 ml of 4% paraformaldehyde fixative. Brains were extracted and post fixed in 4% PLP for one hour. The brains were then cryoprotected successively in 10%, 20%, and 30% sucrose, each for twenty-four hours. Following cryoprotection, brains were sectioned on a freezing microtome at 40 um. Each group consisted of four to six male mice. For immunostaining, the tissue was first blocked in 1% peroxide for 1 hour and then blocked with 3% goat serum and .03% triton-X, in PBS for 1 hour. The tissue is then incubated free floating, in primary antibody (S100B, Sigma; 5-HTT 1 : 1000, generous gift from Dr. Feng Zhou, University of Indiana; 5-HT, 1 : 200, sigma #S5545; F4/80, 1 : 1000, Serotec MCA497R; Tau2, 1 : 500, Sigma #T5530), with 3% normal goat serum and .3% triton-X100, on a cold rotator (4C), for twenty four hours. Tissue was then rinsed in PBS and rotated for 1.5 hour, at room temperature, free floating in secondary IgG (Sigma, 1 : 200) raised against the appropriate species specific for each primary antibody, in PBS with 3% normal goat serum and .3% triton-X100. Next, the tissue was rinsed in PBS and incubated in Vectastain elite avidin biotin complex (Vector, Burlingame, CA), for one hour, rotating at room temperature. Negative control sections were carried out simultaneously, following the same protocol, minus the primary antibodies. The antibody complex was visualized using .05% diaminobenzadine (DAB), with 30% peroxidase added after the tissue soaked for 3 minutes. Tissue was rinsed and mounted onto gelatin coated slides, dehydrated in alcohol, cleared in xylene, and coverslips were applied with permount.

Slides were coded and all images were captured on an Olympus BH-2 microscope, with a Tripix digital camera.

Images were saved on a windows-based computer. In all instances, data was analyzed by raters blind to the condition of the animals.

A.3. Serotonin Transporter Analysis. Estimates of the surface density (Sv) of SERTir fibers were obtained using the cycloid grid intercept method [116]. This method is based on Buffoun's needle principal [117], which states that the length of a object (a needle in Buffon's case, SERTir fibers in this experiment) is directly proportional to the number of random intersections it makes with lines on a grid and inversely proportional to the distance separating the lines on the grid. Mathematically this relationship is described as $P = (2/\text{pi}) \times (\text{l/d})$, where P is the probability of an object-line intersection, $2/pi$ encompasses all possible angles of intersection between the object and the line, l is the length of the objectgh and d is the distance separating the lines on the grid. Cycloids are used in this experiment rather than a strait line grid, because in the case of the serotonin transporter fibers, the fibers are curved and transverse through multiple orientations of the three-dimensions of the tissue. Thus, the cycloid grid compensates for bias created by the cutting orientation [116]. For this experiment, a counting frame with 16 cycloid test lines, each with a length of 21 uM, was superimposed over photomicrographs of the infrapyramidal granule cell layer of the dentate gyrus, which was chosen as the horizontal orientation point. For each animal, the cycloid grid was superimposed over an area of molecular layer of the infrapyramidal blade of the dentate gyrus and intersections between the cycloid lines and SERTir fibers were counted. For each animal, 5 images separated by 240 uM were analyzed, corresponding to Paxino and Franklin plates 41–50 in the mouse brain atlas [118]. At the final magnification (600X), the total cycloid length for the grid was 336 μM. Surface density (Sv) was calculated using the equation $\text{Sv} = 2 \times \text{PL}$, where Sv is the surface area per unit volume of tissue, and PL is total number of intersections, divided by the total length of the probe. A fiber was considered to be in the plane of focus if it was not blurry. In order to insure the accuracy of scoring, three blind raters were given the same set of instructions for what constituted a cycloid intersection. The data were compared using regression analysis and once verified, the three scores for each image were averaged, to yield the average intersections per image. These averages are used to calculate Sv, which is expressed as CM^2 of SERTir fibers, per CM^3 of tissue (CM^2/CM^3). The average Sv for each animal was entered into SPSS (version 9.0) and analyzed using the univariate anova, with condition as fixed factor and surface density of SERT ir fibers, the dependant variable.

In addition to the stereological analysis of SERT fibers, we also performed a densitometric analysis of SERT containing processes using the University of Texas Image Tool program (V. 3.0). Using the threshold setting, the images were assigned a pixel value within which the SERT fibers would be transformed into black pixels and the surrounding neuropil would be transformed into white pixels. This method allowed us to identify only the fibers which stained intensely enough to fall within the threshold range, which were presumed to be those fibers within the range of the plane of focus of the objective lens (Olympus 60X, aperture = .90). The number of black and white pixels was then quantified using the ImageTool program, which digitally automates the counting. For each animal, 5 images were examined from the infrapyramidal blade of the dentate gyrus and averaged, to yield a percentage of black pixels per animal. The averages for each animal were analyzed using the univariate Anova on SPSS (version 9.0).

This method proved to be reliable and consistent with the stereological probe technique in the dentate gyrus. Thus, additional photomicrographs were examined using this technique in the following hippocampal areas: CA1, CA3, stratum radiatum, and lacunosum molecular.

A.4. Analysis of 5-HT Cells in the Adult Raphe Nucleus. The staining protocol was as above, except, the primary antibody was incubated for forty-eight hours. Raphe nucleus sections, separated by 40 uM, containing 5-HTir neurons, were included for cell counts. Images were captured as stated above, and neurons were counted by a blind scorer. Neurons were counted in a 1.058 mm^2 area, containing the dorsal, median, or caudolinear raphe nucleus. The sum of 5-HTir neurons counted for each region was divided by the sum of the number of images counted, to yield an average density of 5-HTir neurons in a 1.058 mm^2 area. The data was analyzed using a multivariate analysis of variance, with condition as the fixed factor, and the dorsal, median, or caudolinear raphe nucleus as the dependant variables.

A.5. Detailed Morphological Analysis of S100B-Labeled Cells. Detailed morphological analysis of S-100B immunoreactive (IR) glial cells in the hippocampus was performed by raters blind to the age and genetic background of the animals. Within the hippocampus, the predominant cell type expressing S-100B IR are astrocytes of varying morphology. The morphology of the astrocytes was defined based on the size of the cell body, complexity of processes, and approximate number. *Size:* cells were defined as small, if cell bodies were less than 4 μm, medium at 4–8 μm, and large if the cell body was greater than 8 μm. *Processes:* cells were classified as simple (no significant processes, similar to cells described as "protoplasmic," immature or Type I), complex (many ramified processes, arranged symmetrically around the cell body, similar to "stellate" or "fibrous"), or atypical (cells with nonsymmetrical processes, such as one large think process). *Number:* cell density was defined as sparse, moderate, or dense based on their relative appearance. *Hippocampal subfields*: in general, area CA2 represented a homogenized zone between CA3 and CA1, demonstrating immunoreactivity for the types of cells seen in both regions. Thus, only areas CA1 and CA3 were analyzed. When referring to CA3 lacunosum moleculare (LAC), this represents the area of LAC which is adjacent to CA3/CA2 region. The rest of LAC is considered to be in the CA1 region. In the dentate gyrus, the morphological appearance of the supra and infrapyramidal blades of the molecular layer of the dentate gyrus was indistinguishable, and thus these are classified together. The following abbreviations are

used to describe the hippocampal subfields: Pyramidal Cell Layer (PYR); Stratum radiatum (SR); Stratum oriens (SO); Lacunosum Moleculare (LAC); Dentate Molecular layers (MOL); Dentate Granule cell layer (DGN); Polymorphic zone (PMZ).

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Review Article

Targeting S100B in Cerebral Ischemia and in Alzheimer's Disease

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S100B is an EF-hand calcium-binding protein that exerts both intracellular and extracellular effects on a variety of cellular processes. The protein is predominantly expressed in the central nervous system by astrocytes, both physiologically and during the course of neurological disease. In the healthy adult brain and during development, constitutive S100B expression acts as a trophic factor to drive neurite extension and to referee neuroplasticity. Yet, when induced during central nervous system disease, the protein can take on maladaptive roles and thereby exacerbate brain pathology. Based on genetic and pharmacological lines of evidence, we consider such deleterious roles of S100B in two common brain pathologies: ischemic stroke and Alzheimer's disease (AD). In rodent models of ischemic brain damage, S100B is induced early on during the subacute phase, where it exacerbates gliosis and delayed infarct expansion and thereby worsens functional recovery. In mouse models of AD, S100B drives brain inflammation and gliosis that accelerate cerebral amyloidosis. Pharmacological inhibition of S100B synthesis mitigates hallmark pathologies of both brain diseases, opening the door for translational approaches to treat these devastating neurological disorders.

1. Introduction

The principal cell types comprising the brain parenchyma are neurons and glial cells. The term "glia" is customarily used to refer to neuroglia (comprised of astrocytes, oligodendrocytes, and more recently, NG2 oligodendrocyte progenitors), Schwann cells, and central nervous system- (CNS-) resident macrophages known as microglia. Occasionally, ependymal cells (ependymoglia) are also classified as glia, as they are differentiated from radial glia [1] and share astrocytic properties [2]. In addition to parenchymal cells, cerebral vascular cells exist and form a physiological barrier in the CNS known as the blood-brain barrier (BBB). Among these cellular constituents, astrocytes greatly outnumber neurons in the brain, making up about 50% of human brain volume [3]. Despite the time-honored concept that astrocytes are "silent partners of the working brain", accumulating evidence has shown that astrocytes are active participants in CNS physiology [4–6], including transport of substances between blood and neurons [3, 4], cerebral blood flow metabolism control [7–10], modulation of synaptic transmission [11–13], synaptogenesis [14–18], and neurogenesis [19–22].

Yet, astrocytes are capable of directly endangering neurons during the course of inflammatory CNS disorders [23, 24]. In fact, acute and chronic CNS disorders often have a component of glial activation, characterized by infiltration of activated microglia and astrocytes into the region of damaged tissue [21, 25–28]. Reactive astrocytes likely exert their effects in collaboration with activated microglia. On the one hand, these cells may exacerbate neuroinflammation by producing a myriad of toxic substances, including cytokines, nitric oxide, prostanoids, and reactive oxygen species; on the other hand, they are capable of exerting beneficial effects by producing neurotrophic substances [3–6, 21, 25, 29, 30]. Much recent attention has been focused on this enigmatic duality so often observed in studies of activated glia within

the broader context of neurological and neurodegenerative diseases.

This paper begins by addressing the double-edged sword of both beneficial and detrimental actions of astrocytic S100B in the CNS. Subsequently, we move on to focus on contributions of reactive astrocytes to glial inflammatory responses in two common neurodegenerative diseases: cerebral ischemia and Alzheimer's disease (AD). Finally, we consider the concept of translating S100B inhibition to the clinic for the treatment of neurodegenerative diseases.

2. Beneficial and Detrimental Actions of S100B in the Central Nervous System

S100 is a large family (over 20 members) of EF-hand (helix E-loop-helix F) calcium-binding proteins, and all but four are clustered on human chromosome 1q21, while the human gene encoding S100B maps to chromosome 21q22 [31–38]. A total of ten S100 family members are expressed in the brain, including S100A1, S100A2, S100A4, S100A5, S100A6, S100A10, S100A11, S100A13, S100B, and S100Z. In addition, mRNA levels of S100A1/S100B are 5-fold higher than S100A6/S100A10 and 100-fold higher than S100A4/S100A13 in the mouse brain. Five of these six family members (S100A1, S100A6, S100A10, S100A13, and S100B) are increased in an age-dependent manner in adult mice [39]. S100B is detected in varying abundance in a limited number of brain cells including astrocytes, maturing oligodendrocytes, neuronal progenitor cells, pituicytes, ependymocytes, and certain neural populations. Although the majority of astrocytic S100B localizes within the cytoplasm, 5%–7% is membrane bound [32, 34, 38, 40–42]. S100B has been implicated in Ca^{2+}-dependent regulation of a variety of intracellular functions such as protein phosphorylation, enzymatic activity, cell proliferation and differentiation, cytoskeletal dynamics, transcription, structural organization of membranes, intracellular Ca^{2+} homeostasis, inflammation, and protection against oxidative damage [31–38, 43–46].

Binding of S100B to receptors on target cells releases intracellular free Ca^{2+} from Ca^{2+} stores via activation of phospholipase C and downstream inositol triphosphate [43]. As overexpression of S100B induces downregulation of p53 protein [47], calcium signaling and S100B may act in cooperation with this pathway, which is implicated in growth inhibition and apoptosis [47–49]. Yet, how elevation of cytosolic Ca^{2+} transduces S100B binding into trophic and proliferative effects on brain cells is still elusive.

During brain development, a temporal correlation has been reported between synaptogenesis and astrocyte differentiation [50]. Numerous findings support the notion that astrocytes regulate the formation, maturation, and maintenance of synapses [14–17]. Astrocytic S100B expression increases in the rodent brain during the first 3 postnatal weeks—a critical period for glial proliferation and synaptogenesis, and it was suggested nearly 40 years ago that the protein likely referees synaptic development *in vivo* [51, 52]. In the adult rodent brain, S100B expression persists at nanomolar concentrations and likely orchestrates neurite extension [53], enhances survival of neurons and promotes synapse formation [54], and exerts protective actions after injury [55, 56].

On the other hand, there is accumulating evidence that S100B may also have detrimental actions in the CNS. Activation of microglia triggers and promotes astrocytic activation through release of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These and other cytokines drive a synergistic relationship between these two types of glial cells via a vicious positive feedback loop known as the "cytokine cycle" [24]. While numerous bioactive substances are part and parcel of this cycle, S100B can be regarded as a major constituent of this brain-damaging feedback loop. In this context, Hu and colleagues demonstrated that S100B at micromolar concentrations induces neuronal damage in a neuron and astrocyte coculture experiment by causing overexpression of inducible nitric oxide synthase and subsequent release of nitric oxide [57]. Lam and colleagues showed that S100B stimulates inducible nitric oxide synthase in rat primary cortical astrocytes through a signal transduction pathway that involves activation of the transcription factor nuclear factor-κB (NF-κB), which is a master regulator of pro-inflammatory responses [58]. Hu and Van Eldik showed that S100B upregulates IL-1β expression in astrocytes [59], and Ponath and coworkers showed that S100B stimulates release of IL-6 and TNF-α from astrocytes [60].

Importantly, numerous secreted S100 proteins (S100B, S100A1, S100A2, S100A4, S100A5, S100A6, S100A7, S100A8/A9, S100A11, S100A12, and S100P) can act in either an autocrine or paracrine fashion through a common receptor: the receptor for advanced glycation endproducts (RAGE), a multiligand receptor that belongs to the immunoglobulin family. Moreover, in cell-based assays, all these family members (except for S100A2 and S100A5) have been shown to trigger RAGE-dependent signaling [38, 61–65]. The fact that the RAGE promoter has functional NF-κB binding sites reinforces the likelihood that this signaling pathway is an important trigger of inflammatory pathogenesis [66]. Moreover, it has been shown that RAGE and S100/calgranulin signaling propagate, recruit, and activate cellular pro-inflammatory effectors [61]. Accordingly, nanomolar concentrations of extracellular S100B can trigger expression of the anti-apoptotic factor Bcl-2 in RAGE-expressing cells, whereas micromolar S100B concentrations induce apoptosis via RAGE activation [67]. In primary microglia, S100B stimulates IL-1β production by activating extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun NH_2 terminal protein kinase (JNK) [68]. There is evidence that S100B-mediated microglial pro-inflammatory responses are RAGE-dependent, as RAGE ligation by S100B induces expression of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) via parallel Ras-Cdc42-Rac1-dependent activation of JNK and Ras-Rac1-dependent stimulation of NF-κB transcriptional activity. Further, S100B engagement of RAGE coordinately stimulates NF-κB and AP-1 transcriptional activity and synergizes with IL-1β and TNF-α to upregulate COX-2 expression [65].

3. Reactive Astrocytes and S100B in Cerebral Ischemia

While there is a wide spectrum of various forms of brain damage, ischemic stroke (cerebral ischemia) is the most prevalent type of brain injury that causes death and long-lasting disability. The pathogenic effects of microcirculatory dysfunction in cerebral ischemia can be divided into two categories: impairment of intraluminal cerebral blood flow and extraluminal effects resulting from alterations in the BBB [69]. Over the past decade, extensive research efforts have been directed toward development of neuroprotective drugs against cerebral ischemia, such as ion channel antagonists, glutamate receptor antagonists, and free radical scavengers. Anti-platelet drugs, anti-thrombotic drugs, tissue plasminogen activators, and free radical scavengers have already come into routine clinical use and clinical trials investigating additional agents are ongoing.

In the clinical setting, S100B is a well-known biomarker that positively associates with severity of brain damage and has been shown to predict prognosis after subarachnoid hemorrhage [70, 71], ischemic brain injury [72, 73], and traumatic brain injury [74]. It is important to note that the above clinical studies are correlative in nature and presume that rise in cerebral spinal fluid and serum concentrations of S100B is due to release of intracellular stores of the protein upon cellular disruption. Thus, the possibility that S100B is synthesized *de novo* by reactive astrocytes and exacerbates infarct evolution was not considered in these reports. In addition, a surge of attention from both physiological and therapeutic standpoints has been directed toward the possible roles of astrocytes in neurometabolic and neurovascular coupling.

After focal cerebral ischemia, brain damage is accompanied by infiltration of reactive astrocytes into the peri-infarct area. Specifically, astrocytes are copiously activated along the outer border of the infarct. Reactive astrocytes are earmarked by increased expression of S100B and glial fibrillary acidic protein (GFAP), appear around 24 hours after the onset of ischemia, and undergo hypertrophy and hyperplasia for a period of weeks thereafter [75–77]. During the chronic disease phase (>168 hours post-ischemia), astrocytes extensively wall off the BBB at the glial limitans [78] (Figure 1), participate in angiogenesis concurrent with development of cerebral edema [29, 79], and possibly drive neurogenesis [19, 22]. On the other hand, when considering the subacute phase (24–168 hours after ischemia onset) of this neurological disorder, it has been difficult to assign a role to reactive astrocytes. This is owed to the long-held belief that infarct expansion after focal cerebral ischemia comes to a complete halt by 12 hours, well before astrocytic activation manifests [80]. However, "delayed infarct expansion" during the subacute phase of cerebral ischemia was later discovered and is now becoming generally accepted. This pathological event has led to a wider recognition of the possible detrimental role of astrocytic activation in the subacute phase of cerebral infarction as a valid research target [81].

With regard to the concept of delayed infarct expansion, Garcia and colleagues used the rat permanent focal cerebral ischemia model and noted for the first time in 1993 that there was a significant increase in infarct area during the time interval between 6 and 72 hours after ischemia onset [76]. In 1996, Du and coworkers followed up by showing that infarction after mild transient focal cerebral ischemia could develop in a delayed fashion in the rat [82]. Using the rat permanent focal cerebral ischemia model, we have shown that after the rapid expansion phase during the first 24 hours post-insult, infarct volume continues to slowly but steadily increase until it reaches a peak at 168 hours. At this time-point, there is a significant increase in infarct volume of ~41% as compared to 24 hours post-insult. Moreover, the occurrence of delayed infarct expansion is associated with astrocytic activation as well as increased abundance of S100B in the peri-infarct area [81]. Clinical research relying on magnetic resonance imaging of ischemic stroke patients has disclosed that infarct expansion occurs during two distinct phases (acute and delayed) [83–85], and the time course and magnitude of ischemic enlargement are quite comparable between patients and the rat permanent focal cerebral ischemia model [81]. The above lines of evidence reinforce the idea that delayed infarct expansion is a viable therapeutic target and may even be as important as acute infarct expansion in terms of disease progression. Moreover, the

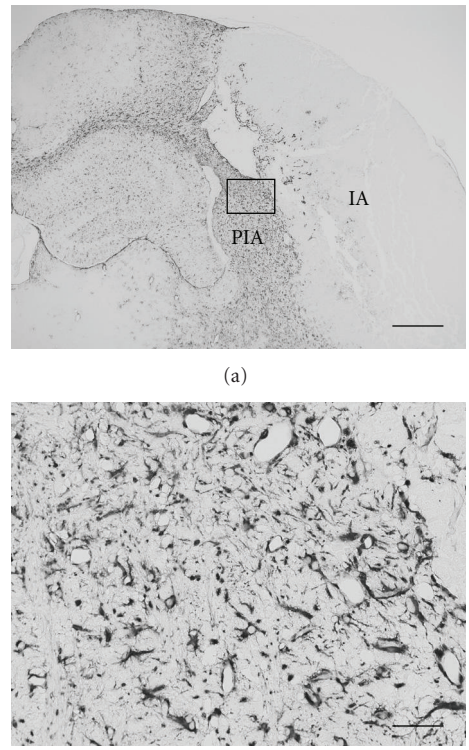


Figure 1: Reactive astrocytes, earmarked by enhanced expression of glial fibrillary acidic protein, hyperplasia, and gemistocytic changes, form a glial barrier in the peri-infarct area of a mouse brain at 7 days post-focal cerebral ischemia. Higher magnification image (b) represents the inset of the lower magnification coronal brain section (a). Abbreviations used: IA: infarct area; PIA: peri-infarct area. Bars denote 500 μm (a) and 50 μm (b).

association among the appearance of numerous peri-infarct reactive astrocytes, increased abundance of S100B in the peri-infarct area, and occurrence of delayed infarct expansion has emerged as an issue of high clinical importance.

Based on the work discussed above, we undertook a series of experiments to investigate the putative causal relationship between S100B and exacerbation of brain damage. Specifically, we forced expression of human S100B or pharmacologically blocked S100B biosynthesis in rodents subjected to ischemic brain injury. We discuss our relevant findings to follow.

3.1. Human S100B Exacerbates Ischemic Brain Damage and Peri-Infarct Gliosis. We sought to evaluate whether forced expression of human S100B in astrocytes may exacerbate brain damage and delayed infarct expansion after permanent focal cerebral ischemia. As a corollary, we aimed to examine whether severity of delayed infarct expansion and peri-infarct gliosis were correlated. To experimentally probe these questions, we utilized transgenic mice overexpressing human S100B (Tg huS100B mice; carrying approximately 10 copies of the human S100B gene under endogenous regulatory control) on an outbred CD-1 genetic background. These transgenic mice overexpress S100B in cortical astrocytes by 4-6-fold over endogenous wild-type levels [86]. We permanently induced focal cerebral ischemia by tandem occlusion of the left common carotid artery and distal segment of the middle cerebral artery using an electrocoagulation method under normothermia [87]. Notable results of this experiment are that Tg huS100B versus control CD-1 mice show significant exacerbation of infarct volume, neurological deficits, and peri-infarct-reactive gliosis (astrocytosis and microgliois) during the time interval between 1 and 7 days after the onset of ischemia, providing evidence for increased susceptibility of Tg huS100B mice to ischemic brain stress. Moreover, reactive gliosis as indicated by increased S100, GFAP, and Iba1 immunoreactivity in the peri-infarct area continued to increase in Tg huS100B mice through to 7 days after focal cerebral ischemia, whereas control mice reach a plateau at 3 days following ischemic insult.

Our interpretation of the above evidence is that pro-inflammatory events associated with S100B-accelerated glial activation in the peri-infarct area contribute to exacerbation of brain damage. In further support of this conclusion, we noted a positive correlation between reactive gliosis along the infarct border and occurrence of delayed infarct expansion in Tg huS100B mice, but no significant relationship between these variables was detected in control animals. Together, these results support the notion that enhanced and prolonged activation of glial cells plays a detrimental role during the subacute phase (1 to 7 days) of focal cerebral ischemia in the rodent brain [81, 87, 88]. Moreover, our results also bolster the hypothesis that astrocyte-derived S100B is a pivotal mediator of these deleterious effects [88–90]. In addition, we found that Tg huS100B mice had higher constitutive levels of S100 as compared with CD-1 mice. The distinction between constitutive and ischemic brain injury-enhanced S100 is important. This is because increased baseline S100 abundance may predispose to worse acute infarct expansion within 1 day after ischemic induction, whereas further induction of S100 expression likely promotes exacerbation of delayed infarct expansion.

As mentioned above, astrocytes and astrocyte-derived S100B seem to play dichotomous roles in progression of various CNS pathologies, although it is still controversial whether, on average, detrimental effects outweigh neuroprotective effects or *vice versa*. Since S100B can be trophic to glia [91], this effect may mechanistically underlie hyperplasia of peri-infarct reactive glia after focal cerebral ischemia in Tg huS100B mouse brains. Interestingly, aged rats show accelerated glial reactivity after cerebral ischemia, which coincides with impaired functional recovery [92]. Thus, worsening of neurological deficit in Tg huS100B mice after focal cerebral ischemia may be caused by inappropriately accelerated "trophic" glial responses.

The above studies adopted a genetic approach to demonstrate *in vivo* evidence that forced expression of human S100B exacerbates brain damage, neurological deficits, and peri-infarct reactive gliosis after ischemic stress. However, whether S100B sits at the epicenter of the damaging cytokine cycle and delayed infarct expansion needed further verification. To definitively validate this hypothesis, we examined whether pharmacological blockade of S100B biosynthesis would produce converse effects on ischemic brain damage. As detailed below, we found support that pharmacological strategies aimed at inhibiting S100B abundance would, in principle, be beneficial to mitigate cerebral ischemia.

3.2. Suppressing S100B Synthesis Mitigates Cerebral Ischemic Brain Damage. To examine if enhanced synthesis of S100B by peri-infarct reactive astroglia played a role in delayed infarct expansion, we undertook a pharmacological approach using a novel agent, arundic acid [ONO-2506, (*R*)-(–)-2-propyloctanoic acid, ONO Pharmaceutical Co. Ltd.], which has been shown to suppress astrocytic S100B synthesis [93]. Yet, arundic acid exerts additional effects both *in vitro* and *in vivo* on other biomolecules, including inhibition of nerve growth factor-β, inducible nitric oxide synthase, and COX-2 expression and increasing glutathione synthesis, mRNA expression of glutamate transporters (glutamate transporter subtype 1: GLT-1 and glutamate/aspartate transporter: GLAST) and GABA receptors ($GABA_A$-R $\beta 1$, $GABA_A$-R $\beta 2$, and $GABA_A$-R $\beta 3$) in activated astrocytes. Yet, it is still unclear whether the agent modulates these other proteins directly or indirectly via its suppressive effect on S100B [93]. To model ischemic brain injury in the rat, we permanently occluded the left middle cerebral artery under normothermia and then divided at a site proximal to the origin of the lenticulostriate arteries [81]. Intravenous administration of arundic acid (10 mg/kg, once a day), initiated immediately after induction of ischemia, significantly reduced infarct volume at 168 hours (but not at 72 hours) in this rat focal cerebral ischemia model. Surprisingly, the agent did not inhibit acute infarct expansion during the initial 24 hours, but almost completely inhibited delayed infarct expansion.

In a subsequent experiment designed to elucidate a therapeutic window, rats were allocated to groups that

received the first administration of drug at 24, 48, or 72 hours after focal cerebral ischemia, and infarct volumes were compared at 168 hours. Treatment with arundic acid commenced at 24 or 48 hours after the induction of ischemia significantly decreased infarct volumes at 168 hours by approximately 43% and 35%, respectively. However, when treatment was initiated later on at 72 hours, we did not observe a protective effect. Collectively, these results show an encouraging liberal therapeutic window, which coincides with the peak of astrocytic S100B synthesis induction after focal cerebral ischemia. Numbers of apoptotic cells in the ischemic hemisphere at 72 hours post-focal cerebral ischemia were markedly decreased by arundic acid treatment. In addition, tissue levels of S100B as well as GFAP in the peri-infarct area were significantly decreased by the agent. Thus, administration of arundic acid led to significant attenuation of astrocytic S100B synthesis, general inhibition of astrocytic activation, and reduced numbers of apoptotic cells in the peri-infarct area. Notably, delayed infarct expansion was almost completely inhibited in this experimental disease model. In addition, we found significant improvement in neurological scores and spontaneous activities as early as one day after the first drug treatment, and these beneficial outcomes continued for several days after treatment. These results support the concept that pharmacological inhibition of astrocytic S100B limits occurrence of delayed infarct expansion after focal cerebral ischemia.

As arundic acid does not significantly inhibit infarct expansion in the rat focal cerebral ischemia model when given beyond the 72-hour time-point, the above results indicate that the compound acts to improve later brain dysfunction through a mechanism that is temporally unrelated to inhibition of subacute infarct expansion. In this regard, it has been reported that transient focal cerebral ischemia is accompanied by relatively widespread and persistent functional disturbances in the peri-infarct region [94, 95]. Thus, improvement in neurological deficit observed after the subacute phase is likely ascribable to the beneficial actions of arundic acid on functional disturbances in the ischemic hemisphere as well as on neural plasticity in both ischemic and non-ischemic hemispheres. That neural functional derangement and delayed infarct expansion can be simultaneously mitigated by arundic acid raises the possibility that both pathologies stem from a common pathogenic mechanism: astrocytic activation. With regard to the relationship between these pathologies, it has been reported that symptoms can regress despite increase in infarct volume [25]. Results of this experiment extend the notion that pharmacological modulation of astrocytic activation via inhibiting S100B biosynthesis can have long-lasting effects on functional recovery after injury, despite only reducing infarct expansion during the initial subacute phase.

The above lines of evidence from (1) a genetic model of forced human S100B expression and (2) pharmacological blockade of S100B biosynthesis bolster a causal relationship between S100B and exacerbation of ischemic brain damage. After cerebral ischemia, astrocytes change in shape and function within a relatively short time-frame, and likely exert their effects at multiple levels and at different phases of infarct evolution. During the subacute phase of focal cerebral ischemia, reactive astrocytes seem to promote delayed infarct expansion by enhancing the viscous cytokine cycle in collaboration with activated microglia. The work detailed above reinforces the idea that astrocytic synthesis of S100B plays a central role in the pathobiology of this disease, and prompts a mechanistic model wherein activated astrocytes, activated microglia, and S100B participate in brain damage and delayed infarct expansion after focal cerebral ischemia (Figure 2). A similar pathogenic mechanism seems to exist during the course of chronic neurodegeneration after focal cerebral ischemia in remote areas such as the thalamus, striatum, and substantia nigra. Since augmented astrocytic S100B synthesis accompanies a diverse number of brain pathologies, we moved on to consider the role(s) of this enigmatic molecule in neurodegenerative disease. In particular, we focused on of the relationship between S100B and AD pathology, as detailed to follow.

4. Alzheimer's Disease, Neuroinflammation, and S100B

AD is the most common progressive dementia of aging and is characterized by memory loss and gradual decline in cognition. AD neuropathological hallmarks include brain deposition of amyloid-β (Aβ) peptide as senile plaques, accumulation of abnormal *tau* protein filaments as intracellular neurofibrillary tangles, extensive neuronal degeneration and loss, profound synaptic loss, and β-amyloid plaque-associated astrocytosis and microgliosis [96–98]. Moreover, augmented expression of S100B has been reported in the brains of patients with Down's syndrome and in AD [99]. In AD, it has been shown that S100B abundance is elevated in activated astrocytes colocalized with β-amyloid plaques, where progressive expression of IL-1α by activated microglia has also been noted [100]. Importantly, it has been reported that astroglial overexpression of S100B actually precedes appearance of neuritic β-amyloid plaques in the PDAPP mouse model of AD [101], suggesting that S100B overabundance may drive cerebral β-amyloidosis as opposed to existing as an epiphenomenon.

Brain Aβ deposition "normally" occurs during the course of senile changes [102], and it is probably a combination of increased accumulation/reduced clearance of the peptide and impaired ability to cope with toxic downstream effects of Aβ that drive AD. Aβ is produced from sequential endoproteolytic cleavage of the type 1 transmembrane glycoprotein, β-amyloid precursor protein (APP), by β- and γ-secretases [103–106]. Recently, it has been hypothesized that newly produced Aβ enters a dynamic equilibrium between soluble and deposited forms in the brain, with continual transport of soluble Aβ out of the brain and into the circulation [107]. In fact, cerebral amyloidosis in AD patient brains might be licensed by an imbalance in this dynamic equilibrium.

Rooted in the "amyloid cascade hypothesis" of AD, which purports that accumulation of cerebral Aβ sets into motion a series of toxic downstream events [108, 109], extensive research efforts have been directed toward

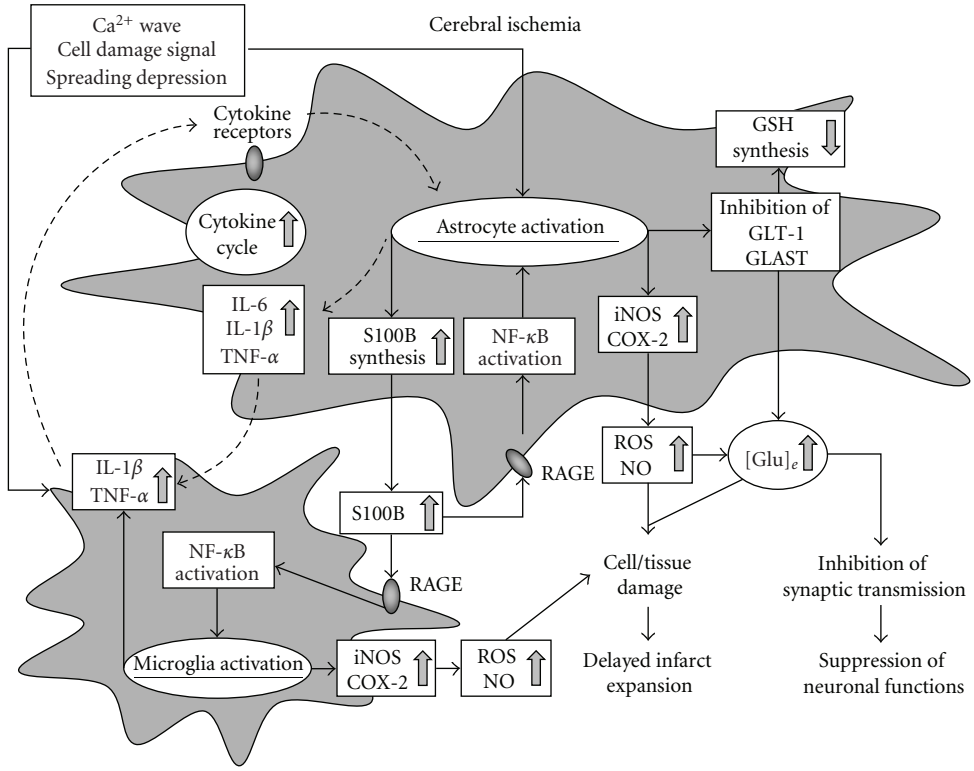


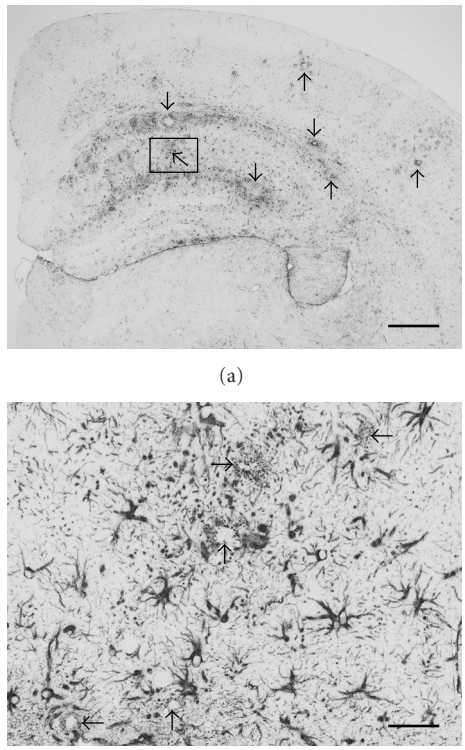
Figure 2: A model for the roles of activated astrocytes, activated microglia, and S100B in delayed infarct expansion after focal cerebral ischemia. The glial cytokine cycle is represented by *dotted lines*. Abbreviations used: COX-2: cyclooxygenase-2; $[Glu]_e$: extracellular glutamate; GLAST: glutamate/aspartate transporter; GLT-1: glutamate transporter subtype 1; GSH: glutathione; IL-1β: interleukin-1β; IL-6: interleukin-6; iNOS: inducible nitric oxide synthase; NF-κB: nuclear factor-κB; NO: nitric oxide; TNF-α: tumor necrosis factor-α; RAGE: receptor for advanced glycation endproducts; ROS: reactive oxygen species.

development of anti-amyloid therapies aimed at reducing cerebral Aβ production (e.g., β- or γ-secretase inhibitors) [110–113] or enhancing Aβ clearance by targeting brain immune/inflammatory mechanisms [27, 28, 114–123]. Unfortunately, however, agents that demonstrated benefit in rodent models of AD have not yet lived up to their promise in the clinic, where cholinesterase inhibitors (e.g., donepezil, rivastigmine, tacrine, and galantamine) and N-methyl D-aspartate antagonist (e.g., memantine) continue to be prescribed. It should be noted, however, that these currently indicated drugs produce only modest symptomatic benefit, especially when administered in the advanced stage of the disease. Thus, research into new drugs based on alternative therapeutic targets has continued at a feverish pace.

Over the past decade, we and others have proposed that inflammatory and immune response pathways are chronically activated in AD patient brains at low levels, and likely play a role in disease progression. "Inflammation" is canonically defined as edema and tissue infiltration of neutrophils, lymphocytes, plasma cells, and macrophages, but these classical pathological findings are not present in the post-mortem AD brain. Yet, accumulating evidence indicates that a variety of factors known to be major participants in inflammatory and immune responses are the norm in AD. Chronic activation of glial cells in and around β-amyloid plaques may be pathoetiologic in AD via production of numerous neurotoxic acute-phase reactants, pro-inflammatory cytokines, and immunostimulatory molecules [24, 28, 124, 125]. However, despite low-level, chronic activation of innate immunity and inflammatory responses in the AD brain, glial cells ultimately fail to clear cerebral β-amyloid plaques.

Astrocytes and microglia are the main innate immune response effectors in the CNS. Imbalances between protective and destructive functions of these cells might impact neurotoxicity and/or synaptotoxicity in the context of neurodegenerative disease. In the AD brain, reactive astrocytes and microglia co-exist in both temporal and spatial proximity with β-amyloid plaques (Figure 3), and it is thought that diffuse β-amyloid deposits attract and activate IL-1β-secreting microglia, which in turn activate astrocytes and promote astrocyte-derived S100B synthesis. This self-propagating neuroinflammatory loop promotes further release of pro-inflammatory cytokines and acute-phase reactants by both activated microglia and astrocytes, leading to production of oxyradicals and nitric oxide, which are toxic at supraphysiologic levels. Aside from further enhancing inflammatory responses, these pro-inflammatory substances likely induce bystander neuronal injury in the AD brain [24, 124].

Given the conspicuous role of brain inflammation and activated astrocytes in AD pathobiology, we undertook



(a)

(b)

Figure 3: Numerous β-amyloid plaque-associated reactive astrocytes (positive for glial fibrillary acidic protein) are shown in a coronal brain section from a Tg2576 Alzheimer's disease model mouse at 19 months of age. Higher magnification image (b) represents the inset of the lower magnification coronal brain section (a). Arrows show β-amyloid plaques that are surrounded by glial fibrillary acidic protein-positive processes in the plaque periphery. Bars denote 500 μm (a) and 50 μm (b).

genetic and pharmacological approaches to target S100B in order to explore a putative causal relationship between S100B and progression of AD-like pathology. Our relevant findings are reviewed and discussed in the following sections.

4.1. Overexpression of Human S100B Exacerbates Alzheimer's Disease-Like Pathology. Over the past decade, numerous transgenic mouse models have been constructed using mutations in human APP and/or presenilin-1 that cause autosomal dominant early-onset AD [126]. To date, at least five lines that bear mutant human APP genes, that is, Tg2576, PDAPP, APP23, TgCRND8, and J20, have been reported and are widely used. These transgenic mouse lines differ in terms of genetic characteristics (e.g., different mutations, promoters, and/or genetic backgrounds), yielding different transgene expression levels and varying severity of AD-like pathology. Given its wide usage as a mouse model of AD-like pathology, we adopted Tg2576 mice originally developed by Karen Hsiao and colleagues in the mid-90s [127]. This mouse line overproduces human $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ and develops progressive β-amyloid deposits and learning and memory impairment beginning at 9-10 months of age [127–129]. To examine a possible role of S100B in the progression of AD-like pathology, we undertook a genetic approach to overproduce S100B by crossing transgenic mice expressing human S100B (TghuS100B mice) [86] with Tg2576 animals [127] to yield four genotypes of littermates: Tg2576, Tg2576-huS100B, TghuS100B, and wild-type. We then examined AD-like pathology in aged animals, including brain parenchymal and cerebral vascular β-amyloid deposits, Aβ levels, β-amyloid deposit-associated gliosis (astrocytosis and microgliosis), and pro-inflammatory cytokines.

We initially noted that the huS100B transgene exacerbated age-dependent cerebral amyloidosis, including brain parenchymal and cerebral vascular β-amyloid deposits in bitransgenic mice. We also undertook a biochemical approach to measure different forms of Aβ peptides. Consistent with histological results, we noted elevated soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ abundance. In brain areas examined early on during the course of Aβ deposition (at 9 months of age), we observed that Tg2576 mice had mainly dot-like β-amyloid deposits ($<5\,\mu$m in maximum diameter); by contrast, Tg2576-huS100B mice had large-sized β-amyloid plaques ($>50\,\mu$m in maximum diameter) that were already present at this early age. In addition, as we did not observe β-amyloid deposits in Tg2576 or Tg2576-huS100B mice at an even earlier age (7 months), the main difference between bigenic and singly-transgenic Tg2576 mice is higher plaque burden in the former, but not that amyloid deposition is initiated earlier in Tg2576-huS100B mice. Our findings can thus be interpreted as accelerated AD-like pathology by up to 4 months of age in TghuS100B-Tg2576 versus Tg2576 mice.

We also noted augmented astrocytosis and microgliosis, elevated levels of endogenous mouse S100 expression, and increased levels of pro-inflammatory cytokines (TNF-α and IL-1β) as early as 7–9 months of age in bigenic animals—prior to the onset of frank cerebral amyloid deposits in Tg2576 mice. Others have reported that gliosis is consequent upon progressive cerebral amyloid burden in AD model mice, given that glial activation is proportional to β-amyloid plaque load [130–132]. However, our finding that gliosis precedes increased β-amyloid plaque deposition in Tg2576-huS100B mice instead suggests that S100B-induced glial inflammatory responses drive accelerated β-amyloid load in these animals. This conclusion is strengthened by our findings that Tg2576-huS100B mice have significantly enhanced astrocytosis and microgliosis as compared to Tg2576 animals, both during initiation of cerebral amyloidosis (at 9 months of age) and at an age prior to β-amyloid deposition (at 7 months of age). However, it should be noted that we also measured pro-inflammatory cytokine mRNAs including TNF-α, IL-1β, IL-6, and mouse S100B at 7 months of age, and were not able to detect any consistent differences in these cytokines between the four groups of littermates. Thus, while the huS100B and Tg2576 transgenes synergize on increasing glial surface activation markers prior to formation of β-amyloid plaques (i.e., at 7 months of age), these two transgenes do not seem to cooperatively affect expression of pro-inflammatory cytokines until 9 months of age in this model.

The role of inflammatory responses and glial activation in the AD pathological process is multifarious, and studies

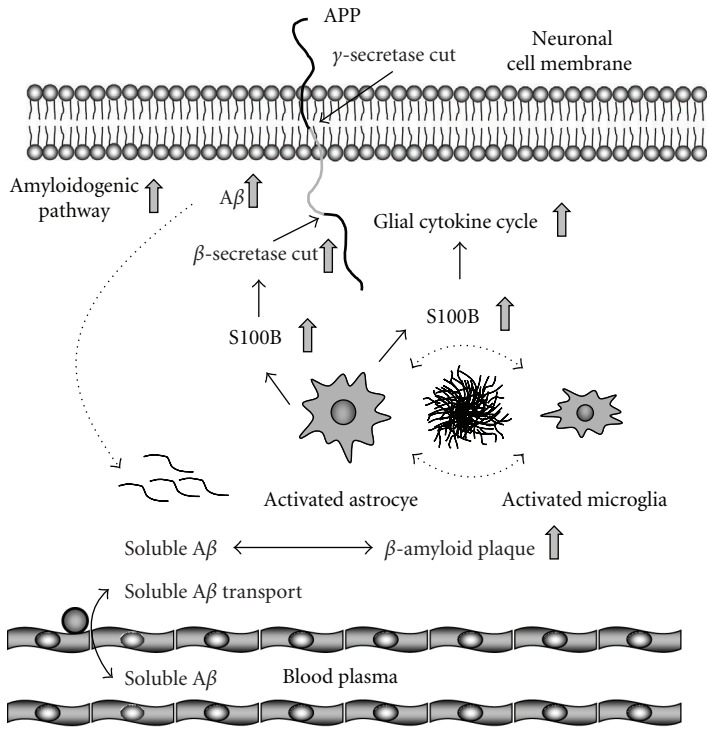


Figure 4: Diagrammatic representation of brain amyloid-β (Aβ) production/clearance, the harmful actions of S100B on Aβ production, and resultant effects on brain and plasma Aβ levels. In the amyloidogenic pathway, Aβ is primarily produced in neurons from sequential endoproteolytic cleavage of the type 1 transmembrane glycoprotein, β-amyloid precursor protein (APP), by β- and γ-secretases. Subsequently, soluble Aβ is secreted into the extracellular space, and then enters into a dynamic equilibrium between soluble and deposited (insoluble, β-amyloid plaque) forms. Continual transport of soluble Aβ occurs into and from the plasma. S100B enhancement of β-secretase activity promotes Aβ production, resulting in higher soluble and deposited Aβ in the brain. In addition, S100B promotes a damaging glial cytokine cycle through activation of astrocytes and microglia, resulting in enhancement of the amyloidogenic pathway.

have shown both beneficial and harmful effects of glial activation on AD-like pathology in mouse models depending on the stimulus [90, 114–117, 122, 132]. These results have led to the conclusion that there are various forms of gliosis in the context of AD, and not all forms of glial activation are damaging—some are likely even therapeutically advantageous [26, 123, 133]. The lines of evidence presented above suggest that brain pro-inflammatory events associated with S100B-dependent glial activation in close proximity to β-amyloid plaques lead to exacerbation of AD-like pathology, supporting the notion that chronic and prolonged activation of glia is detrimental in the context of neurodegenerative disease [81, 87, 134]. Such findings lend further support to the hypothesis that the astrocyte-derived protein, S100B, exacerbates the pro-inflammatory cytokine cycle, AD-like pathology, and associated brain injury [88–90]. A model depicting brain Aβ production/clearance, the harmful actions of S100B on Aβ production, and resultant effects on brain and plasma Aβ levels is shown in Figure 4.

Astrocyte-derived S100B promotes neurite extension and contributes to synaptogenesis and synapse remodeling in the developing and in the mature brain under physiological conditions [31, 32, 34, 38]. However, in the context of brain pathology such as AD, S100B may confer maladaptive neuritic changes in β-amyloid plaques. Supporting this notion, it has been reported that astrocytic overexpression of S100B drives dystrophic neurite outgrowth, culminating in the formation of neuritic plaques in Down's syndrome [135], and that S100B induction precedes appearance of neuritic β-amyloid plaques in the PSAPP AD mouse model [101]. Thus, S100B may drive conversion of diffuse, nonfibrillar β-amyloid deposits to neuritic β-amyloid plaques [30]. It is noteworthy that, while β-amyloid plaque morphometric analysis disclosed significant increases in small, medium, and large plaque size subsets in Tg2576-huS100B mice, we consistently noted the greatest exacerbation in medium- and large-sized plaques. Further, the huS100B transgene produced a number of "gigantic" β-amyloid plaques (>150 μm) in aged bigenic mice. These data can be interpreted as consistent with a role for S100B in the maturation of β-amyloid deposits.

In addition to affecting cerebral parenchymal Aβ burden, the huS100B transgene also promotes cerebral vascular β-amyloid deposits. While Aβ synthesis has classically been regarded to predominantly take place at neuronal synapses, it has recently been reported that reactive astrocytes surrounding β-amyloid plaques express β-secretase in AD patients and in Tg2576 mice [136, 137]. Thus, the huS100B transgene may directly impact astrocytic amyloidogenic APP metabolism. This would explain both the exacerbation of parenchymal and cerebrovascular Aβ deposits in bigenic

mice. But, does S100B accelerate cerebral amyloidosis by directly promoting amyloidogenic metabolism of APP? To evaluate this possibility, we examined amyloidogenic β-carboxyl terminal fragment (β-CTF; also known as C99) and amino-terminal APP cleavage products (soluble APPβ) and noted increases in both species in Tg2576-huS100B versus Tg2576 mice. We also found increased levels of β-site APP cleaving enzyme 1 (BACE1; commonly known as β-secretase), and enhanced enzymatic activity in brain homogenates from Tg2576-huS100B mice. Thus, in addition to its ability to promote brain inflammatory responses, our data show that S100B has a previously unappreciated role in directly promoting amyloidogenic APP processing.

The results discussed above using an *in vivo* genetic approach can be interpreted as definitive evidence that forced expression of human S100B exacerbates AD-like pathology, including brain parenchymal and cerebral vascular β-amyloid deposits, Aβ levels, β-amyloid deposit-associated astrocytosis and microgliosis, and pro-inflammatory cytokines [138]. These data suggest that inhibition of S100B represents a novel therapeutic target for AD. To further explore this tantalizing possibility, we took a pharmacological approach to blocking S100B biosynthesis, as detailed below.

4.2. Arundic Acid Ameliorates Cerebral Amyloidosis and Gliosis in Alzheimer Transgenic Mice. We have previously shown that arundic acid negatively regulates S100B synthesis by suppressing mRNA expression in activated astrocytes [93]. In addition, arundic acid exerts further effects both *in vitro* and *in vivo* on other biomolecules as described above. To examine whether inhibiting reactive astrocyte-derived S100B might impact progression of AD-like pathology in the Tg2576 AD mouse model [127], we orally administered the S100B biosynthesis blocker, arundic acid, to Tg2576 mice for 6 months, commencing at 12 months of age (when β-amyloid plaques are initially present in this AD mouse model). Strikingly, arundic acid significantly suppressed cerebral amyloidosis in treated Tg2576 mice. Importantly, β-amyloid deposits were significantly decreased in arundic acid-treated Tg2576 mice, irrespective of size. Concurrent reductions in brain levels of both soluble and insoluble Aβ species corroborated our histopathological observations. Notably, β-amyloid plaque-associated reactive astrocytosis and microgliosis were also significantly inhibited, suggesting that arundic acid treatment arrested the feed-forward pro-inflammatory cytokine cycle that would be expected to enable astrocytic activation of microglia.

In terms of a direct mechanism for the beneficial effects of arundic acid on reducing AD-like pathology, a number of possibilities deserve consideration. Firstly, the drug might alter APP expression in Tg2576 mice, either by affecting endogenous murine APP levels or by modulating the hamster prion promoter-driven mutant human APP transgene [127]. Yet, APP expression in brain homogenates was comparable between arundic acid-treated and vehicle-treated Tg2576 mice, making this explanation seem unlikely. Secondly, as long-term administration of indomethacin, a key inhibitor of the inflammatory mediator NF-κB, significantly reduces cerebral amyloidosis in Tg2576 mice [139], and NF-κB is downstream of S100B [58], it is possible that arundic acid-induced inhibition of NF-κB activity may mitigate amyloidosis in Tg2576 mice. In fact, we previously observed that lipopolysaccharide-induced activation of NF-κB in cultured astrocytes was significantly attenuated by arundic acid [93]. Thus, it seems likely that NF-κB inhibition is at least partly responsible for arundic acid-induced reduction of Aβ pathology in this scenario. Thirdly, biochemical evidence of decreased brain levels of soluble and insoluble Aβ—against a backdrop of unaltered APP production—suggests inhibition of amyloidogenic APP metabolism in arundic acid-treated Tg2576 mice. Whether this effect occurs via direct inhibition of β-secretase, or via an indirect mechanism, remains to be elucidated. Because, as mentioned above, astrocytes express β-secretase and have been directly implicated in amyloidogenic APP metabolism in the context of AD [136, 137], a direct mechanism of action remains possible.

Lastly, in addition to the pro-inflammatory effects of astrocytic S100B, its effects on neural reparative processes might be related to β-amyloid plaque development. It has been proposed that synaptic dysfunction occurs in both the prodromal and the clinical phases of AD [98], and excessive neuroplastic burden has been postulated to be a prime mover in the disease process [140]. It has been reported that astrocytic filopodia in tripartite synapses sense alterations in synaptic transmission, leading to their activation [141]. Activated astrocytes contribute to both reparative and destructive actions, which are considered to be at least partially mediated by altered levels of S100B [142]. Thus, pharmacological suppression of astrocytic S100B biosynthesis by arundic acid may have dual roles in delaying disease progression: in brain regions with relatively low levels of S100B, arundic acid might inhibit neurite extension in response to cerebral amyloidosis, and thereby decrease neuroplastic burden. By contrast, in brain regions where S100B is highly expressed, the agent may suppress the pro-inflammatory autotoxic loop [24, 30] and thereby reduce β-amyloid plaque maturation.

5. Concluding Remarks

In this paper, we have considered beneficial physiological functions and detrimental roles of the astrocyte-derived protein S100B in cerebral ischemia and in AD. Importantly, we propose that both of these neurological diseases share a common pathogenic mechanism—maladaptive astrocytic activation—where S100B acts as a perpetrator of neuroinflammation and neurotoxicity. Using both genetic and pharmacological approaches, we have produced evidence supporting the idea that enhanced and prolonged activation of astrocytes plays a detrimental role in the pathogenesis of cerebral ischemia and AD, and that astroctye-derived S100B is at the epicenter of these damaging cellular responses. In agreement with these lines of evidence, another group has demonstrated that S100B transgenic and knockout mice show worsening and attenuation, respectively, of ischemic brain damage [143]. In terms of AD, there is evidence that S100B transgenic mice show enhanced susceptibility to

neuroinflammation and neuronal dysfunction induced by intracerebroventricular infusion of human β-amlyoid [144]. These and other studies unequivocally suggest that inhibiting astrocytic activation by pharmacological blockade of S100B biosynthesis may be a valuable therapeutic strategy to combat ischemic stroke and to delay onset and/or progression of AD. As with any pharmacological approach, it is important to note that there are advantages and disadvantages to modulating astrocytic activation via targeting S100B. Since available data are limited using genetic approach(es) to elucidate the possible role(s) of S100B in CNS health and disease, further studies using S100B and RAGE knockout mice, either singly or doubly-deficient for these genes, are warranted. The availability of selective S100B biosynthesis inhibitors such as arundic acid is expected to further translational research for neurological and neurodegenerative diseases, and perhaps other disorders, such as inflammatory bowl disease, which are S100B overexpresssion-related. Such future studies will go on to address the tantalizing possibility of next-generation therapeutics for ischemic stroke and AD aimed at the pleiotropic S100B pathway.

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Review Article

The S100B/RAGE Axis in Alzheimer's Disease

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Increasing evidence suggests that the small EF-hand calcium-binding protein S100B plays an important role in Alzheimer's disease. Among other evidences are the increased levels of both S100B and its receptor, the Receptor for Advanced Glycation Endproducts (RAGEs) in the AD diseased brain. The regulation of RAGE signaling by S100B is complex and probably involves other ligands including the amyloid beta peptide (Aβ), the Advanced Glycation Endproducts (AGEs), or transtheyretin. In this paper we discuss the current literature regarding the role of S100B/RAGE activation in Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly [1]. AD patients suffer from a progressive decline of cognitive functions that include language, personality, and memory impairments.

The pathological hallmarks of the disease are characterized by the presence of senile plaques (SPs), neurofibrillary tangles (NFTs), and severe gliosis in the cerebral cortex and the hippocampus [2]. Senile plaques result from the accumulation of extracellular amyloid-β (Aβ) fibrils [3] and contain elevated levels of zinc and copper ions [4]. Neurofibrillar tangles are mainly constituted of intracellular, abnormally phosphorylated tau protein [5–7]. AD brain is also characterized by increases in inflammatory responses, oxidative stress, dysregulation of calcium homeostasis [8], and by elevated levels of several S100 calcium-binding proteins namely S100B, S100A6, S100A9, and S100A12 [9–12].

Neurons, microglia, and endothelial cells, surrounding the senile plaques express higher levels of the receptor for advanced glycation endproducts (RAGEs) as the pathology progresses [13, 14]. Although its exact role in AD remains to be clearly established, RAGE appears to initiate several signal transduction cascades in response to ligands, related to AD including Aβ, AGEs, transthyretin, and S100 proteins. The present paper will focus and discuss the current knowledge on the role of S100B/RAGE axis in AD.

2. The Receptor for Advanced Glycation Endproducts

RAGE is an immunoglobulin-like cell surface receptor that is often described as a pattern recognition receptor due to the structural heterogeneity of its ligands. RAGE was initially identified as receptor for the advanced glycation endproducts (AGEs) [15, 16]. AGEs are formed by nonenzymatic modification of proteins or lipids by reducing carbohydrates, are highly heterogeneous (reviewed in [17]), and are often found elevated at sites of inflammation where they can trigger RAGE-dependent oxidative stress and NF-κB activation. NF-κB activation leads to increased RAGE expression because of the presence of NF-κB response elements within the promotor region of RAGE [18]. Activation of RAGE in turn results in sustained NF-κB activation [19]. Positive feedback loops between RAGE, oxidative stress, and inflammation can thus develop [20]. In this view high levels of AGEs have been found at site of inflammation and colocalize with neurofibrillar tangles and senile plaques in AD brain [21–23].

A second group of RAGE ligand is formed by amyloid-forming proteins or peptides such as Aβ peptide [13], and transthyretin (TTR) [24]. The amyloid β-peptide results from amyloid precursor protein (APP) processing by the beta and gamma secretases. Aβ accumulation in the brain plays a key role in the development of the disease [13, 25]. RAGE has been shown to mediate the transport of Aβ through the neuronal cell membrane and blood brain barrier [13, 26, 27].

In contrast, TTR has been suggested to have a protective effect in AD by binding to Aβ in a chaperone-like manner [28].

RAGE can also be activated by amphoterin (High Mobility Group Box 1, HMGB1) that plays a role in neuronal development and cancer [29]. Although it also plays a role in inflammation, we will not discuss the putative role of amphoterin in AD in this paper [30].

Another group of RAGE ligand is constituted by the S100 proteins. S100 proteins are small EF-hand calcium-binding proteins that regulate calcium homeostasis and modulate various enzymes involved in cellular functions such as cell growth, differentiation, and metabolism (reviewed in [31–33]). Twenty one members of S100 proteins have been described [34]. They all share high amino acid and structural homologies. Among them S100B, S100A6, S100A9 and S100A12 have been linked to Alzheimer's Disease [9–12].

Various alternatively spliced isoforms of RAGE exist [16, 35]. The two prevalent isoforms appear to be the full-length RAGE (RAGE) and the secreted isoform RAGE_v1 [36]. Full-length RAGE is composed of an extracellular part (314 aa), a single transmembrane spanning helix (27 aa), and a short cytosolic domain (41 aa) (Figure 1) [16]. The extracellular part of RAGE contains an Ig-like V-domain (residues 24–127) and two constant Ig-like C type domains frequently referred to as C1 (residues 132–230) and C2 domains (residues 239–320). RAGE possesses two N-glycosylation sites, one adjacent to the V-domain (residue 26) and the second one within the V domain (residue 81) (Figure 1) [16, 37]. Recent studies suggest that glycosylation may modulate the interaction of certain AGEs with RAGE [38, 39]. The RAGE_v1 splice isoform lacks the transmembrane and cytoplasmic portion and is released in the extracellular space (Figure 1) [36, 40–42]. The distribution and relative expression of the different RAGE isoforms are tissue specific. The full-length RAGE isoform is present at low levels in most adult tissues but at relatively high levels in lungs [43]. The truncated variant RAGE_v1 appears to be the prevalent isoform in endothelial cells and in human brain (Figure 1) [41, 44]. Interestingly, the soluble form of RAGE (sRAGE) can also be produced by proteolytic cleavage [45–47]. sRAGE produced either by splicing or shedding has been suggested to play the role of a decoy that interacts with free circulating RAGE ligand. RAGE_v1 expression is reduced in hippocampal neurons of AD patients. This could potentially lead to a sustained RAGE activation [48, 49]. In this view, sRAGE formed as a result of proteolysis could prevent Aβ peptide transport across the blood brain barrier and protect against Alzheimer's disease [50]. In the last five years, soluble RAGE has emerged as a new biomarker with potential clinical and therapeutic applications (reviewed in [51, 52]) and polypeptides based on RAGE_v1 are currently tested in clinical trials for their therapeutic effects against deleterious effects triggered by RAGE activation by its ligands.

However, the role of sRAGE and its regulation appears to be very complex. Indeed recent studies aiming at comparing the concentration of sRAGE in the serum of patients versus controls in various pathophysiological conditions have shown both negative and positive correlation between

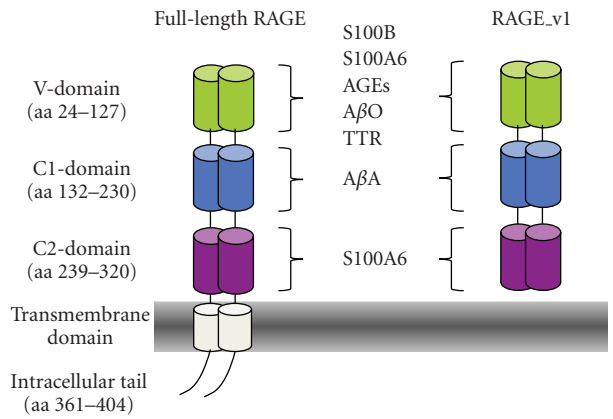

Figure 1: Schematic representation of the two main RAGE isoforms, full-length RAGE and RAGE_v1. Full-length RAGE is an immunoglobulin like receptor with one variable-like domain (V) and two constant-like domains (C) comprising residues. A short transmembrane domain anchors RAGE to the cell surface. A 41 residues intracellular tail is critical for signal transduction. RAGE_v1 does not possess the transmembrane domain and the intracellular tail. It is soluble in the circulation and plays the role of decoy to antagonize the activation of full-length RAGE by its ligands. A soluble form of RAGE can also be generated by proteolysis. S100B, AGEs Aβ oligomers, and TTR bind to RAGE V domain. Aβ aggregates binds to RAGE C1 domain. S100A6 binds both to the V and C2 domain but exerts its cellular effects preferentially through the C2 domain. The exact oligomerization states of full-length RAGE and RAGE_v1 are currently unknown. RAGE is arbitrarily represented as a dimer.

the concentration of sRAGE and the severity of the disease ([53, 54] and reviewed in [55]). Moreover, blocking RAGE function might not be beneficial in all pathologies. Indeed RAGE has been shown to modulate the regeneration of peripheral nerves in a mouse model of axotomy and blockade of RAGE signaling using sRAGE resulted in impaired regeneration in these animals [56–58]. Although a large number of studies with rodent models of human diseases have demonstrated the short-term benefit of treatment with sRAGE (reviewed in [52]), the long-term effects of such treatments remain also to be studied. The role of sRAGE as a decoy that would neutralize the excess of RAGE ligands also needs to be reconsidered at the view at recent studies showing very low concentrations (10–50 pM) of sRAGE in the serum of both patients and healthy individuals [53–55].

In order to understand the role of RAGE in the various RAGE-related pathologies including AD, it is important to understand how the different RAGE ligands interact with the receptor. Binding and activation of RAGE by S100B was first demonstrated in HUVEC cells [59]. In recent years, our laboratory and others have studied in detail the interaction of S100B with RAGE. S100B interacts preferentially with the V domain of RAGE and might involve multimerization of the receptor [60–62]. The V domain of RAGE is also the binding site of AGEs and TTR [63–66].

Aβ-RAGE interactions are more complex since Aβ exhibits several conformational states. Aβ is generated

by proteolytic cleavage of the transmembrane β-amyloid precursor protein (APP) (reviewed in [67]). The resulting 1-40 or 1-42 amino acid Aβ peptides can form soluble oligomers (AβO), beta-sheet containing fibrils, and insoluble aggregates (AβA) [25, 68–74]. It is now believed that the synaptic dysfunction and neuronal death observed in AD patients are caused mainly by Aβ oligomers and Aβ fibrils [25, 71–73, 75–78]. We recently showed that the interaction of Aβ with RAGE is driven by conformational states of Aβ. Indeed AβO and AβA were found to bind to distinct domains of RAGE, the V-, and C1-domain, respectively. Furthermore, AβO RAGE interaction was found to modulate ERK activity and to induce neuronal death [25].

Although S100B, AGEs, and AβO interact with the V domain of the receptor, it is currently not known if they interact within the same region of the V domain. Future studies will answer this question.

3. RAGE in Alzheimer's Disease

RAGE is up-regulated in the brain of Azheimer's disease and triggers the generation of proinflammatory cytokines at the blood brain barrier [13, 27]. The role of RAGE in AD has been demonstrated in cell culture and in animal models. In various cell types that include neurons, endothelial cells, and microglia, engagement of RAGE by Aβ can lead to the formation of reactive oxygen species (ROS), the activation of NF-κB, or the expression of cell adhesion molecules mediating the recruitment of inflammatory cells [79, 80]. Neurons overexpressing RAGE showed higher susceptibility to Aβ-induced cell death than control cells [81]. Several models of transgenic mice have been used to demonstrate the role of RAGE in AD. The double transgenic RAGE/APP mouse model combines the overexpression of RAGE with the expression of mutants of APP [82, 83]. RAGE/APP mice show impaired spatial learning and memory capabilities, reduced basal synaptic transmission and long-term potentiation (LTP) compared to their single transgenic littermates. At the cellular level, these mice show reduced density of cholinergic fibers and synapses, characteristics often associated with AD-like pathology [83]. At the molecular level, RAGE/APP mice show enhanced activation of inflammation and stress-related MAP kinases and of the transcription factors NF-κB [83]. Despite these evidences the role of RAGE in Alzheimer's disease is still to be understood in detail. Indeed, recent experiments performed on RAGE (-/-) arcA-beta double transgenic animals showed that RAGE deletion could not prevent the decline in cognitive performance of the mice nor the age-related cerebral accumulation of Aβ peptides [84]. These discrepancies may be due to differences in the mouse models used in the distinct studies. The Arc mutation is characterized by a change in amino acid within the Aβ peptide sequence and thus may generate distinct peptide conformations that have less or no affinity for RAGE. Interestingly, the arcBeta transgenic mice showed reduced clearance of Aβ accross blood vessels [85]. This could reflect a decrease in the binding capacity of the arcAbeta for another of its receptor, LRP that has been shown to mediate brain efflux of Aβ [86].

4. S100B

S100B is a member of the S100 protein family mainly expressed in the CNS [87]. Animal studies using S100B transgenic mice revealed that S100B plays important roles in spatial and fear memory, learning capabilities, and epileptogenesis [88–90].

Unlike other members of the S100 protein family, the gene of S100B is located on human chromosome 21 [91, 92]. S100B possesses two Ca^{2+}-binding sites of the EF-hand type, defined as a helix-loop-helix motif connected by a central hinge region. The C-terminal domain contains the classical EF-hand with a canonical 12 amino acid Ca^{2+}-binding loop whereas the N-terminal domain contains the S100B specific 14 amino acid Ca^{2+}-binding loop [93, 94]. S100B binds two calcium ions per subunit with moderate affinity (2–20 μM) [95]. Binding of calcium to the EF-hands triggers structural changes that allow the interaction with target proteins [32, 96]. Besides calcium, S100B also binds zinc (K_D = 0.1–1 μM) and copper (K_D = 2.2 μM), two metal ions highly abundant in senile plaques [32, 96–98]. Interestingly, binding of zinc to S100B results in higher affinity for both calcium and S100B's target proteins. The extracellular function of S100B may thus be altered in the brain of AD patients due to the high levels of zinc and copper [99].

S100B interacts with various intracellular targets. These targets have been extensively described in previous reviews [98, 100, 101]. S100B interacts with elements of the cytoskeleton (microtubules, type III intermediate filaments), with enzymes of the glycolytic pathway (fructose 1,6-bisphosphate aldolase, phosphoglucomutase), and with the tumor suppressor p53. S100B also regulates calcium homeostasis, protein phosphorylation and degradation [100]. S100B is mainly found as homodimer but can also form active tetramers, or hexamers exhibiting distinct functions [61, 102–104]. Furthermore, S100B is also able to interact with S100A1. This protein complex exhibits distinct physiological functions compared to S100B or S100A1 homodimers [32, 61, 102–105].

Besides its known intracellular function, S100B can also be secreted in the extracellular space where it acts as a cytokine. The secretion of S100B occurs via both the classical endoplasmic reticulum-Golgi pathway and an alternative pathway involving cytoskeletal tubulin [106, 107].

High levels of extracellular S100B have been detected in various clinical conditions that include brain trauma, ischemia and neurodegenerative, and inflammatory and psychiatric diseases [108, 109]. S100B is also a well-established prognostic marker for melanoma and high serum concentration of S100B correlate with poor prognosis [110, 111].

In the brain, S100B is actively secreted from astrocytes in the extracellular medium (Figure 2) [112]. S100B release is driven by the developmental stage of the astrocytes [112], and metabolic stress (oxygen, serum, or glucose deprivation) [113]. S100B can also be released in response to external stimuli such as glutamate [114], serotonin [115], the pro-inflammatory cytokines TNF-alpha [116] and IL-1beta [117], beta-amyloid peptides [118], 1-methyl-4-phenyl 1,2,3, and 6 tetrahydropyridine (MPTP) [119],

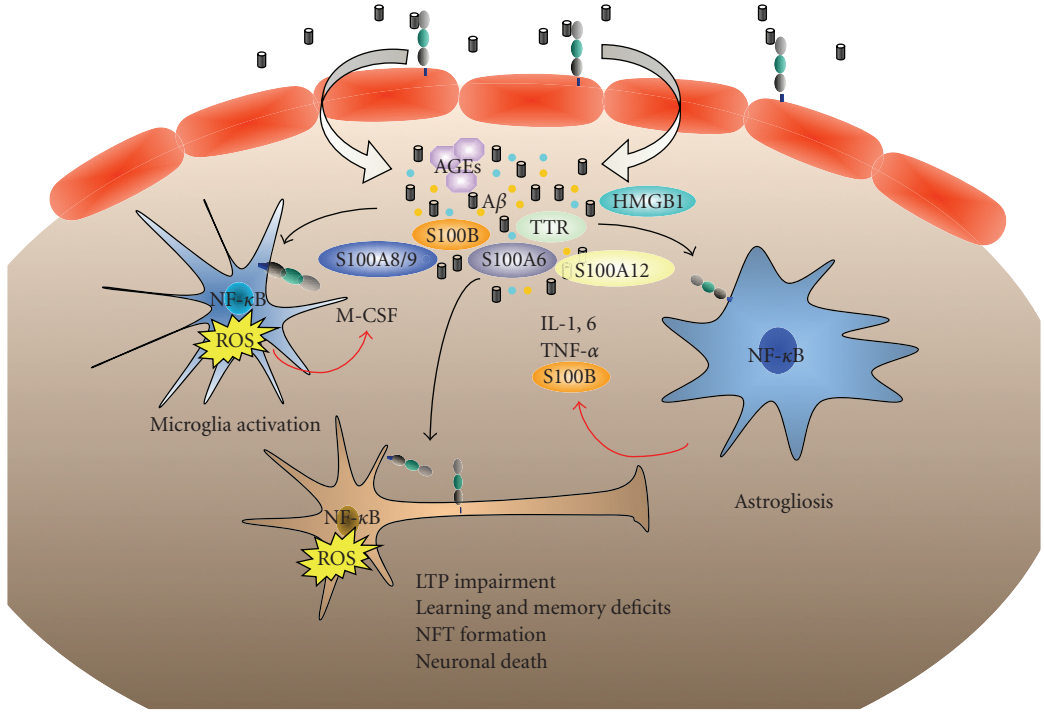

Figure 2: Crosstalk between RAGE and its ligands in Alzheimer's disease. RAGE mediates Aβ brain influx and accumulation. Aβ directly or indirectly triggers dysregulation of calcium homeostasis thereby activating the S100 proteins. RAGE-mediated activation of glia cells results in the activation of NF-κB driven gene transcription, and the release of inflammatory cytokines such as IL-1, IL-6, TNF-α, IL-1β, M-CSF and S100B. The brain of AD patients becomes the site of intense inflammation and oxidative stress that facilitates formation of AGEs. S100B, Aβ and AGEs as well as other RAGE ligands including TTR, HMGB1, S100A6, S100A8/A9, and S100A12 accumulate in the brain during the course of the disease. Secreted S100B and chronic RAGE activation trigger several AD-associated neuropathological features including microglia activation, the production of reactive oxygen species (ROS), neurite degeneration, NFT formation, and neuronal apoptosis ultimately leading to memory impairment.

forskolin, lysophosphatidic acid [120], and the plant natural antioxydants resveratrol and epicatechin [121, 122] and by the increase of calcium concentration [107].

Extracellular S100B has been shown to modulate the activity of neurons, microglia, astrocytes, monocytes, and endothelial cells (Figure 2). On neurons, S100B triggers trophic or toxic effects, depending on its concentration. Nanomolar concentration of S100B is neuroprotective, induces neurite outgrowth, and triggers glial cell proliferation in a RAGE dependent manner, whereas micromolar concentration of S100B is neurotoxic [120, 123–126]. At the molecular level, nanomolar concentration of S100B induces the upregulation of the antiapoptotic factor Bcl-2 resulting in neuroprotection. In contrast, when present in micromolar concentration S100B induces the up-regulation of caspase 3 through the activation of the oxidant stress-dependent MEK/ERK pathways, leading to apoptosis [126]. In addition, S100B can also modulate the toxicity of other extracellular molecules. In rat hippocampal neurons low concentration of S100B protects the cells against the toxic effect of N-methyl-D-aspartate, through the activation of NF-κB and possibly through the engagment of RAGE [127]. S100B also protects astrocytes and microglia against toxicity of trimethyltin [128]. Similar protection is observed in LAN-5 neuroblastomas, in the presence of Aβ peptide [129]. Importantly, in these cells, higher concentration of S100B (micromolar) potentiates the toxicity of Aβ peptide.

S100B activates astrocytes in an autocrine manner and triggers the release of TNF-α and IL-6, probably through the activation of RAGE [130] leading to cellular inflammation (Figure 2).

Extracellular S100B can also stimulate endothelial cells, resulting in perpetuated activation of NF-κB and the up-regulation of vascular cell-adhesion molecule (VCAM-1) and the monocyte chemoattractant protein 1 (MCP-1) through the engagement of RAGE [59, 131]. Stimulation of endothelial cells by S100B results in adhesion and transendothelial migration of monocytes, leading to further inflammation in adjacent tissue [132]. Engagement of RAGE by S100B could thus contribute to the chronicity of inflammation observed to Alzheimer's disease [19].

5. S100B in Alzheimer's Disease

A role of S100B in AD is suggested by a large number of clinical studies showing elevated levels of S100B in the brain or cerebrospinal fluid of AD patients [9, 108, 133–137]. Furthermore, studies showed enhanced susceptibility to neuroinflammation and neuronal dysfunction after infusion

of Aβ in transgenic mice overexpressing S100B supporting a role for S100B in AD [138]. Additional supporting evidence comes from recent studies using double transgenic mice over-expressing S100B and carrying mutation in APP (Tg2576/S100B) [139]. Over-expression of human S100B in these mice promotes brain inflammation as shown by astrogliosis and microgliosis and enhances Aβ generation from APP.

Evidence also suggests a role of S100B in the formation of neurofibrillar tangles. Hyperphosphorylated tau protein is the main component of neurofibrilar tangles. S100B binds directly to tau protein in the presence of calcium resulting in the inhibition of its phosphorylation by Ca^{2+}/Calmodulin-dependent kinase II [140]. Intriguingly, extracellular S100B has also been found to promote RAGE-dependent hyperphosphorylation of tau protein through the modulation of the JNK and Wnt pathways [141]. Thus, S100B exhibits opposite effects depending on its localization. In AD patients S100B is actively released and may promote the hyperphosphorylation of tau protein and the development of neurofibrillary tangles in a RAGE dependent manner [129, 142, 143]. The secretion of S100B itself might be triggered by RAGE endocytosis [144, 145].

Thus, it is tempting to speculate that the role of S100B in Alzheimer's disease is mediated by RAGE and numerous studies mentioned in this paper support this hypothesis. Targeting specifically RAGE/S100B interaction in the brain might be beneficial to AD patients. Another interesting therapeutic approach may be to inhibit the binding of both S100B and Aβ to the V domain of RAGE using specific antibodies or small molecules.

6. Other RAGE Ligands in Alzheimer's Disease

Besides S100B, RAGE can also be engaged by other ligands that are all relevant in Alzheimer's disease.

S100A6 is another member of the S100 protein family. S100A6 is upregulated in astrocytes of animal models and in patients with AD [10]. High levels of this protein were also found in the senile plaques of AD patients [10]. The exact role of S100A6 in AD is currently unknown but our recent studies suggest that S100A6 might play a role through RAGE. Indeed we recently showed that S100A6 interacts with both the V- and C2-domains of RAGE *in vitro*. However, in contrast to S100B, the cellular effects triggered by S100A6 appeared to occur via the C2-domain only [62]. Two other S100 family members, S100A8/A9 and S100A12 may play a role in AD as well by participating in inflammatory-mediated events contributing to neurodegeneration. High levels of S100A9 and S100A12 have been found in microglia of patients suffering from sporadic AD [11, 12]. As with S100B, the effects triggered by S100A8/A9 and S100A12 could involve RAGE. Indeed, these two cytokine-like S100 proteins have been shown to interact with RAGE and to trigger RAGE-dependent cellular signaling [59, 146, 147] leading to sustained inflammation [24, 28, 148–153]. Thus, RAGE can be engaged by distinct ligands associated with AD.

Beside S100 proteins the senile plaques also contain elevated levels of AGEs, and TTR. The role of TTR in AD is suggested from both in vitro experiments and animal models studies [151–153]. RAGE interacts with both soluble and fibrillar TTR [149, 150]. TTR might have a protective effect in AD by binding to Aβ in a chaperone-like manner [28]. In AD settings, production of cytokines as a result of local inflammation would suppress TTR expression and reduce its protective role. However in other conditions TTR could also trigger NF-κB activation through RAGE resulting in sustained inflammation and cellular stress [24, 150].

7. Synergistic Effects between RAGE Ligands

Recent cell-based experiments have shown synergistic effects between the different RAGE ligands. In cultured neurons, AGEs and Aβ act synergistically resulting in increased APP and RAGE expression [142]. In microglia, Aβ acts as an amplifier of the inflammatory response when cells are preactivated with AGEs [143]. In endothelial cells, only AGEs pretreated cells could respond to stimulation by S100A8/A9 [154]. As mentioned earlier, S100B can also potentiate the toxic effect of Aβ in LAN-5 neuroblastomas [129].

8. Conclusion

Alzheimer's disease is a complex disease involving many molecular partners including RAGE and S100B. Following the large number of promising studies where blockade of RAGE could reverse a large number of symptoms in animal models, RAGE became a well-pursued therapeutic target. We mentioned earlier in the paper that polypeptides based on the sequence of sRAGE were currently evaluated in clinical trials [155]. Small molecule compounds are also currently in phase 2 clinical trials (Pfizer: PF-04494700 [156]). Targeting RAGE would be beneficial to treat chronic RAGE-dependent pathologies. However, the recent studies on the role of RAGE in peripheral nerve regeneration also suggest that care must be taken when blocking RAGE signaling.

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Review Article

S100B Serum Levels in Schizophrenia Are Presumably Related to Visceral Obesity and Insulin Resistance

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Elevated blood levels of S100B in schizophrenia have so far been mainly attributed to glial pathology, as S100B is produced by astro- and oligodendroglial cells and is thought to act as a neurotrophic factor with effects on synaptogenesis, dopaminergic and glutamatergic neutrotransmission. However, adipocytes are another important source of S100B since the concentration of S100B in adipose tissue is as high as in nervous tissue. Insulin is downregulating S100B in adipocytes, astrocyte cultures and rat brain. As reviewed in this paper, our recent studies suggest that overweight, visceral obesity, and peripheral/cerebral insulin resistance may be pivotal for at least part of the elevated S100B serum levels in schizophrenia. In the context of this recently identified framework of metabolic disturbances accompanying S100B elevation in schizophrenia, it rather has to be attributed to systemic alterations in glucose metabolism than to be considered a surrogate marker for astrocyte-specific pathologies.

1. Significance of S100B as Surrogate Marker for Glial or Blood-Brain Barrier Dysfunction in Schizophrenia

S100B is a secretory protein which is implicated in various intracellular and extracellular functional processes. Previous studies have indicated its involvement in the regulation of protein phosphorylation, cytoskeleton assembly, Ca^{2+} homeostasis, transcription factors, and glucose metabolism [1]. Blood levels of S100B levels are increased in schizophrenia, as summarized in a recent meta-analysis of 13 studies involving 420 patients with schizophrenia and 393 control subjects [2]. Serum S100B reaches high effect sizes in schizophrenia compared to controls (mean ± SD: 2.02 ± 1.78), as confirmed by including only studies investigating drug-free patients (mean ± SD: 1.94 ± 1.33; $n = 7$). Elevated S100B levels were associated with acute exacerbations or deficit symptoms and have frequently been attributed to glial damage and dysfunction or blood-brain barrier leakage [2–5]. S100B is produced by astrocytes and oligodendrocytes and is acting as a neurotrophic factor with effects on synaptogenesis, dopaminergic and glutamatergic neutrotransmission [6–9]. Whitaker-Azmitia et al. observed a loss of dendrites in mice overexpressing S100B [10]. Interestingly, schizophrenia is indeed associated with certain haplotypes, leading to an increased S100B expression [11], and postmortem studies are suggestive of a progressive reduction of neuropil [12]. Therefore, a causal link between the aforementioned finding of elevated S100B levels and schizophrenia might be considered.

A recent analysis of cerebrospinal fluid (CSF) from first onset schizophrenia cases observed increased levels of S100B, without indications for impaired glial or neuronal cell integrity, as assessed by simultaneous measurement of non-secretory glial and neuronal proteins (glial fibrillary acidic protein/GFAP, myelin basic protein/MBP, neuron specific enolase/NSE) [13]. This finding speaks against the assumption of glial damage during acute psychosis, but

could be interpreted as indirect evidence for an activated glial synthesis and release of S100B during acute psychosis. Of note, S100B has also been detected outside the nervous system, for example, in adipose and chondroid tissues, lymphocytes, melanocytes, the myocardium, and vascular endothelial/smooth muscle cells [8].

In conclusion, previous findings support the hypothesis that S100B is involved in the pathogenesis of schizophrenia, but elevated levels of this protein may not exclusively reflect brain- or glial-specific pathologies. Recently, Marchi et al. suggested that serum S100B is an ideal marker of blood-brain barrier integrity, because with a molecular weight of 21 kDa (S100B dimer) it may not penetrate through an intact blood-brain barrier [14]. Furthermore, its concentration is high in central nervous system fluids and low in blood. Indeed, serum levels of S100B were directly correlated with an experimental opening of the blood-brain barrier [14–16]. However, unlike in patients suffering from stroke, traumatic brain injury, or inflammatory brain disorders, there is no clear experimental evidence for a disruption of the blood-brain barrier in schizophrenia. Therefore, it remains unclear if serum levels of S100B are reflecting blood-brain barrier integrity also in schizophrenia.

2. Altered Peripheral and Cerebral Glucose Metabolism in Schizophrenia

Recent studies indicate novel interpretations of previous S100B findings in the context of disturbances in energy metabolism in schizophrenia (see below). Therefore, this section is briefly summarizing current knowledge on schizophrenia-related alterations in glucose metabolism.

Schizophrenia is characterized by a 20% higher mortality rate compared with the general population. Important contributing factors are an increased risk for type 2 diabetes and metabolic syndrome (defined by the American Heart Association as presence of three or more of the components: abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, prothrombotic state, and proinflammatory state). Weight gain and impaired glucose tolerance have been mainly attributed to side effects of atypical antipsychotic medication, such as clozapine and olanzapine [17–19]. However, impaired fasting glucose tolerance has also been reported in drug naïve schizophrenia cases [20–25] and unaffected siblings [26], suggesting disease-inherent abnormalities in peripheral glucose metabolism.

Cerebral insulin signaling seems to be likewise affected in schizophrenia [27, 28], probably causing disturbances in neural glucose uptake and utilization, as revealed by measurements of elevated CSF glucose levels [29], in vivo fluorodeoxyglucose positron emission tomography (FDG-PET) and functional magnetic resonance imaging (fMRI) studies [30–32]. State-dependent alterations in cerebral glucose-metabolism and functional disconnection have been found in brain regions which are involved in the pathophysiology of schizophrenia (e.g., of the prefrontal cortex, thalamus and mediotemporal lobe). The mediotemporal region and the hippocampus in particular appear to be of specific importance for cognitive impairment in schizophrenia. Studies addressing neuronal activation using fMRI have demonstrated that schizophrenic subjects show impaired patterns of hippocampal activity in novelty detection, declarative learning, and memory tasks [33–35]. Notably, it has been demonstrated that hippocampus-dependent memory performance can be improved by the administration of glucose in rodents and humans [36, 37]. In humans it has been shown that this effect is more pronounced when the task is cognitively demanding [37], or when the cognitive resources that can be applied for it are sparse, as is the case in the elderly and in schizophrenia patients [38, 39]. Of note, low rodent insulin-like growth factor 1 (IGF-1) concentrations were associated with impaired glucose metabolism in brain areas involved in learning and memory [40]. In schizophrenic subjects, clozapine has been shown to normalize disease-related IGF-1 deficits [41–43], and glucose administration boosts mediotemporal as well as dorsolateral prefrontal neuronal activity during the encoding into declarative memory [44, 45].

3. Adipose Tissue as a Potential Source of S100B

Adipocytes appear to be an important source of serum S100B since the concentration of S100B in adipose tissue is as high as in nervous tissue [46–49]. This fact has been barely considered during the past years. Remarkably, S100B is closely linked to the regulation of cellular energy metabolism. An immunoelectron-microscopic study suggested that S100B may be involved in the regulation of lipolysis [50]. The release of S100B from adipocytes is reduced by insulin, and activated by physiological factors such as stress (catecholamines and adrenocorticotropic hormone (ACTH)) or fasting [51–53]. Interestingly, a study on streptotocin-induced diabetes in Sprague-Dawley rats revealed a 2-fold increase in S100B protein levels in both brain and white fat tissue [54]. Therefore, an increased adipose tissue mass or changes of insulin metabolism such as insulin resistance most probably play a major role in increased S100B levels in schizophrenia too, given the increased prevalence of obesity and metabolic syndrome in patients and their first-degree relatives (see above) [55, 56].

4. S100B Serum Is Correlated with Body Mass Index and A-FABP in Healthy Human Subjects

Indeed, a recent study showed a close correlation between body mass index (BMI) and serum S100B levels [57]. This study assessed S100B serum levels in 60 adult subjects (36 female, 24 male, age 22–58 years) with a BMI between 18–45 kg/m^2 without a prior history of neuropsychiatric disorders. S100B levels correlated with the BMI ($r = 0.538$, $P < .001$), levels of leptin ($r = 0.683$, $P < .001$), and adipocyte-type fatty acid-binding protein (A-FABP; $r = 0.801$, $P < .001$) (Figure 1(a)). Accordingly, follow-up single group comparisons of BMI groups showed that S100B levels in obesity were significantly higher than in overweight ($P =$

.006, Cohen's $d = 2.25$), and normal weight subjects ($P = .001$, Cohen's $d = 2.90$), or in overweight higher than in the latter ($P = .049$, Cohen's $d = 0.65$) (Figure 1(b)). A stepwise regression analysis showed that of the variables age, leptin, and A-FABP only the latter was significantly predicting S100B ($P < .001$). Correspondingly, new evidence from population studies and experimental animal models indicates that serum A-FABP is a powerful new risk marker for predicting metabolic syndrome and arteriosclerosis [58].

Effect sizes as measured by Cohen's d (see upper paragraph) indicated medium ($0.5 < d < 0.8$) to strong effects ($0.9 < d$) of BMI on S100B blood levels. This finding may explain previous reports indicating a direct relationship between S100B blood levels with body weight in anorexia nervosa: Effect sizes that were obtained from data given for anorexic subjects by Ehrlich et al. [59] before and after weight gain (i.e., mean ± SD of BMI: initially 14.5 ± 1.3, after >10% weight gain 17.1 ± 0.9; mean ± SD of S100B levels: initially 0.095 ± 0.041 μg/l, finally: 0.128 ± 0.063 μg/l) and by Holtkamp et al. [60] after 21 weeks of weight gain (mean ± SD of BMI: initially 14.8 ± 1.3, finally 17.0 ± 1.2; mean ± SD of S100B levels: initially 0.077 ± 0.023 μg/l, finally 0.107 ± 0.035 μg/l) were 0.81 and 1.3, respectively. In conclusion, S100B blood levels are directly related to BMI across an extensive range of nutritional states spanning from starvation to extreme obesity. Importantly, the effect sizes that BMI exerts on S100B blood levels in neuropsychiatrically healthy subjects (obesity compared to overweight: Cohen's $d = 2.25$; obesity compared to normal weight: Cohen's $d = 2.90$) are well within the range of effect sizes observed in schizophrenia (all studies: 2.02 ± 1.78; studies with drug-free patients: 1.94 ± 1.33) [2]. In conclusion, the correlation of S100B levels with other adipose-related measures, such as leptin and A-FABP, indicates that BMI or waist-to-hip ratio adjustments are strongly advised for future clinical studies examining peripheral blood S100B levels in order to avoid misinterpretation of results.

5. Elevated S100B Levels in Schizophrenia Are Presumably Associated with Visceral Obesity and Insulin Resistance

A recent study thus assessed S100B in both medicated and drug free schizophrenic patients along with the BMI, measures of glucose utilization and adipokine levels [61]. The subjects were comprised of 26 inpatients with acute paranoid schizophrenia and 32 control subjects, which did not differ significantly regarding age, gender, BMI and smoking habits (Table 1). Eleven patients were unmedicated for at least 6 weeks before admission (Table 2); 15 were already put on atypical antipsychotics (amisulpride, aripiprazole, clozapine, olanzapine, quetiapine, risperidone, or ziprasidone) for 26 ± 21 days, but still suffered from acute psychosis. Only benzodiazepines were allowed as additional psychotropic medication (for ≤6 days). Blood was collected between 9:00 and 11:00 AM (nonfasted). S100B and the following adipose-related factors were determined from serum samples: leptin, monocyte chemotactic protein 1 (MCP-1), hepatocyte growth factor (HGF), resistin, plasminogen activator inhibitor 1 (PAI-1), tumor necrosis factor alpha (TNF-α) and high-sensitivity C-reactive protein (hs-CRP). Levels of glucose, triglycerides and C-peptide[1] were also assessed.

In control subjects, circulating S100B concentrations correlated significantly with the BMI ($r = 0.540$, $P = .001$), as in the abovementioned study in 60 subjects without a prior history of neuropsychiatric disorders [57]. Moreover, adipokines (leptin: $r = 0.545$, $P = .001$, HGF: $r = 0.441$, $P = .012$, resistin: $r = 0.377$, $P = .033$) and C-peptide/glucose ratios (an estimate of insulin resistance, $r = 0.432$, $P = .014$) predicted S100B levels. In contrast, circulating S100B levels in schizophrenia subjects were neither correlated with the BMI (drug free: $r = -0.108$, $P = .751$; with drug: $r = -0.007$, $P = .981$) nor with levels of leptin, HGF and resistin (Figure 2). It has to be clarified by future studies, if the different finding in schizophrenia may be explained by a predominant visceral fat distribution, which is not adequately assessed by the BMI. This idea is supported by our finding of MCP-1-correlated S100B levels in the patient group ($r = 0.673$, $P = .023$). MCP-1 is known to be particularly related to the visceral fat mass [62].

As illustrated in Figure 3 and Table 1, acutely ill schizophrenic subjects showed elevated S100B levels ($P = .012$). Indications of insulin resistance were revealed by increased glucose ($P < .001$), C-peptide levels ($P = .002$) and C-peptide/glucose ratios ($P = .006$). S100B and BMI were elevated in medicated schizophrenic patients ($P = .041/P < .001$), but controls with a BMI ≥ 25 were also found to show increased S100B levels ($P = .025$) and comparable correlations held true when adipokines were considered as predictors of S100B levels. A disease specific increase of S100B could however be demonstrated for closely BMI-matched drug free patients ($P = .028$, Table 2). Similarly, the finding of disease-related insulin insensitivity persisted when controlling for effects of medication, smoking or stress (ANCOVA).

These results are suggestive of insulin resistance in schizophrenia that may result in an increased release of S100B from adipose tissue. Commonly observed weight gain upon neuroleptic treatment would thus appear on the basis of an increased metabolic vulnerability in patients due to primary insulin resistance, which is also present independent of medication.

6. Studies on Insulin-Regulated S100B Release from Adipose Tissue Are Challenged by Findings in CSF and Brain Tissue

Schizophrenia-related disturbances in S100B expression are not specific for adipose tissue, but have also been observed in CSF and brain tissue (see above) [13, 63, 64]. Given the general distribution of energy consumption with the brain having the highest glucose turnover, especially under normal or resting conditions, a cerebral insulin resistance appears plausible, as opposed to a disease that primarily or exclusively

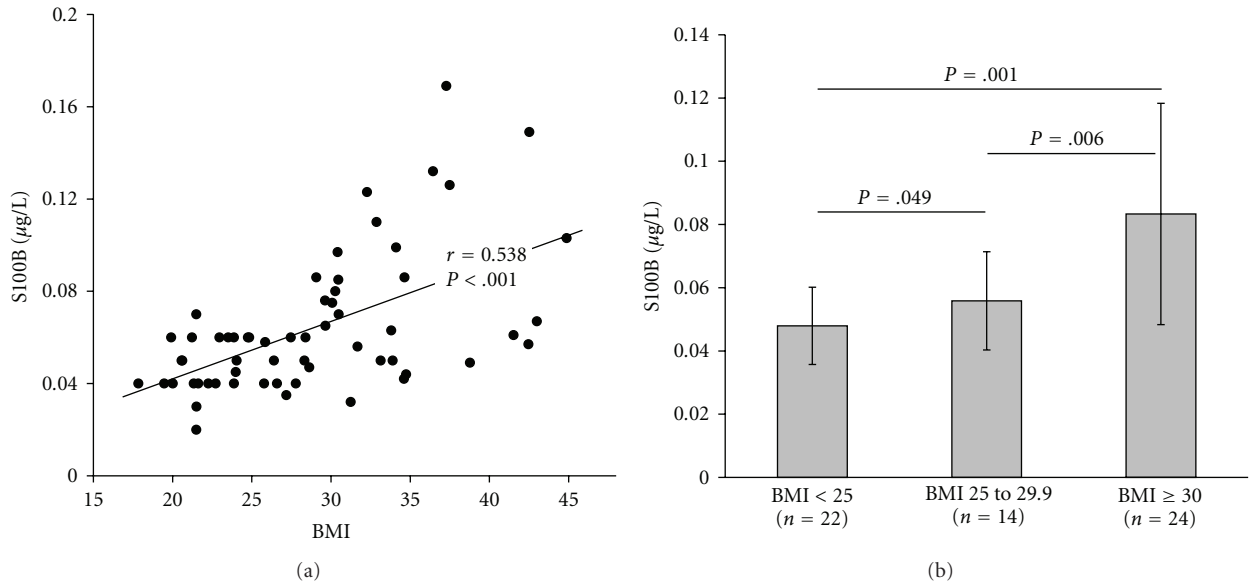

Figure 1: [57]: S100B serum levels were closely correlated with the BMI (a). Obese subjects (BMI ≥ 30) showed significantly elevated S100B levels in comparison to normal weight (BMI < 25) or overweight (BMI 25.0–29.9) subjects (b). *Annotation:* (a) r = Pearson correlation coefficient; (b) data are given as mean ± standard deviation (SD).

Table 1: Demographics and clinical measurements of control subjects and patients with paranoid schizophrenia. *Annotation:* data are given as mean ± standard deviation (SD), n.a. = not applicable, [a]ANOVA, [b]Chi-square-test, [c]ANCOVA, ↑↓ positive or negative influence on measures, bold *P*-values were significant, underlined *P*-values remained significant after Bonferroni correction.

Demographic data	Controls (n = 32)	Paranoid schizophrenia (n = 26)	*P*-values Schizophrenia versus controls		
Age [y]	34.4 ± 10.8	34.7 ± 11.3	0.914[a]		
Duration of disease [y]	—	8 ± 9	n.a.		
Male/Female [n]	20/12	17/9	0.820[b]		
BMI [kg/m^2]	24.3 ± 3.8	27.7 ± 5.7	0.196[a]		
Smokers/non-smokers [n]	14/18	16/10	0.178[b]		
Clinical data/measures	Mean ± SD	Mean ± SD	Influence of diagnosis (uncorrected ANOVA)	Influence of diagnosis (ANCOVA)	Influence of BMI (ANCOVA)
PANSS total score	—	84.8 ± 11.2	n.a.	n.a.	—
PANSS positive score	—	20.1 ± 4.9	n.a.	n.a.	—
PANSS negative score	—	22.1 ± 6.5	n.a.	n.a.	—
PANSS general score	—	42.7 ± 5.6	n.a.	n.a.	—
S100B [μg/L]	0.052 ± 0.018	0.072 ± 0.038	**0.012**[a] ↑	**0.039**[c] ↑	0.384[c]
hs-CRP [mg/L]	1.76 ± 2.89	3.11 ± 2.68	0.073[a] ↑	0.510[c]	**0.001**[c] ↑
TNF-α [ng/L]	2.38 ± 1.62	3.45 ± 2.44	0.052[a] ↑	0.193[c]	0.102[c]
Leptin [μg/L]	9.62 ± 15.25	18.24 ± 22.96	0.093[a] ↑	0.628[c]	< **0.001**[c] ↑
MCP-1 [ng/L]	285.6 ± 155.3	397.0 ± 233.3	**0.034**[a] ↑	0.409[c]	< **0.001**[c] ↑
HGF [μg/L]	2.44 ± 1.92	3.83 ± 3.37	0.054[a] ↑	0.488[c]	< **0.001**[c] ↑
Resistin [μg/L]	6.09 ± 2.11	6.31 ± 1.97	0.681[a]	0.893[c]	0.469[c]
PAI-1 [μg/L]	1.47 ± 0.81	2.35 ± 1.50	**0.006**[a] ↑	**0.046**[c] ↑	**0.048**[c] ↑
Triglycerides [mg/dL]	146.1 ± 68.2	221.3 ± 160.1	**0.020**[c] ↑	0.088[c] ↑	0.124[c]
Glucose [mg/dL]	82.9 ± 18.6	104.3 ± 21.6	< **0.001**[a] ↑	< **0.001**[c] ↑	0.894[c]
C-peptide [pmol/L]	1907.8 ± 1305.6	2572.8 ± 1376.5	< **0.001**[a] ↑	**0.002**[c] ↑	0.213[c]

Table 2: Demographics and clinical measurements of *drug free patients* with paranoid schizophrenia compared with *control subjects which were closely matched for BMI and smoking habits*. Annotation: data are given as mean ± standard deviation (SD), n.a. = not applicable, [a]ANOVA, [b]Chi-square-test, ↑↓ positive or negative influence on measures, bold *P*-values were significant.

Demographic data	Controls ($n = 11$)	Paranoid schizophrenia: drug free ($n = 11$)	Schizophrenia *versus* controls *P*-values
Age [*y*]	34.7 ± 12.0	35.1 ± 13.2	0.947[a]
Duration of disease [*y*]	—	8.5 ± 10.8	—
Male/Female [*n*]	7/4	7/4	1.000[b]
BMI [kg/m^2]	23.5 ± 3.4	24.5 ± 5.5	0.621[a]
Smokers/non-smokers [*n*]	6/5	7/4	0.665[b]
Measures	Mean ± SD	Mean ± SD	
S100B [μg/L]	0.046 ± 0.014	0.073 ± 0.033	**0.028**[a] ↑
hs-CRP [mg/L]	1.47 ± 1.64	2.50 ± 2.51	0.267[a]
TNF-α [ng/L]	2.51 ± 1.95	2.95 ± 1.79	0.584[a]
Leptin [μg/L]	11.82 ± 16.91	13.74 ± 12.49	0.766[a]
MCP-1 [ng/L]	314.9 ± 190.0	308.8 ± 166.2	0.936[a]
HGF [μg/L]	2.32 ± 1.85	2.84 ± 2.11	0.543[a]
Resistin [μg/L]	5.71 ± 1.80	6.70 ± 1.83	0.214[a]
PAI-1 [μg/L]	1.49 ± 0.75	2.30 ± 1.55	0.139[a]
Triglycerides [mg/dL]	144.3 ± 47.2	205.6 ± 102.3	0.093[a] ↑
Glucose [mg/dL]	81.1 ± 12.2	105.7 ± 25.0	**0.011**[a] ↑
C-peptide [pmol/L]	1219.0 ± 680.1	2760.3 ± 1657.0	**0.013**[a] ↑

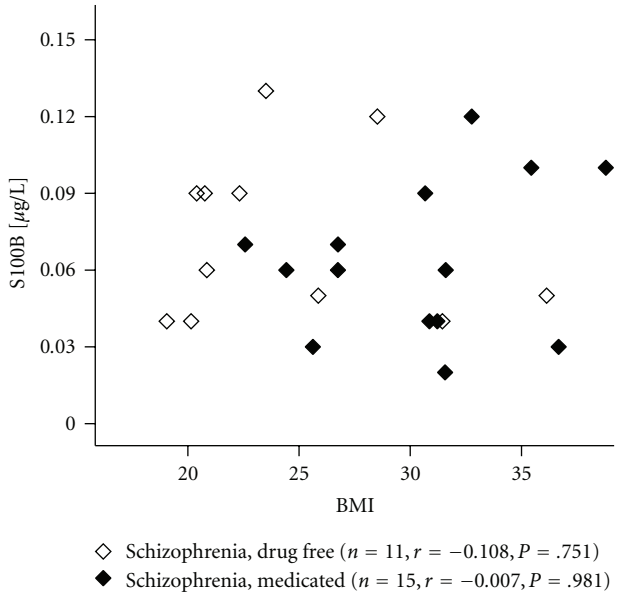

Figure 2: S100B levels in drug free and medicated schizophrenic patients were not correlated with the BMI. *Annotation:* r = correlation coefficient.

affects peripheral adipose tissue [65, 66]. This hypothesis is in line with observations of a dysfunctional cerebral insulin receptor signaling in dorsolateral prefrontal cortex tissue from patients with schizophrenia [27]. Strikingly, insulin has been shown to downregulate S100B expression in astrocyte cultures and rat brain [54, 67, 68], in analogy to the above-mentioned observations in adipose tissue [53, 69]. Previous cell culture experiments have shown that the expression and release of S100B from astrocytes and oligodendrocytes is stimulated by cellular glucose deprivation [6, 70]. A similar condition may occur in the brains of schizophrenia patients due to impaired glial glucose uptake as a consequence of insulin resistance.

Therefore, altered S100B protein expression in schizophrenia probably indicates systemic disturbances in cellular energy supply (e.g., by disrupted peripheral and cerebral insulin signaling) rather than adipocyte- or glia-specific pathologies. Upregulated insulin levels might represent a compensatory effort coming up against these insulin receptor disturbances. Indeed, a better psychopathology profile has been observed in acutely ill schizophrenic patients with higher insulin levels, potentially compensating these insulin receptor disturbances [71]. These considerations are offering promising new approaches in the therapy of schizophrenia-related metabolic and psychopathological alterations, for example the usage of glitazones, a group of insulin sensitizing drugs [72, 73].

7. Potential Relationship of S100B to the Metabolic Syndrome in Schizophrenia

According to the American Heart Association, the metabolic syndrome is characterized by a group of cardiovascular risk factors in one person (http://www.americanheart.org/presenter.jhtml?identifier=4756). They include:

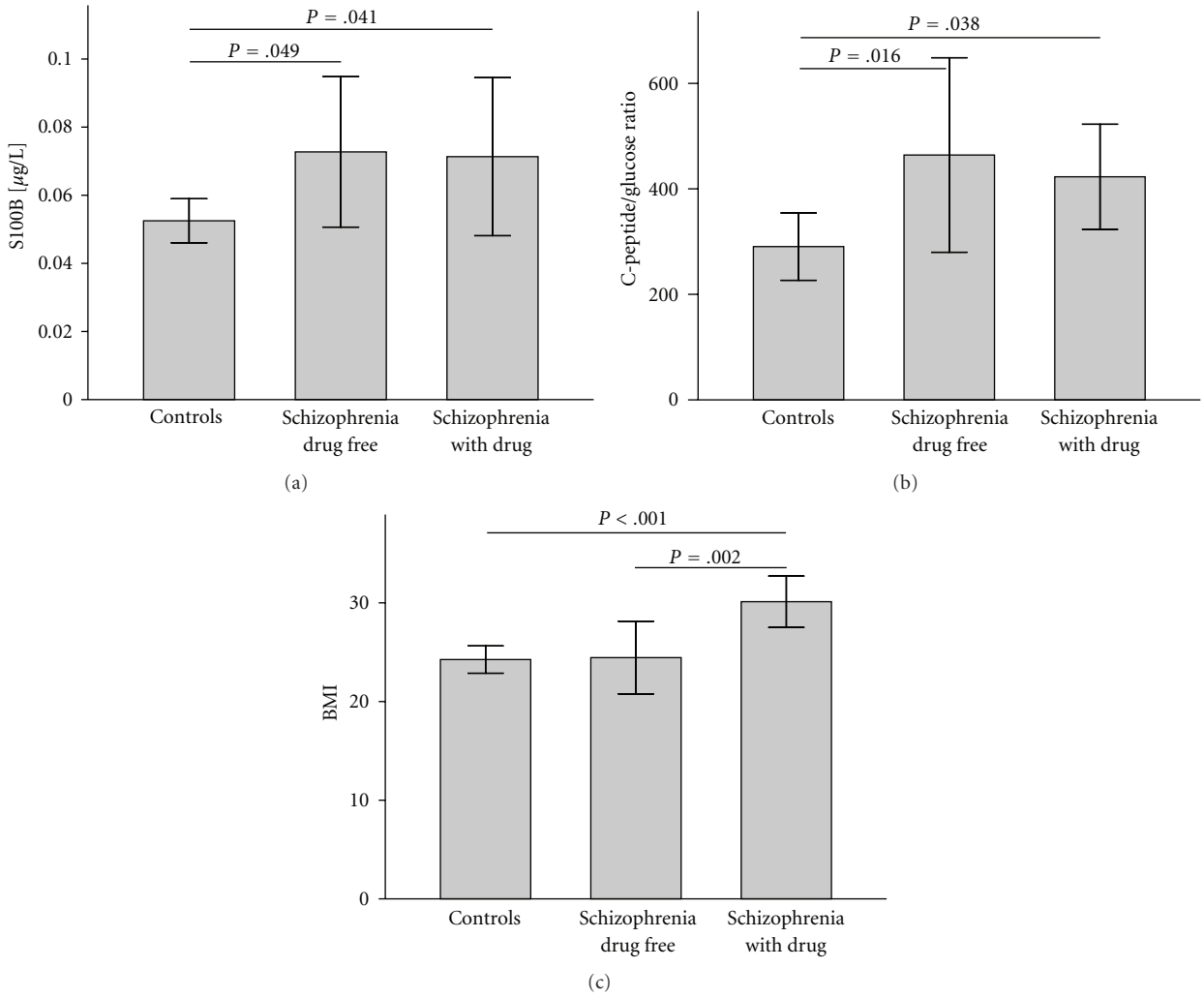

Figure 3: [61]: Elevated S100B serum concentrations (a) and an increased C-peptide/glucose-ratio (b), indicating insulin resistance, were schizophrenia-related. However, increases of BMI were primarily a consequence of antipsychotic medication (c). *Annotation:* Data are given as mean with 95% confidence intervals. Only *P*-values of significant group differences are displayed.

(i) *Abdominal obesity:* excessive fat tissue in and around the abdomen, elevated waist circumference: men—equal to or greater than 40 inches (102 cm); women—equal to or greater than 35 inches (88 cm).

(ii) *Elevated blood pressure:* equal to or greater than 130/85 mm Hg.

(iii) *Insulin resistance or glucose intolerance:* fasting glucose equal to or greater than 100 mg/dL.

(iv) *Atherogenic dyslipidemia:* blood fat disorders—high triglycerides (greater than 150 mg/dL), low HDL cholesterol (men—less than 40 mg/dL; women—less than 50 mg/dL) and high LDL cholesterol—that foster plaque buildups in artery walls.

(v) *Prothrombotic state:* e.g., high fibrinogen or PAI-1 in the blood.

(vi) *Proinflammatory state:* e.g., elevated CRP in the blood.

Interestingly, we previously observed an upregulation of the S100B/AGE scavenger sRAGE (soluble receptor of advanced glycation end products) during reconvalescence from acute schizophrenia [74]. Benefits of sRAGE have also been found in cardiovascular and metabolic disorders, which are associated with increased AGE levels, such as diabetes mellitus and arteriosclerosis [75, 76]. A closer look at the data from our recent study in Molecular Psychiatry reveals that some of the abovementioned metabolic risk factors were present in schizophrenia cases, but not all of them were linked to elevated S100B serum levels (Table 1) [61].

(i) *Abdominal Obesity:* We did not measure waist circumferences in this study, but blood concentrations of MCP-1 were elevated in the schizophrenia group ($P = .034$), indicating an increased visceral fat mass [62], and correlating with levels of S100B ($r = 0.673$, $P = .023$).

(ii) *Elevated Blood Pressure:* This was an exclusion criterion of the study. Therefore, the relation of hypertension to S100B could not be analyzed.

(iii) *Insulin Resistance or Glucose Intolerance:* Elevated glucose and C-peptide levels were observed in the schizophrenia cohort. C-peptide/glucose ratios predicted S100B levels ($r = 0.432$, $P = .014$).

(iv) *Atherogenic Dyslipidemia:* Triglyceride levels were slightly elevated in schizophrenic subjects ($P = .020$), but were not correlated with S100B concentrations. HDL and LDL cholesterol were not systematically assessed.

(v) *Prothrombotic State:* Schizophrenia cases showed higher PAI-1 levels ($P = .006$) that were not correlated with S100B.

(vi) *Proinflammatory State:* There was a trend towards elevated hs-CRP and TNF-α levels in schizophrenia. However, these inflammatory parameters were not related to S100B levels.

It remains unclear whether S100B may be considered as a predictor of metabolic syndrome in schizophrenia. This topic has to be elucidated by future studies in drug-naïve and prodromal schizophrenia cases with follow-up blood takes and clinical examinations in larger samples, comparing different standardized treatment regimens. Apart from the measurement of fasting glucose, triglyceride and HDL cholesterol levels in patients and controls, it will be necessary to assess additional clinical parameters such as blood pressure and waist circumference for a clear identification of patients at risk.

8. Summary and Conclusion

Several studies reported on elevated blood levels of S100B in schizophrenia, which have been attributed to glial pathology [2]. However, considerable amounts of S100B are released from adipose tissue during lipolysis. The release of S100B from adipocytes is reduced by insulin, and activated by physiological factors such as stress (catecholamines and adrenocorticotropic hormone) or fasting [46–48, 51–53]. Our recent observations in healthy human subjects are suggesting a close relation between serum S100B levels and the BMI, or levels of the adipose-derived factors leptin, HGF, resistin and A-FABP [57, 61]. Of note, previous studies showed that serum A-FABP is a powerful marker for predicting metabolic syndrome and arteriosclerosis [58].

Given the increased prevalence of visceral obesity and insulin resistance in schizophrenia, we recently analyzed the relation of serum S100B levels to the BMI and adipose derived hormones in acute paranoid schizophrenia [61]. The physiological relation of S100B to the BMI and the abovementioned adipose-derived factors was disrupted in schizophrenia. Several reasons may be hypothesized:

(1) Schizophrenia-related disturbances in adipose tissue distribution, such as increased visceral fat which is better assessed by the waist-to-hip ratio instead of BMI. Interestingly, blood concentrations of MCP-1 were indeed elevated in the schizophrenia group, indicating an increased visceral fat mass [62], and correlated with levels of S100B (see above).

(2) Schizophrenia-related disturbances in adipose tissue metabolism, such as an increased release of S100B, together with triglycerides and free fatty acids due to a predisposition to insulin resistance. Indeed, elevated glucose and C-peptide levels were observed in the schizophrenia cohort and C-peptide/glucose ratios predicted S100B levels (see above).

(3) An increased release of S100B from brain tissue, as suggested by histological and CSF studies [13, 63, 64]. Notably, insulin has also been shown to downregulate S100B expression in astrocyte cultures and rat brain [54, 67, 68]. However, an animal study in Wistar rats suggests that the concentration of S100B may be differentially regulated in the periphery and the central nervous system: lipolysis following fasting was linked to an increased release of S100B to serum, while cerebrospinal fluid levels of S100B were not significantly altered [51]. This topic awaits further clarification.

(4) Drug effects on cellular S100B production and release.

(a) Glial cell culture experiments have shown that antipsychotic drugs can *directly* affect glial S100B release. Increased amounts of S100B were found in the supernatants of astroglial C6 cells treated with very high doses of risperidone [77]. In contrary, treatment of astroglial C6 and oligodendroglial OLN-93 cells with haloperidol and clozapine at a concentration corresponding to the assumed therapeutic dose range of these drugs reduced the release of S100B in vitro [78]. Other S100B-expressing cell types, like adipocytes, have not been tested yet in this context.

(b) Alternatively, the potential influence of atypical antipsychotics on S100B via changing metabolic factors should be considered as a more *indirect mechanism*. Among the second-generation antipsychotics, clozapine and olanzapine are associated with the highest risk of weight gain, as well as changes in insulin sensitivity and lipid metabolism, which increase the risk of diabetes and cardiovascular disease [17–19].

(5) Adrenaline, noradrenalin or ACTH-enhanced release of S100B from adipose tissue due to a schizophrenia-related activation of the endocrine stress axis [51–53]. This idea is supported by our finding of free cortisol index $(FCI)^2$-correlated S100B levels in untreated acutely ill patients (unpublished results: $r = 0.611$, $P = .046$).

In conclusion, there is evidence for a novel link between S100B and disturbances of energy metabolism in schizophrenia, resulting in an increased release of S100B from brain and adipose tissue. Such systemic alterations in glucose metabolism may also affect glial S100B release. An upregulation of S100B may be a compensatory phenomenon, increasing intracellular energy supply by activating glycolysis (fructose-1,6-bisphosphate aldolase) and glycogenolysis (phosphoglucomutase) [79, 80]. Future studies in larger samples may focus on fasted drug naive schizophrenic subjects and the comparison of different standardized treatment regimens to further elucidate the suggested link between S100B and abnormal energy metabolism.

Abbreviations

ACTH:	Adrenocorticotropic hormone
A-FABP:	Adipocyte-type fatty acid-binding protein
BMI:	Body mass index
C6:	An astroglial cell line from rat
CBG:	Cortisol-binding globulin
CSF:	Cerebrospinal fluid
FCI:	Free cortisol index
FDG-PET:	Fluorodeoxyglucose positron emission tomography
fMRI:	Functional magnetic resonance imaging
GFAP:	Glial fibrillary acidic protein
HDL:	High-density lipoprotein
HGF:	Hepatocyte growth factor
hs-CRP:	High-sensitivity C-reactive protein
IGF-1:	Insulin-like growth factor 1
LDL:	Low-density lipoprotein
MBP:	Myelin basic protein
MCP-1:	Monocyte chemotactic protein 1
NSE:	Neuron specific enolase
OLN-93:	An oligodendrocytic cell line from rat
PAI-1:	Plasminogen activator inhibitor 1
S100B:	Member of a family of proteins that are 100% soluble in ammonium sulfate at neutral pH
SD:	Standard deviation
sRAGE:	Soluble receptor of advanced glycation end products
TNF-α:	Tumor necrosis factor alpha.

Endnotes

1. C-peptide is generated in the pancreas when proinsulin is split into insulin and C-peptide. It has no metabolic function. However, since C-peptide and insulin are secreted in equimolar amounts, the immunoassay of C-peptide permits the quantitation of insulin secretion. Moreover, C-peptide measurement has several advantages over immunoassays of insulin [81]: First of all, approximately 50% of the portal vein concentration of insulin is rapidly degraded by the liver whereas C-peptide extraction by the liver is only about 12%. As a result, peripheral venous levels of C-peptide more accurately reflect pancreatic insulin secretion than do peripheral insulin levels. Unlike insulin, which has significant uptake at target tissues, C-peptide is metabolically fairly inert. It is primarily removed from the circulation by the kidney, both by degradation and by urinary excretion. The half-life of C-peptide in the circulation is between two and five times longer than that of insulin. Therefore, C-peptide levels are a more stable indicator of insulin secretion than the more rapidly changing insulin levels. A very clear practical advantage of C-peptide measurement arising from its relative metabolic inertness as compared to insulin is that C-peptide levels in peripheral venous blood are about 5-6 times greater than insulin levels.
2. The FCI is a surrogate marker for free cortisol, which is the biologically active component. It is calculated by dividing the serum total cortisol [nmol/L] by the cortisol-binding globulin (CBG) level [mg/L].

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Research Article

A Comparison of the Dynamics of S100B, S100A1, and S100A6 mRNA Expression in Hippocampal CA1 Area of Rats during Long-Term Potentiation and after Low-Frequency Stimulation

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The interest in tissue- and cell-specific S100 proteins physiological roles in the brain remains high. However, necessary experimental data for the assessment of their dynamics in one of the most important brain activities, its plasticity, is not sufficient. We studied the expression of S100B, S100A1, and S100A6 mRNA in the subfield CA1 of rat hippocampal slices after tetanic and low-frequency stimulation by real-time PCR. Within 30 min after tetanization, a 2–4 fold increase of the S100B mRNA level was observed as compared to the control (intact slices) or to low-frequency stimulation. Subsequently, the S100B mRNA content gradually returned to baseline. The amount of S100A1 mRNA gradually increased during first hour and maintained at the achieved level in the course of second hour after tetanization. The level of S100A6 mRNA did not change following tetanization or low-frequency stimulation.

1. Introduction

Although the S100 proteins are known to have a broad spectrum of intra- and extracellular functions, the roles they play in the central nervous system, in general, and, particularly, in mediating one of the most important brain activities, its plasticity, are largely unknown. Most S100 proteins undergo conformational changes upon calcium binding, which allows them to interact with target proteins [1]. They are differentially expressed in a variety of cell types and tissues, and are thought to play unique roles, despite a high degree of sequence homology they share and a significant overlap of their expression patterns. For example, in the brain, S100A1 is predominantly expressed in neurons, S100B expression maps to the astrocytes of hippocampus and cortex, as well as to the subpopulations of oligodendrocytes and neurons, while S100A6 is mainly detected in neurons of the restricted brain areas (amygdala and entorhinal cortex) and in some astrocytes [2–5]. At least several S100 proteins affect cell growth, for example, S100B and S100A1, which interact with the tumor suppressor p53 [6, 7].

Among S100 proteins, S100B is the most studied one in connection with the neuronal plasticity, although the data available is contradictory. Antiserum against S-100 protein prevents the long-term potentiation (LTP) in the CA1 region of rat hippocampal slices, suggesting a positive role of S100B in the manifestation of LTP [8]. In contrast to this observation, another set of experiments indicate at a rather negative influence of S100B on LTP [9, 10]. Transgenic mice overexpressing the human S100B protein exhibited the impaired hippocampal LTP and spatial learning [9]. Conversely, an enhanced LTP was observed in the hippocampal

CA1 region of mutant mice devoid of S100B, while the perfusion of hippocampal slices with the recombinant S100B protein reversed the LTP in the slices from mutant mice to the wild-type level [10]. The expression of both S100A6 and S100B proteins has been shown to be modulated in the course of human brain development [11]. Higher levels of S100B have been detected in the sera of patients after brain trauma or ischemia, as well as those suffering from Alzheimer disease or Down syndrome [12]. Overexpression of S100A6 has also been observed in patients suffering from Alzheimer disease or amyotrophic lateral sclerosis [13, 14]. S100A1 plays a certain role in modulating innate fear and exploration of novel stimuli [15].

The long-term brain plasticity requires the changes in gene expression [16, 17]. Complex spatiotemporal patterns of the expression of new growth factors, ion channels, structural molecules, and other proteins specify the alterations of neuronal circuitry. With the purpose of studying the role of gene expression in mediating the mechanisms of neuronal plasticity, we compared the dynamics of mRNA expression for three highly homologous S100 proteins with different brain localizations after the LTP-inducing tetanization or the low-frequency stimulation (LFS), which did not induce LTP, in hippocampal CA1 area of rats.

2. Materials and Methods

Experiments were conducted on hippocampal slices prepared routinely. Wistar male rats (weight 180–220 g) were decapitated, the brains were rapidly removed and placed into ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF): 126 mM NaCl, 4 mM KCl, 1.24 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.3 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, pH 7.4. Left hippocampus was dissected for removal and cut into 400 μm-thick slices perpendicular to the longitudinal axis, using a chopper. Four consecutive slices from dorsolateral region of the left hippocampus were transferred to a submerged recording chamber (volume 7 mL). Slices were continuously perfused with the fresh, oxygenated aCSF warmed to 32–33°C at a rate of 2 mL/min. For extracellular recordings, the recording electrode filled with aCSF (resistance 1–5 MΩ) was placed in the hippocampal CA1 pyramidal cell layer. To stimulate Schaffer's collaterals, a bipolar electrode with a tip diameter of 50 μm filled with aCSF was placed in the CA1 stratum radiatum. Intensity of stimulation was adjusted to get p-spike amplitude ~50% of the maximal response.

Two separate series of experiments were carried out: the first, to test efficiency of the stimulation protocols in our hands, and the second, to obtain the samples for real time PCR analysis. While testing the stimulation protocol efficiency, a single slice from each animal was used. Tetanization (4 series × 1 s, 100 Hz, with 30 s intervals) or low-frequency stimulation (400 stimuli during 91 s, i.e., at 4.4 Hz) were performed 2.5 hr postdissection. Control slices were not treated by tetanization or LFS protocols and received only test stimulation in these experiments.

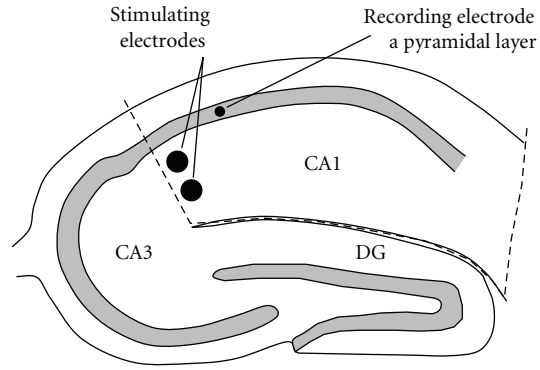

Figure 1: The region of the hippocampal slice (CA1) used for mRNA sample preparation. CA1, CA3, DG—hippocampus subfields. Hatched line indicates the direction of sections made to separate the studied region.

Sample preparation for real time PCR analysis was as follows. The slice, which was used as a baseline control for the mRNA content normalization remained intact throughout the entire incubation period (4.5 hrs). Three other slices were tetanized (or subjected to LFS) 120, 60, or 30 min prior to the end of incubation. Electrodes were placed onto the slices 10 min before and were removed immediately after the stimulation. At the end of incubation, slices were transferred to an ice-cold oxygenated aCSF, the CA1 field was rapidly dissected from each slice (as shown in Figure 1) and placed into liquid nitrogen. Five relevant slices from different animals were pooled to prepare each mRNA sample.

Total RNA was isolated from frozen slices using Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories) according to the manufacturer's protocol. The RNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm (corrected for background at 320 nm), and the RNA integrity was assessed by running the sample on a denaturing agarose (1%) gel and visualization of 18S and 28S rRNA bands. As much as 0.5 μg of total RNA was used for single-stranded cDNA synthesis. First strand cDNA synthesis was carried out using iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's protocol. Gene expression levels were assayed by real-time PCR using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories). Real-time PCR experiments were performed with IQ5 Real-Time PCR instrument (Bio-Rad Laboratories). The "house-keeping" gene, β-actin, was used as an endogenous internal control for normalization. The gene-specific primer sequences for S100B, S100A1, S100A6, and β-actin amplification were as follows: S100B F: 5′-TTGCCCTCATTGATGTCTTCCA-3′, R: 5′-TCTGCCTTGATTCTTACAGGTGAC-3′; S100A1 F: 5′-CCATGGAGACCCTCATCAAT-3′, R: 5′-TTCTGG-ACATCCAGGAAGC-3′; S100A6 F: 5′-CTTCTCGTGGCT-ATCTTCC-3′, R: 5′-ACTGGACTTGACTGGGATAG-3′; β-actin F: 5′-ACCCACACTGTGCCCATCTA-3′, R: 5′-CGGAACCGCTCATTGCC-3′.

The optimal annealing temperature for each primer set was determined prior to the experimental sample analysis.

Duplicate real-time PCR reactions were run for each sample containing SYBR Green master mix, 300 nM forward and reverse primers, and 25 ng cDNA template in 25 μL of a total reaction volume. The following standard real-time PCR conditions were used: one cycle of 95°C for 3 min and 40 cycles of 95°C for 15 s, the primer specific annealing temperature (58°C) for 20 s, 72°C for 20 s, optical data were collected at 80°C for 10 s. After PCR experiments, the dissociation curve was established using the built-in melting curve program to confirm the presence of a single PCR product, which was then confirmed by gel electrophoresis. The fold change in the target gene, normalized to β-actin and relative to control, was calculated based on PCR efficiency (E) and Ct.

Data is expressed as the mean ± S.E.M and tested for statistical significance using Student's t-test (for the comparison of the amplitudes of p-spikes after tetanization or LFS with the baseline), paired t-test (for the comparison of mRNA amounts in the samples from stimulated and control/nonstimulated slices), and repeated measures ANOVA (for the comparison of mRNA dynamics after tetanization and LFS).

3. Results

As shown in Figure 2, the employed tetanization protocol caused a significant increase in the responses in CA1 field to the stimulation of Schaffer's collaterals. The response level remained increased throughout the entire course of observation (3 hrs). The same amount of 4.4 Hz stimuli (LFS) applied within the same time frame did not cause any potentiation.

Tetanization increased the S100B mRNA level drastically and rapidly (Figure 3). As early as 30 min after tetanization, a substantial increase of S100B mRNA level was observed (286 ± 27%, $P_t = .006$, $n = 4$) in treated specimens, as compared to the control (nonstimulated) slices. Then S100B mRNA level gradually returned to baseline (218 ± 26% at 60 min, $P_t = .02$, $n = 4$) and, within 120 min after tetanization, it was not differing from the control values (128 ± 14%, $P_t = .14$, $n = 4$). LFS did not cause any changes of the S100B mRNA level at studied time points (Figure 3). 2-Way repeated measures ANOVA (group(2) × time(3)) showed highly significant difference in the amount and dynamics of S100B mRNA between tetanization and LFS (group effect $F_{(1,5)} = 21$, $P < .006$; group-time interaction $F_{(2,10)} = 19$, $P < .001$).

The S100A1 mRNA content, conversely, increased slowly (during the first hour) and was maintained at the achieved level in the course of the second hour after tetanization (Figure 3) (30 min, 138 ± 30%, $P_t = .15$, $n = 3$; 60 min, 177 ± 10%, $P_t = .004$, $n = 3$; 120 min, 179 ± 1%, $P_t \ll .001$, $n = 3$). 2-Way repeated measures ANOVA (group(2) × time(3)) showed highly significant difference in the amount of S100A1 mRNA between tetanization and LFS (group effect $F_{(1,4)} = 314$, $P \ll .001$; group-time interaction $F_{(2,8)} = 3.7$, $P = .07$).

Analysis of the S100A6 mRNA content did not reveal any difference between treated and control samples at all time points studied both after tetanization and following LFS (Figure 3). Correspondingly, 2-Way repeated measures ANOVA (group(2) × time(3)) showed insignificant differences between tetanization and LFS in respect to the amount or dynamics of S100A6 mRNA after stimulation (group effect $F_{(1,5)} = 0.4$, $P = .56$; group-time interaction $F_{(2,10)} = 0.1$, $P = .89$).

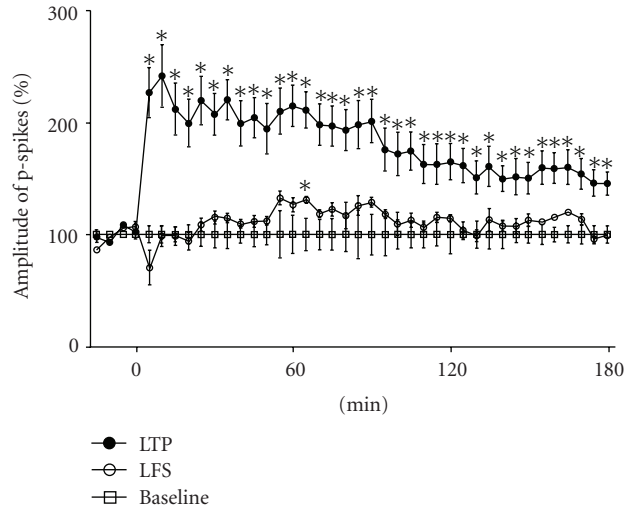

Figure 2: The influence of tetanization and low-frequency stimulation on the amplitude of p-spikes in pyramidal layer of CA1. P-spikes were elicited by stimulation of Shaffer's collaterals. Abscissa, the time from the onset of tetanization (LTP, long-term potentiation) or low-frequency stimulation (LFS). Ordinate, the amplitude of p-spike, first, normalized to the average amplitude of p-spikes in 4 responses prior to tetanization ($n = 5$) or LFS ($n = 3$), and then, to the mean amplitude of responses in the control group (baseline, $n = 4$). $^*P_t < .05$, against baseline, Student's t-test.

4. Discussion

Gene transcription is required for establishing and maintaining the enduring form of long-term potentiation. The products of coordinated expression of a multitude of genes enable the stable modification of synaptic efficiency and neuronal excitability [18, 19]. In the past years, the classical view that astrocytes play a relatively passive role in brain function has been overturned, and it has become increasingly clear that signaling between neurons and astrocytes may play a crucial role in the information processing performed by the brain [20]. Investigation and characterization of not only neuronal, but also the glial transcriptional profiles after tetanic stimulation will provide a better understanding of the processes underlying the long-term changes in response to neuroglial activation.

We showed that LTP-inducing tetanization of Schaffer's collaterals, but not LFS, evokes increases in S100B and S100A1 mRNA levels in hippocampal CA1 area of rats. This result suggests that tetanization-induced changes in S100B and S100A1 mRNA amounts were not associated with mechanical or electric damage of slices with electrodes,

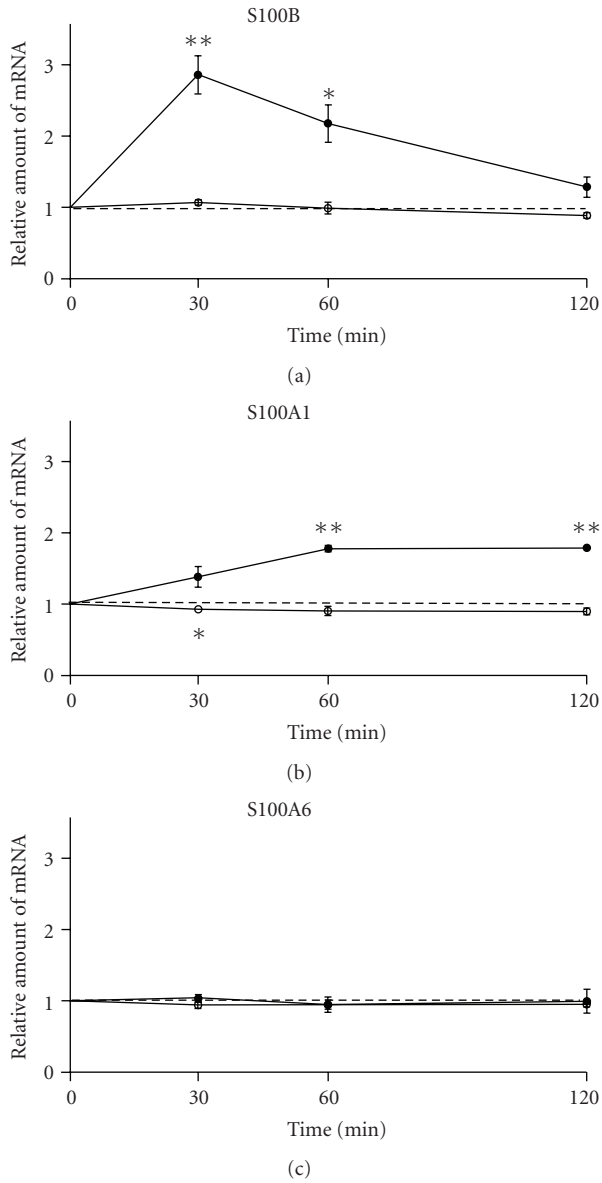

Figure 3: The dynamics of S100B, S100A1, and S100A6 relative mRNA amounts in hippocampal CA1 area of rats after tetanization and low-frequency stimulation. Ordinate, relative amount of mRNA normalized to relative amount of mRNA in control samples (nonstimulated slices). Abscissa, time after stimulation (min). S100B: $n1 = 4$, $n2 = 3$; S100A1: $n1 = 3$, $n2 = 3$; S100A6: $n1 = 4$, $n2 = 3$ for tetanization (black circles) and low-frequency stimulation (white circles), respectively. $^{**}P_t < .01$, $^{*}P_t < .05$, against control samples, Student's paired t-test.

but might rather be relevant to the processes underlying neuronal plasticity. The expression of S100B in astrocytes is upregulated by brain-derived neurotrophic factor (BDNF) [21], which is thought to play a crucial role in LTP mechanisms [22]. BDNF activity during LTP initiation might possibly participate in the induction of a rapid and transient increase of S100B mRNA content after tetanization, although a number of other mechanisms of S100B upregulation exist [23], including the spillover of glutamate from activated synapses. Extracellular S100B evokes an increase in intracellular free calcium concentration in glial and neuronal cells [24]. Calcium is involved in a number of cellular processes including the regulation of transcription. It is, therefore, possible that increased S100B protein secretion from astrocytes associated with neuronal activation [25] might induce S100B gene transcription.

In contrast to our results, LTP induction in mouse dentate gyrus slightly decreased S100B mRNA content [19], however, the reasons for that discrepancy remain unclear. It would be interesting to compare the baseline expression of S100B mRNA in our control (nonstimulated slices) samples ($35 \pm 5\%$ of the β-actin mRNA, $n = 7$) with those in mice. Chang et al. [19] used minislices of dentate gyrus dissected from the whole hippocampal slices immediately after their preparation, while we worked on whole slices and dissected CA1 immediately before freezing. Supposedly, a more extensive injury to the slices, as in [19], might be responsible for a more prominent basal expression of S100B and, thereby, might occlude its further increase after LTP.

Functional significance of the S100B mRNA increase after tetanization in our experiments is not yet clear. It would be worthwhile to investigate whether S100B protein content increases along with its mRNA expression. High frequency neuronal activation stimulates S100B secretion in hippocampal slices [25]. Whether the enhanced mRNA expression simply replenishes the pool of S100B protein or a further increase of the protein above baseline takes place remains to be elucidated.

As mentioned above, experimental data concerning the influence of S100B protein on LTP is contradictory. Natural sources of this discrepancy arising from the complexity of the biochemical machinery, which S100B is involved in, were discussed elsewhere [23]. Still another reason for contradictions could be the immanent instability of acute slices. Cutting hippocampal slices for in vitro experiments causes prolonged disturbance of the background genetic activity, which might never reach steady state. For example, the expression of interleukin-1β mRNA can increase steadily with the time over at least 7 hours after slice preparation [26]. Changing background genetic activity could influence the mechanisms of plasticity. A degree of LTP in hippocampal slices was reported to decrease during survival [27]. Unfortunately, this issue is commonly neglected by researchers. Thus, when a varying amount of slices from one animal is used in experiments and processed serially, it is difficult to exclude artifacts associated with the differences in times of slice survival in the compared experimental groups.

As most of the other S100 proteins, S100A1 is a multifunctional regulator. With respect to LTP mechanisms, an implication of S100A1 in the Ca^{2+}-dependent regulation of synaptic vesicle trafficking and, ultimately, in the regulation of presynaptic function and plasticity [28] might be relevant. Though the data on brain S100A1 secretion is lacking so far, it is known to induce RAGE-dependent growth of neurites and promote neuronal survival in physiological concentrations [29]. Furthermore, it is shown that RAGE activation results in a fast CREB phosphorylation and its

translocation into the nucleus [30], the factor being involved in LTP mechanisms [17].

We did not observe any changes in S100A6 mRNA following LTP induction. Obviously, this fact alone does not exclude S100A6 participation in LTP mechanisms, but, rather, reflects the peculiarities of the protein cellular distribution and/or expression regulation. As S100B, S100A1, and some other S100 proteins, S100A6 is a RAGE ligand and might be involved in mechanisms of cell survival [31].

Our results indicate that glial gene expression response to the neuronal activation can be quite appreciable, although functional significance of the observed increment in S100B mRNA content after tetanization remains to be clarified. We hope that further investigation of this phenomenon will extend our knowledge about neuroglial cooperation during LTP formation.

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Research Article

S100b Counteracts Neurodegeneration of Rat Cholinergic Neurons in Brain Slices after Oxygen-Glucose Deprivation

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Alzheimer's disease is a severe chronic neurodegenerative disorder characterized by beta-amyloid plaques, tau pathology, cerebrovascular damage, inflammation, reactive gliosis, and cell death of cholinergic neurons. The aim of the present study is to test whether the glia-derived molecule S100b can counteract neurodegeneration of cholinergic neurons after oxygen-glucose deprivation (OGD) in organotypic brain slices of basal nucleus of Meynert. Our data showed that 3 days of OGD induced a marked decrease of cholinergic neurons (60% of control), which could be counteracted by 50 μg/mL recombinant S100b. The effect was dose and time dependent. Application of nerve growth factor or fibroblast growth factor-2 was less protective. C-fos-like immunoreactivity was enhanced 3 hours after OGD indicating metabolic stress. We conclude that S100b is a potent neuroprotective factor for cholinergic neurons during ischemic events.

1. Introduction

Alzheimer's disease (AD) is characterized by beta-amyloid plaque depositions, tau pathology, inflammation, cerebrovascular damage, and cell death of cholinergic neurons. The lack of cortical acetylcholine directly correlates with cognitive impairment. Loss of function and degeneration of cholinergic neurons in the basal forebrain has been a central theme in AD research, leading to cholinergic neuroprotection strategies for therapy [1]. Loss of trophic support by target neurons in the cortex has been considered as one mechanism for cholinergic degeneration [1]. Furthermore, cholinergic neurons show pathological changes such as accumulation of neurofibrillary tangles and axonal abnormalities early during aging and AD [2–4]. Changes of the basal forebrain are already found in the presymptomatic phase of AD using neuroimaging [5, 6]. Exogenous trophic factors can support cholinergic neurons in vitro and in vivo and replacement strategies have been under consideration for treating AD. Nerve growth factor (NGF) is so far the most potent trophic factor to protect cholinergic neurons against neurodegeneration [7]. However, other trophic molecules are also of considerable interest, such as, for example, S100b.

S100b is a calcium-binding protein predominantly produced in astrocytes [8], but also oligodendrocytes have been shown to express the protein [9]. S100b is released from glial cells and reaches surrounding neurons on which it exerts trophic functions under physiological conditions [8, 10, 11]. Neuroprotection against glutamate toxicity has been shown in neuronal cell culture models [12]. S100b also activates microglial cells and stimulates iNOS or IL1β via the NF-kappaB pathway [13, 14]. RAGE (receptor for advanced glycosylation end products

(AGE)) has also been linked to cellular activation by S100b [15]. S100b has been also linked to AD, because astrocytes are strongly activated in AD and in the brain and tissue levels of S100b are increased [16]. It was generally thought that the astroglial response serves protective functions, including the increased production of trophic factors and uptake of beta-amyloid peptides, but negative

aspects of astrocytic activation have also been uncovered [17]. It was postulated that excessive production of S100b could cause damage to neurons in the brain of AD patients [16, 18]. In fact, overexpression of human S100b exacerbated brain damage after ischemia-hypoxia [19, 20], and arundic acid ameliorated ischemic damage by preventing astrocytic overproduction of S100b [21]. Taken together, at low (nanomolar) concentrations S100b exerts trophic functions on neurons, whereas at high (micromolar) concentrations the protein stimulates cytokine production which in turn may trigger apoptosis [10].

It has been suggested that chronic neurovascular damage and moderate ischemia-hypoxia (silent strokes) may play a role in the progression of AD [22]. Moderate ischemia and hypoxia can stimulate astrocytes [23]. In the brain afflicted by AD, loss of trophic support coexists with hypoxic conditions stimulating glial cells, resulting in complex interactions that may involve S100b. Ischemic damage of neurons provokes reactions of astrocytes whereby immunohistochemical labeling for S100b is detected in reactive astrocytes and in damaged neurons [24].

The aim of the present study is to explore the role of S100b on cholinergic neurons in our well established organotypic brain slice culture model in which cholinergic basal nucle**us** of Meynert (nBM) neurons are provided with NGF for two weeks. Slices incubated without NGF display loss of choline acetyltransferase (ChAT) labeling after two weeks [25] which is an indicator of neurodegeneration. Oxygen-glucose deprivation (OGD) of slice cultures has become an established model for "ischemia-like" conditions [26–28]. In this report, we show that prolonged OGD induces loss of ChAT signals of 2-week old nBM neurons and that S100b can protect against the detrimental effects of OGD. No evidence for a negative effect of S100b itself was detected. The present study suggests that S100b plays a protective role for cholinergic neurons undergoing ischemia-hypoxia stress.

2. Materials and Methods

2.1. Organotypic Brain Slices. Cholinergic neurons in organotypic brain slices were cultured as described by us in detail [25, 29]. Briefly, the basal nucleus of Meynert of postnatal day 8 (P8) rats was dissected under aseptic conditions, 400 μm slices were cut with a tissue chopper (McIlwain, USA), and the slices were placed on 30 mm Millicell-CM 0.4 μm pore membrane culture plate inserts (7-8 slices per membrane). Slices were cultured in 6-well plates at 37°C and 5% CO_2 with 1.2 mL/well of slice medium (50% MEM/HEPES (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech, Austria), 25% Hanks' solution (Gibco), 2 mM $NaHCO_3$ (Merck, Austria), 6.5 mg/mL glucose (Merck), and 2 mM glutamine (Merck), pH 7.2) including 10 ng/mL nerve growth factor (NGF) for 2 weeks. It is well-established that the 400 μm brain slices become thinner during the 2 weeks of incubation and have a thickness of approximately 100 μm after 2 weeks. Slices which did not flatten were removed from the experiments. For the experiment the 2-week old slices were cultured for 3 days without NGF and then transferred to different media: (1) medium with normal 33.2 mM glucose or (2) medium with low (6.15 mM) glucose. Recombinant S100b from bovine brain (Calbiochem) was added to the medium at concentrations from 0.1–50 μg/mL. Alternatively, 10 ng/mL NGF or 2 ng/mL fibroblast growth factor-2 (FGF-2) was added. It has to be noted that slices could not be cultured in medium without any glucose, because then slices become shrunken and cannot be evaluated.

2.2. Oxygen-Glucose Deprivation (OGD). Slices in the wells (with low glucose) were transferred to a Modular Incubator Hypoxia Chamber (MIC-101, Billups-Rothenberg, Inc., Del Mar, CA, USA) connected to a flow meter. The chamber was sealed and a mixture of 95% N_2/5% CO_2 was flushed at a flow rate of 25 L/min for 5 minutes. The in- and outports of the chamber were closed, and the closed air tight system was placed at 37°C in an incubator for 1, 2, or 3 days. Measurements by the manufacturer indicate that the pO_2 reached a nadir of 35 mm Hg after 6 hr. At the end of the experiment the outlet port was slowly opened, the chamber opened, and the culture wells taken out and slices fixed for 3 hours with 4% paraformaldehyde at 4°C. Control slices were kept at normal 5% CO_2/air conditions in the incubator at 37°C.

2.3. Immunohistochemistry. Immunohistochemistry using the avidin-biotin technique was performed to detect cholinergic neurons as described [30, 31]. All incubations for immunohistochemistry were performed free-floating at 4°C for 2 days including 0.1% Triton, which allows good penetration of the antibody into the slices from both sides. Fixed slices were washed for 30 minutes with 0.1% Triton/PBS (T-PBS) at room temperature and pretreated for 20 minutes with 5% methanol/1% H_2O_2/PBS. Then the slices were rinsed three times for 10 minutes with PBS, blocked with 20% horse serum/0.2% BSA/T-PBS, and then incubated with the primary antibody against ChAT (1 : 750, Millipore) or c-fos (1 : 1000, Santa Cruz) in 0.2% BSA/T-PBS for 2 days at 4°C. Slices were washed and incubated with secondary biotinylated anti-goat (ChAT) or anti-rabbit (c-fos) antibody (1 : 200, Vector Laboratories), for 1 hour at room temperature. After rinsing three times in PBS, slices were incubated in avidin-biotin complex solution (ABC; Elite Standard PK 6100, Vector Laboratories) for 1 hr, then washed three times in 50 mM Tris-buffered saline (TBS), and the signal was detected using 0.5 mg/mL 3,3′diaminobenzidine (DAB) in TBS with 0.003% H_2O_2 as substrate. Slices were then rinsed in PBS and mounted on gelatine-coated glass slides.

2.4. Quantitative Analysis and Statistics. All neuronal counts were based on individual sections and show total number of neurons per slices. The number of microscopically detectable immunoreactive $ChAT^+$ neurons was counted in the whole slice visualized under a 20× objective by an investigator blinded to the treatment code. Multistatistical analysis was obtained by one-way ANOVA, followed by a subsequent

Fisher PLSD posthoc test by comparing controls against the respective treatments, where $P < .05$ represents statistical significance.

3. Results

When brain slices were cultured for 2 weeks in medium with normal glucose levels containing 10 ng/mL NGF, approximately 140 cholinergic nBM neurons/slice were detectable (Table 1; Figures 1(a) and 1(c)). This number did not change when slices were incubated without NGF for further 3 days (Table 1). Reduction of glucose to 6 mM (glucose deprivation) did not significantly change the number of cholinergic neurons after 1-2-3 days of incubation (Table 1). Slices exposed to hypoxia at normal (high) glucose did not show a reduced number of ChAT$^+$ neurons after 1-2-3 days of incubation (Table 1). However, when slices were incubated in low glucose and hypoxia (oxygen-glucose deprivation, OGD), the number of ChAT$^+$ neurons significantly decreased to about 70 neurons/slice after 3 days but not after 1 or 2 days of incubation (Table 1; Figure 1(b)).

In order to test whether S100b counteracts degeneration of cholinergic neurons caused by OGD for 3 days, slices were incubated with 50 μg/mL of S100b during the exposure. S100b counteracted the decrease of ChAT$^+$ neurons after 3 days of OGD (Table 1). By comparison NGF also counteracted loss of ChAT$^+$ neurons but was less potent (Table 1). FGF-2 only slightly counteracted the OGD effect (Table 1). Incubation of slices with S100b alone did not exert any toxic effect on ChAT expression in cholinergic neurons (Table 1).

In order to verify stressful effects of OGD on neurons, slices were stained for c-fos immunoreactivity at 3 and 24 hours of OGD (Figures 1(d)–1(f)). In controls without OGD, immunohistochemistry showed very few c-fos positive nuclei (Figure 1(d)). The number and intensity of c-fos positive nuclei significantly ($P < .001$) increased at 3 hours of OGD (Figure 1(e) and 1(f)) and thereafter returned to control levels at 24 hours of OGD.

Table 1: Cholinergic neurons after glucose-oxygen deprivation and neuroprotection with S100b.

	Treatment	ChAT$^+$ neurons	p1	p2
Day 0		136 ± 17 (14)	—	
+1d	GD	128 ± 17 (5)	ns	
	OD	131 ± 27 (6)	ns	
	OGD	113 ± 22 (6)	ns	—
	OGD + S100b (50 μg/mL)	121 ± 34 (3)		ns
+2d	GD	130 ± 21 (5)	ns	
	OD	124 ± 21 (8)	ns	
	OGD	110 ± 25 (6)	ns	—
	OGD + S100b (50 μg/mL)	138 ± 25 (6)		ns
+3d	(-)	138 ± 10 (18)	—	
	S100b (50 μg/mL)	139 ± 18 (19)	ns	
	GD	122 ± 16 (13)	ns	
	OD	135 ± 12 (12)	ns	
	OGD	81 ± 8 (28)	***	—
	OGD + S100b (50 μg/mL)	130 ± 11 (26)	ns	***
	OGD + S100b (1 μg/mL)	89 ± 10 (14)	**	ns
	OGD + S100b (0.1 μg/mL)	92 ± 12 (14)	*	ns
	OGD + FGF-2 (2 ng/mL)	102 ± 9 (12)	$P = .06$	ns
	OGD + NGF (10 ng/mL)	94 ± 12 (18)	*	ns

Brain slices of the nBM were cultured for 2 weeks with 10 ng/mL nerve growth factor (NGF), then for 3 days without NGF and then treated under oxygen-glucose deprivation (OGD) with or without S100b or NGF or fibroblast growth factor-2 (FGF-2) for 1–3 days. Values are given as mean ± SEM (n in parentheses). Statistical analysis was performed by one-way ANOVA with a subsequent Fisher PLSD posthoc test and compared against the untreated control (p1) or against OGD (p2) (*$P < .05$; **$P < .01$; ***$P < .001$; ns not significant). GD, glucose deprivation; OD, oxygen deprivation; OGD, oxygen and glucose deprivation.

4. Discussion

In the present study we show that cholinergic neurons of the basal nucleus of Meynert undergo neurodegeneration when exposed during 3 days of oxygen-glucose deprivation in a slice culture model. This neurodegeneration could be counteracted by recombinant S100b which is an endogenous glial protein.

4.1. Cholinergic Neurons in Brain Slices. The cholinergic neurons of the basal forebrain are a functionally homogeneous population, localized in the medial septum/diagonal band of Broca and basal nucleus of Meynert (nBM), provide the major cholinergic innervation to the hippocampus/amygdala and neocortex, respectively, and play a crucial role in cognition and memory. In neurodegenerative disorders such as AD, these neurons are dysfunctional and can further degenerate in the progression of the disease [32]. To study the mechanisms involved in neurodegenerative processes, as well as neuroprotective strategies, the organotypic brain slice model has been established [25, 33–37]. Detection of cholinergic neurons was performed using the immunohistochemical marker for the enzyme ChAT, which is expressed in the cell bodies and nerve fibers. A decreased number of ChAT$^+$ neurons directly correlates with neurodegeneration of cholinergic neurons. In our brain slice model cholinergic nBM neurons are axotomized and the number of ChAT$^+$ neurons is markedly decreased without exogenous NGF. Application of recombinant NGF counteracts this degeneration, displaying a slight neuroprotective effect at a concentration of >0.1 ng/mL NGF and full activity at >1 ng/mL NGF [37]. Thus, our brain slice model may allow to study the cholinergic phenotype or the degeneration of the majority of cholinergic neurons, which is seen by shrunken neurons and loss of nerve fibers and a decrease of the ChAT$^+$ immunoreactivity. In order to test whether S100b exerts

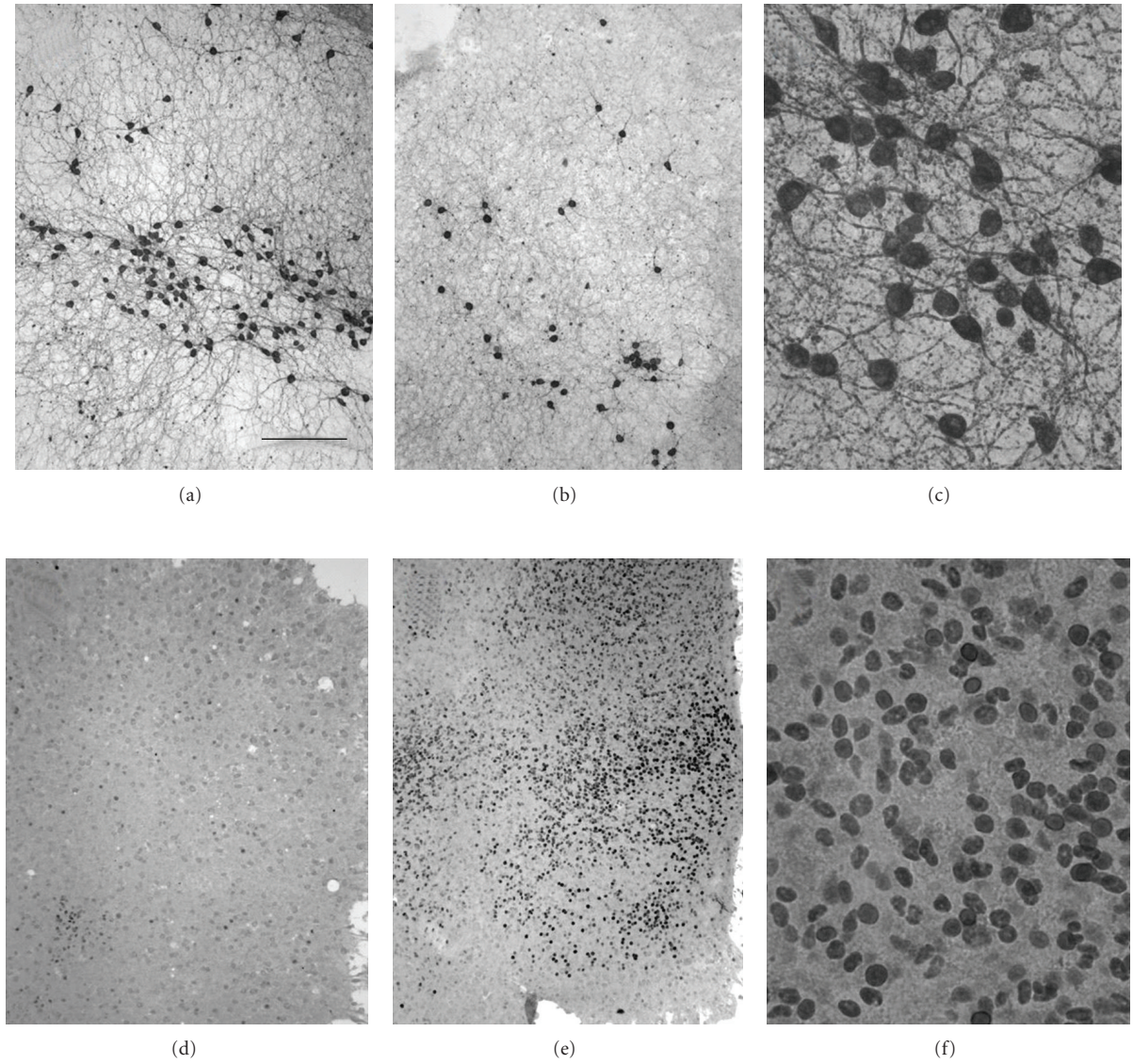

Figure 1: Immunohistochemistry for cholinergic choline acetyltransferase (ChAT) positive neurons (a)–(c) and c-fos immunoreactive nuclei (d)–(f) in organotypic brain slices of the basal nucleus of Meynert. Slices show control stainings before ((a), (c), (d)), or 3 days (ChAT$^+$, (b)) or 3 hours (c-fos+, (e) and (f)) after oxygen-glucose deprivation. Scale bar in $A = 300\,\mu$m ((a), (b), (d), and (e)) and $75\,\mu$m ((c) and (f)).

toxicity on cholinergic neurons, 2-week slices were exposed to a relatively high dose of 50 μg/mL (2.38 μM) of S100b for 3 days, but S100b did not affect the cholinergic neurons in our slice model. It is unlikely that a toxic effect would have shown with short exposures of 1 or 2 days.

4.2. Oxygen-Glucose Deprivation as a Model of Ischemia. The OGD model in slice cultures has been widely used to study "ischemia-like" conditions [26–28]. Most of the studies have used a strong and transient insult in slice cultures of the hippocampus to model the selective vulnerability to ischemia that is observed in vivo after cardiac arrest [26]. Neuronal damage is typically evaluated by dye-uptake and imaging techniques to quantify treatment effects [28]. The present model used a mild and more prolonged OGD impact (i.e., 1–3 d) to simulate the blood flow disturbances in the brain afflicted by vascular changes in AD. The evaluation was focused on the cholinergic neurons in the nBM using a high-resolution approach with neuronal counting. OGD in slice culture eliminates the influence of blood flow, which otherwise is a complex variable in animal models of brain ischemia. The observation that neurons are more vulnerable to ischemia (= combined loss of oxygen and glucose) than to either hypoglycemia or hypoxia alone has been made in multiple experimental conditions and probably applies to the clinical situation as well. One has to recognize that experimental OGD induces strong reductions but not total removal of glucose and/or oxygen. Since the slices are a stable system, the effects of OGD alone are very weak

after 3 days. We observed reductions of cholinergic neurons but no widespread cell damage. In the case of glucose deprivation (GD), endogenous stores are not completely exhausted within three days and alternative metabolites may be used. We have data showing that glucose reduction alone for 2 weeks markedly reduced cholinergic neurons. Oxygen deprivation (= hypoxia) is tolerated because of anaerobic glycolysis based on glucose stores which last for three days. Oxygen deprivation longer than 3 days is problematic, because the pH markedly decreases due to lactate production.

It should be noted that vascular networks persist in the slice culture in the absence of blood flow [35, 38]. The slice model should provide the opportunity to study reactions of vascular cells to ischemia-like conditions in the absence of intraluminal inflammatory cells. This is an important aspect, because endothelial cells can produce potentially damaging factors for cholinergic neurons [36]. In order to show that cellular stress is initiated soon after onset of oxygen-glucose deprivation, we analyzed the prototypic immediate early gene c-fos, which is upregulated within minutes in response to a strong stimulus, including ischemia, and rapidly downregulated in most experimental systems, even if the stimulus persists. For this purpose it was sufficient to show that c-fos was increased at 3 hours.

4.3. S100b Protects Cholinergic Neurons. The present finding that S100b protected cholinergic neurons in the slice culture model against OGD supplements previous reports of a protection of hippocampal neurons from glucose deprivation [39]. At low concentrations, S100b exerts multiple positive effects on neurons, including control of protein phosphorylation, regulation of energy metabolism and function of the cytoskeleton [8, 10]. Autocrine effects on astrocytes have also been described [10]. The role of S100b stimulation of the RAGE receptor in protection remains to be understood [10]. While there are reports of damaging effects of S100b in high concentrations [10], our experimental data do not suggest a negative influence on cholinergic neurons in vitro, at least for 3 days. In our study we tested different concentrations of 0.1–50 μg/mL but only the highest dose of 50 μg/mL (=2.38 μM) provided protection against oxygen-glucose deprivation. It has been reported that oxygen-serum-glucose deprivation induces release of S100b in the nanomolar concentration from astrocytes after 24 hours [11]. In our slice experiments 0.1 μg/mL S100b did not provide protection. Indeed, we assume that in our model of oxygen-glucose deprivation, the intrinsic astrocytes do release S100b but this may be insufficient to provide rescue of cholinergic neurons in this insult. A 100-fold higher dose was required to provide protection in our model system with oxygen-glucose deprivation but it is difficult to compare concentrations obtained in different experimental systems. Our organotypic brain slice cultures have a thickness of 100-150 μm by two weeks after explantation and it seems likely that a higher dose is necessary for diffusion deep into slices, so that concentrations reached at the level of the cell-bodies several tens of microns deep in the slice may be much lower.

4.4. An Expanded Role for Astrocytes in Alzheimer's Disease. Glial cells surrounding the basal forebrain cholinergic neurons are known to provide important paracrine trophic support, including NGF, FGF-2, epidermal growth factor, brain-derived neurotrophic factor, and ciliary neurotrophic factor. S100b could be an additional trophic factor supplied by local glial cells to cholinergic neurons, and theoretically, a decline of S100b production could contribute to cholinergic dysfunction. So far, most studies have focussed on the activation of astrocytes in the AD brain which are often related to senile plaques [40]. However, a reduction of glial function could be part of the complex pathophysiology of AD and improving glial function should be protective [17]. It is notable that two of the major risk genes for late-onset AD, that is, APOE and CLU/Clusterin [41], have important functions in astrocytes [42, 43], and gene variants could relate to reduced glial functions. Thus, complex interactions between the primary processes in AD (beta-amyloid plaque depositions, tau pathology), moderate ischemia-hypoxia, and genetic variations of glial reactions can be envisaged. In fact, it has been reported that S100b protects LAN-5 neuroblastoma cells against beta-amyloid-induced neurotoxicity at lower nanomolar doses, while S100b was toxic to LAN-5 cells at micromolar doses [44]. In their study [44] individual cells were exposed directly to S100b, whereas in our model the protein had to diffuse into the slices. In fact, neurons or glial cells in isolation on culture dishes do not have protection from surrounding cells and are exposed to high levels of radical oxygen species. Our slice model may incorporate these endogenous protective mechanisms. One could argue that our model using a tissue explant is closer to the in vivo situation than cell cultures. Another explanation is also that the recombinant S100b is derived from the bovine gene, while we use rat brain slices, and a mismatch in protein sequence and/or altered dimeric/monomeric forms may contribute to a lower functional efficiency on rat brain S100b sensitive receptors.

In conclusion the present study shows that 3 days of oxygen-glucose deprivation induced a marked decrease of cholinergic neurons, which could be counteracted by S100b. We conclude that S100b is a potent neuroprotective factor for cholinergic neurons after ischemic events.

Abbreviations

AD: Alzheimer's disease
ChAT: Choline acetyltransferase
nBM: Basal nucleus of Meynert
NGF: Nerve growth factor
OGD: Oxygen-glucose deprivation.

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Clinical Study

The Passage of S100B from Brain to Blood Is Not Specifically Related to the Blood-Brain Barrier Integrity

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Following brain injury, S100B is released from damaged astrocytes but also yields repair mechanisms. We measured S100B in the cerebrospinal fluid (CSF) and serum (Cobas e411 electrochemiluminescence assay, Roche) longitudinally in a large cohort of patients treated with a ventricular drainage following traumatic brain injury (TBI) or subarachnoid hemorrhage (SAH). Statistical analysis was performed with SPSS software applying the Mann-Whitney rank sum test or chi-test where appropriate. S100B in CSF and serum was significantly increased following TBI ($n = 71$) and SAH ($n = 185$) for at least one week following injury. High S100B levels in CSF and serum were inconsistent associated with outcome. The passage of S100B from CSF to blood ($100^{*}\text{serum}_{\text{S100B}}/\text{CSF}_{\text{S100B}}$) was significantly decreased although the albumin quotient suggested an "open" blood-CSF barrier. Events possibly interfering with the BBB did not affect the S100B passage ($P = .591$). In conclusion, we could not confirm S100B measurements to reliably predict outcome, and a compromised blood-CSF barrier did not affect the passage of S100B from CSF to serum.

1. Introduction

There is a desire for a reliable indicator to accurately determine the extent of brain injury and consequent prognosis. The measurement of putative biochemical markers, such as the S100B protein, has been proposed in this role. Furthermore, such a biomarker would aid in identifying events contributing to secondary brain damage and monitoring the success of therapeutic interventions.

Over the past decade, numerous studies have reported a positive correlation between S100B levels in blood or cerebrospinal fluid (CSF) and impaired neurological function following traumatic brain injury (TBI) [1], intracerebral hemorrhage [2], stroke [3], perinatal brain damage [4], septic encephalopathy [5], bacterial meningitis [5], or even in major depression [6], and extracranial injuries [7]. Furthermore, S100B levels have been used to monitor therapeutic effects such as the application of hypertonic saline in TBI [8] or of naloxone in epilepsia [9]. However, considerable evidence indicates that S100B is not only a biomarker of brain damage but also represents ongoing neuroregeneration [10]. Moreover, contradictory data interpretation exists with regard to the contribution of an altered blood-brain barrier (BBB) to S100B serum levels [11, 12].

Although in cell cultures the injury-induced S100B release continues to increase up to 48 hours [13, 14], in patients S100B serum levels have been reported to be highest directly after the injury and become normalized within 24 hours in a high percentage of cases, even in those patients with a bad outcome [15]. The underlying mechanism describing the passage of S100B from brain to blood following acute brain injury has not yet been clarified, nor does an unequivocal data interpretation exist regarding cerebral S100B levels and their correlation to serum S100B levels.

Opposite to the BBB, the compartmental barrier within the ventricles is not at the level of the blood vessels but

between the epithelial cells that form the inner CSF-facing surface of the choroid plexus. Since the choroid plexus are of mesodermal origin, their leaky capillaries are one of the exceptions to the rule that almost all capillaries in the central nervous system (CNS) form tight junctions between their endothelial cells thereby establishing the BBB. CSF is directly secreted by the choroid plexus into the ventricles constituting a blood ultrafiltrate and is also derived from the extracellular fluid (ECF). The ECF communicates reasonably freely with the ventricular CSF through normal nonbarrier spaces between ependymal cells [16]. CSF production is measured by dilution studies, and a total volume of 130 ml CSF in men is renewed every 5–7 hours. The classic view of CSF removal is to pass through the arachnoid villi into the venous sinuses by bulk flow [17], but alternatively CSF may pass into the blood vessels driven by a mixing or pulsatile flow [18].

Although the functional assessment of the dynamics of protein passage from blood to brain or *vice versa* in CNS disorders is of general interest, in patients the means of quantification are limited. Since blood and CSF are readily accessible, calculating respective ratios is reasonable. The $\text{albumin}_{\text{CSF}}/\text{albumin}_{\text{serum}}$ quotient (Q_A) has been established as the "golden standard" for the assessment of blood-CSF barrier dysfunction [19, 20] although it is occasionally mistaken to measure BBB permeability. The 66 KD protein albumin is synthesized peripherally, is not catabolised within the CNS, and does not readily diffuse across an intact BBB. In adults, normal values are defined as a $Q_A \leq 0.007$, and a damaged or open blood-CSF barrier is defined as mild ($Q_A = 0.007$–0.01), moderate ($Q_A = 0.01$–0.02), and severe ($Q_A \geq 0.02$), respectively [21]. Applying the Q_A, it is important to note that the ratio has been established in lumbar CSF. Extensive studies on the dynamics of blood- and brain-derived proteins across the blood-CSF barrier support the view that blood-CSF barrier dysfunction is a biophysical concept of increased molecular flux with decreasing CSF flow rate rather than a morphological "leakage" model [22].

Thus, interpreting the serum and CSF levels of the 22 kD dimeric neurotrophic protein S100B following acute brain damage is a dual challenge. Firstly, reasonable evidence exists that S100B is not only passively released by damaged astrocytes but also actively secreted and acts in a positive paracrine manner to foster neuronal repair or regeneration. Secondly, protein reabsorption accompanying CSF turnover emerges as a possibility. Facing the limitations of in vitro and in vivo experimental models, we confined ourselves to data collection in the clinical setting of acute brain injury. In particular, the purpose of the present study was (i) to examine the temporal profile of S100B release into CSF and blood in a large cohort of patients following TBI and subarachnoid hemorrhage (SAH) longitudinally, (ii) to calculate the ratio of S100B in the CSF/serum in order to estimate the passage from CSF to blood, and (iii) to correlate the respective S100B levels with the neurological function and recovery as well as with specific events known to interfere with the BBB integrity.

2. Methods

2.1. Subjects. The study was approved by the Ethics Committee of our hospital and was conducted in compliance with the Declaration of Helsinki. Informed consent was obtained from the next of kin of the patient. Patients were included unless one of the following exclusion criteria was present: age <18 years; pregnancy or nursing state; primary central nervous disorders (e.g., meningitis, neoplasm, or known epilepsy); expected to die within the first 48 hours; melanoma; severe burns, orthopaedic surgery, or cardiac bypass surgery. Patients were enrolled into two groups, one presenting with isolated TBI requiring ventriculostomy and catheter placement ($n = 71$) and the other presenting with SAH ($n = 185$). Normal values of S100B in serum and CSF in healthy controls had been established in the past in [12]. Since the normal values in this study were established in the lumbar CSF of control patients, we applied a 3.5 correction factor according to the CSF flow rate model of Reiber [22].

In the study patients, ventriculostomy catheters were placed as part of the clinical care within 12 hours of admission. Ventriculostomy catheters are typically placed to monitor intracerebral pressure in patients with severe TBI (Glasgow Coma Scale [GCS] score ≤8) and an intracranial injury on computed tomography (CT) scan. However, these catheters are also placed in those with severe TBI and a normal CT if two or more of the following factors are present: age >40 years, unilateral/bilateral motor posturing, and systolic blood pressure <90 mmHg [23]. In some cases, subjects with initial GCS scores above 8 received ventriculostomy catheters because of subsequent clinical deterioration and were included in the study. Subjects were eligible for inclusion as a SAH subject if the diagnosis was confirmed by CT or an abnormal lumbar puncture.

Extracranial and brain injury were documented by CT, neurological function by the GCS and Glasgow outcome Score (GOS), as well as intensive care scores (APACHE and/or SAPS). All events potentially interfering with S100B passage were recorded: hypotonia (mean blood pressure < 65 mmHg), hypoxia (SpO2 < 90%), hyperthermia (>38°C), increased intracranial pressure (ICP > 20 mmHg), treatment with mannitol, increased cerebral blood flow velocity (>80 cm/s), and treatment modalities as aneurysm surgery or coiling, change of ventricular drainage, shunt implantation, or tracheotomy.

2.2. Sample Collection and Processing. In TBI and SAH subjects, we collected blood and CSF samples daily at 8 AM for up to 4 weeks postinjury. For all blood draws, 4 ml of venous or arterial blood were drawn into serum separator tubes and centrifuged at 3000 rpm for 10 minutes at room temperature. The cellular components were discarded and the serum stored at −80°C until used for assays. CSF was collected at the time of blood draws. For each CSF sample, 5–10 ml were collected into a 15 ml polypropylene tube, immediately placed on ice, and centrifuged at 3000 rpm for 10 minutes at room temperature. The cellular components were discarded and the remaining sample stored at −80°C until used for assays. Serum S100B concentrations were measured with

the Cobas e411 S100 electrochemiluminescence assay, Roche Diagnostics. S100B concentrations in the serum samples were determined using the standard curve generated from the absorbance of the standards. The lower detection limit of this assay is 0.005 μg/l, the upper limit 39 μg/l. In order to quantify the S100B passage from CSF into blood, we calculated the S100B ratio in serum originating from CSF ($= 100^{*}\text{serum}_{\text{S100B}}/\text{CSF}_{\text{S100B}}$).

Albumin was analysed by immunochemical nephelometry, BN Prospec, Siemens Diagnostics, as described elsewhere [24]. The standard calibration curve for CSF measurements was also applied for serum measurements following an instrumented dilution either by x400 or x2000. Analysis of the blood-CSF barrier function was determined using the $\text{CSF}_{\text{albumin}}/\text{serum}_{\text{albumin}}$ ratio (Q_A). Daily albumin values were measured in CSF and serum, and the Q_A was calculated. Since our albumin analyses were performed in ventricular CSF, a correction factor for a ventricular to lumbar CSF gradient of 1 : 2.5 was applied, and a relevant disturbance of the blood-CSF barrier function was assumed if the Q_A exceeded 0.0028 [21, 22].

2.3. Statistical Analysis. All values are given as mean ± SEM. The statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL) and R (open source software). Group comparisons, such as TBI versus SAH, or good (GOS 4 + 5), moderate (GOS 3), and worse (GOS 1 + 2) outcome were made applying the Mann-Whitney rank sum test since no normal distribution was present. Correlation analysis between S100B values and the Q_A was performed using the nonparametric Kendall's tau_b Pearson correlation test. The chi-test was used to compare the incidence of events associated with an increase or decrease of S100B levels. Significance was defined as $P < .05$.

3. Results

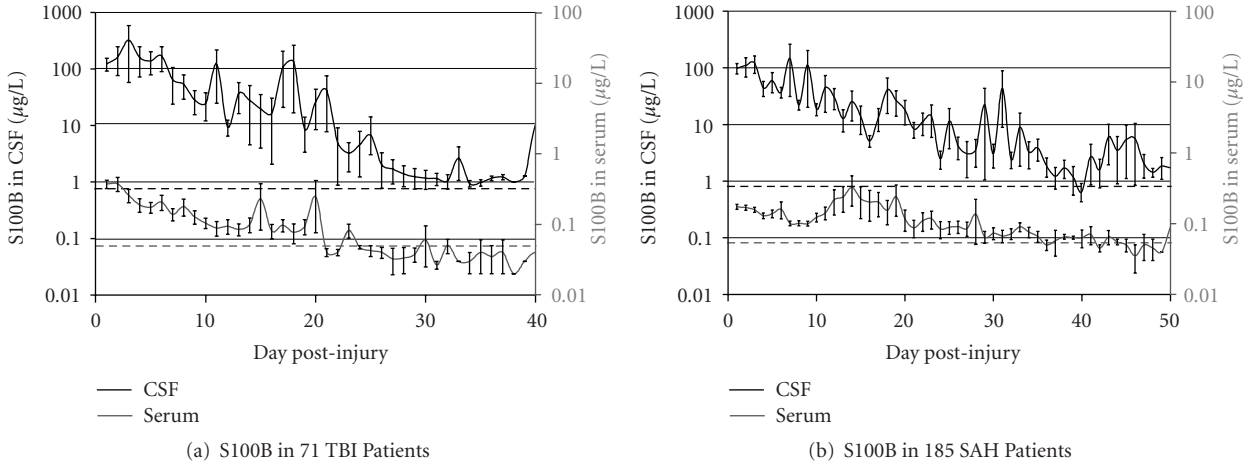
We included 71 patients admitted for TBI and 185 patients following SAH. The normal S100B values had been established in control patients undergoing pituitary surgery treated with a lumbar drainage at 0.07 μg/l in serum and at 2.87 μg/l in CSF applying a 3.5 correction factor to normalize for the negative ventricular-lumbar CSF gradient of brain-derived proteins. S100B in CSF was significantly increased up to day 7 following TBI ($64.98 \pm 272.39\,\mu$g/l, $P = .024$) and SAH ($146.90 \pm 1374.86\,\mu$g/l, $P = .009$) and in serum up to day 8 following TBI ($0.16 \pm 0.30\,\mu$g/l, $P = .032$) and up to day 14 following SAH ($0.33 \pm 1.12\,\mu$g/l, $P = .016$). The time course of the S100B concentration in serum and CSF in 185 SAH patients and 71 TBI patients is displayed in Figures 1(a) and 1(b). Statistical comparison between the different types of acute brain injury by the Whitney-Mann rank sum test revealed that S100B in CSF and serum was significantly higher following TBI than SAH for the first 5 days ($P < .05$).

3.1. S100B Serum/CSF Ratio and the Blood-CSF Barrier. In order to quantify the S100B passage from CSF into blood, we compared the ratio $\text{serum}_{\text{S100B}}/\text{CSF}_{\text{S100B}}$. In the control patients, S100B in serum comprised around 2.8% of the respective CSF concentration, applying a 3.5 correction factor for the negative ventricular to lumbar CSF gradient. The S100B ratio in SAH and TBI patients was significantly reduced for the first 4 days ($P < .05$). In accordance to the literature, we quantified the ratio QA, respectively, $\text{CSF}_{\text{Albumin}}/\text{serum}_{\text{Albumin}}$ reflecting the passage of albumin from blood to the brain. The Q_A was significantly increased over the investigation period (day 1: 0.015 ± 0.012, day 10: 0.016 ± 0.013) as compared to normal values (Q_A [normal for ventricular CSF] ≤ 0.0028, $P < .05$). There was no correlation between the S100B ratio and the Q_A (day 1: $r = 0.233$, $P = .615$, day 7: $r = 0.110$, $P = .860$).

3.2. Prediction of Outcome. Within the groups, there was no consistent correlation between S100B concentrations in either serum or CSF and neurological function as assessed by GCS. The statistical analysis of outcome prediction and S100B levels revealed inconsistent findings, especially in TBI patients. SAH patients with worse outcome (GOS 1 + 2) had significantly higher S100B serum levels on day 2 ($P = .042$), day 3 ($P = .042$), and day 4 ($P = .031$) as compared to moderate outcome (GOS 3) and on day 5 ($P = .006$), day 7 ($P = .004$), day 11 ($P = .012$), day 12 ($P = .008$), day 13 ($P = .003$), day 14 ($P = .003$), day 15 ($P = .032$), and day 17 ($P = .036$) as compared to good outcome (GOS 4 + 5). SAH patients with worse outcome had significantly higher S100B CSF levels on day 1 ($P = .011$), day 2 ($P = .010$), and day 3 ($P = .010$) as compared to moderate outcome and on day 5 ($P = .011$), day 7 ($P = .021$), day 10 ($P = .003$), day 12 ($P = .012$), and day 13 ($P = .028$) as compared to good outcome. SAH patients with worse outcome had a significantly impaired S100B CSF/serum passage on day 3 ($P = .026$) as compared to moderate outcome and on day 10 ($P = .039$) as compared to good outcome. TBI patients with worse outcome had significantly higher S100B serum levels on day 2 ($P = .019$) as compared to good outcome. TBI patients with worse outcome had significantly *lower* S100B CSF levels on day 3 ($P = .016$) as compared to moderate outcome. TBI patients with worse outcome had a significantly *improved* S100B CSF/serum passage on day 3 ($P = .016$) as compared to moderate outcome.

3.3. Events Affecting S100B Levels. In 30 patients, we analyzed events possibly interfering with the BBB in detail (Figure 2). Neither hypotonia (mean blood pressure < 65 mmHg) nor hypoxia (SpO2 < 90%), hyperthermia (>38°C), increased intracranial pressure (ICP > 20 mmHg), treatment with mannitol, increased cerebral blood flow velocity (>80 cm/s), or treatment modalities as aneurysm surgery or coiling, change of ventricular drainage, shunt implantation or tracheotomy did affect the passage of S100B or albumin through the blood-CSF barrier ($P = .591$). The incidence of events is displayed in Figure 3.

3.4. Contribution of Extracerebral S100B Sources. In TBI patients, the contribution of extracerebral sources to S100B



(a) S100B in 71 TBI Patients

(b) S100B in 185 SAH Patients

Figure 1: Mean S100B levels in CSF (a) and serum (b) following subarachnoid hemorrhage (SAH) or traumatic brain injury (TBI). The values are given as mean ± SEM.

levels was assessed. One patient suffered from a femur fracture contributing to excessively elevated S100B serum levels on admission (1.22 μg/l). However, S100B was cleared from serum on the following day (0.25 μg/l), and CSF levels on admission remained below mean values (36.98 μg/l), and for the total investigation period. In two other patients with a fracture of the clavicle, S100B serum levels were not affected. Furthermore, S100B is expressed in relevant concentrations in adipose tissue [25], and S100B serum levels were reported to correlate with the BMI and speculated to be closely linked to an altered energy metabolism in diabetic patients [26]. In our study, obesity did not significantly affect S100B serum levels while in diabetic patients, S100B serum levels were significantly higher than in nondiabetic ones ($P = .013$).

4. Discussion

To the best of our knowledge, this is the first study to investigate concurrent concentrations of S100B in serum and CSF in more than 250 patients following acute brain injury longitudinally for up to 4 weeks. As it has been iterated by a plethora of authors, we found some correlation of high S100B levels and worse outcome, but far from any prerequisite of unequivocal outcome prediction. Opposite to common reasoning, in our study the passage of S100B from CSF to serum was impaired following acute brain injury. We could not confirm any contribution of a compromised blood-CSF barrier to S100B serum levels.

4.1. Release of S100B into CSF. Neither the role of the glial protein S100B in the acutely injured brain nor the release into the ECF and the subsequent passage to the CSF and blood has been established. From *in vitro* injury, we learned that S100B is released into the culture medium [13, 14]. The ECF communicates with the CSF through normal nonbarrier spaces [16], presumably allowing S100B to pass freely from the ECF to the CSF. Thus, it is reasonable to assume that S100B CSF concentration measured following human brain injury reflects the S100B release into the ECF. The injury-induced S100B release in cell cultures displayed an upward slope over the investigation period [14] demonstrating an active stimulated release contributing to the total S100B concentration measured. *In vivo* data suggest a S100B release due to learning and memory processes [27, 28]. In patients, electroshock therapy did not affect S100B serum levels [29], but acute psychosis resulted in an increased concentration of S100B in CSF and serum [30]. The long-lasting increased S100B levels in CSF found in our patients following SAH or TBI are unlikely to result purely from brain injury. Furthermore, we were unable to verify any consistent correlation of S100B CSF levels and injury severity or outcome. However, since a strong S100B immunopositivity of the ependymal and choroid plexus epithelia has been observed although the functional consequences have not been elucidated yet, we cannot exclude a contribution from these cells to the S100B CSF concentration [31]. A limitation of our study is comparing the ventricular CSF measurements from our patients with lumbar CSF measurements from controls subjects of a previous study. To eliminate any interference of ventricular-lumbar protein gradients, we applied an estimated "correction factor" of 3.5 for S100B and of 0.4 for albumin [22]. Taken together, the literature and our findings imply an active stimulated S100B release into CSF reflecting neuronal-glial activation, synaptic plasticity, or neuroregeneration rather than to result from injured cells.

4.2. Brain-CSF-Blood Barrier. Increased S100B concentrations in the blood have been attributed to the passage through an impaired BBB following brain injury [26], whenever an extracerebral origin of S100B was excluded [26, 32, 33]. However, contradictory data interpretation exists with regard to the contribution of an altered BBB to S100B serum levels [12].

The BBB prevents diffusion of most water-soluble molecules over 500 Da. Although the $\text{albumin}_{\text{CSF}}/\text{albumin}_{\text{serum}}$ quotient (Q_A) has originally been described for

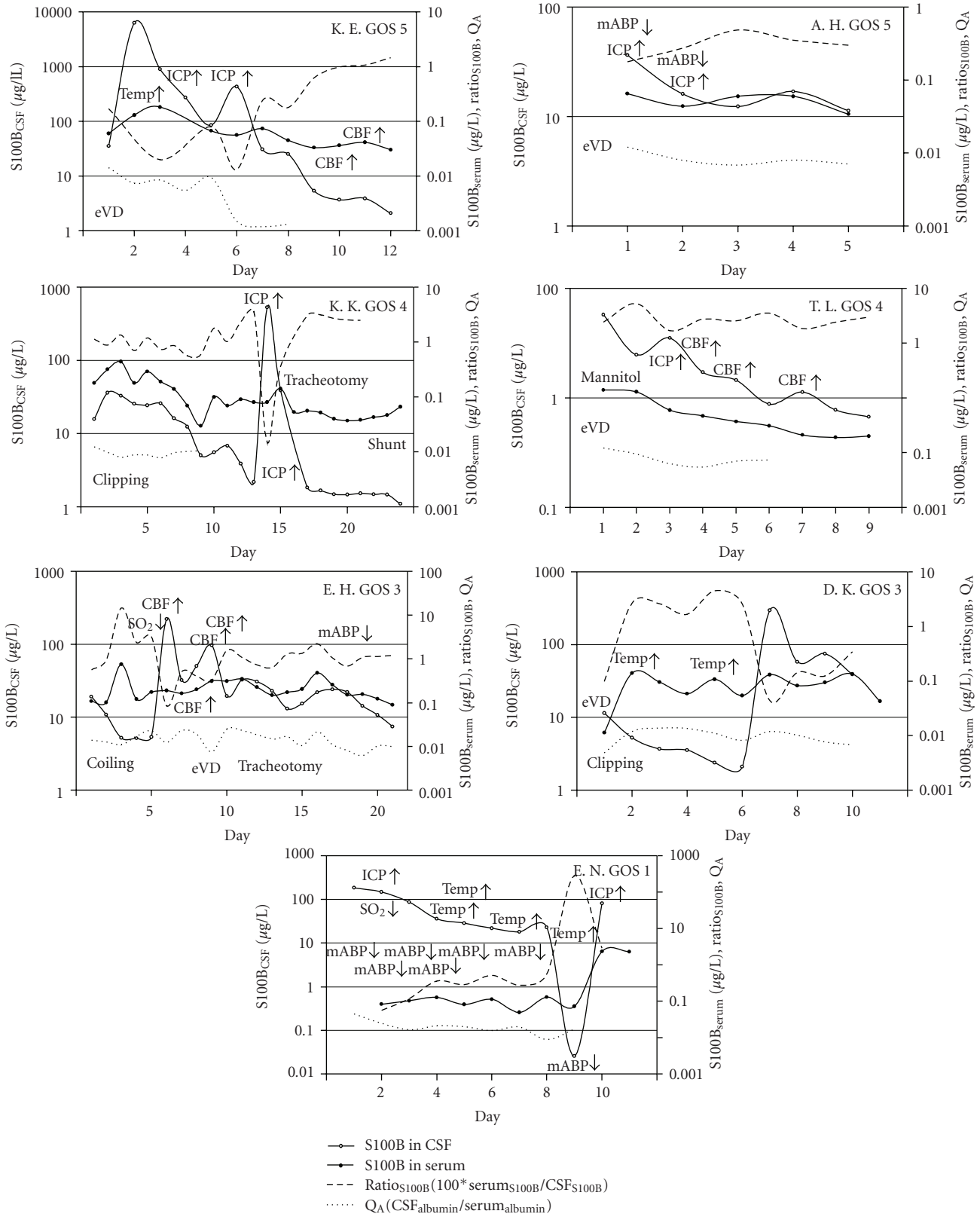


FIGURE 2: For individual patients, S100B in serum and CSF as well as the ratio $serum_{S100B}/CSF_{S100B}$ and the $Q_A CSF_{Albumin}/serum_{Albumin}$ are displayed as well as events affecting the blood-brain barrier or blood-CSF barrier integrity. Note that the values are displayed on a logarithmic scale. GOS, Glasgow Outcome Score.

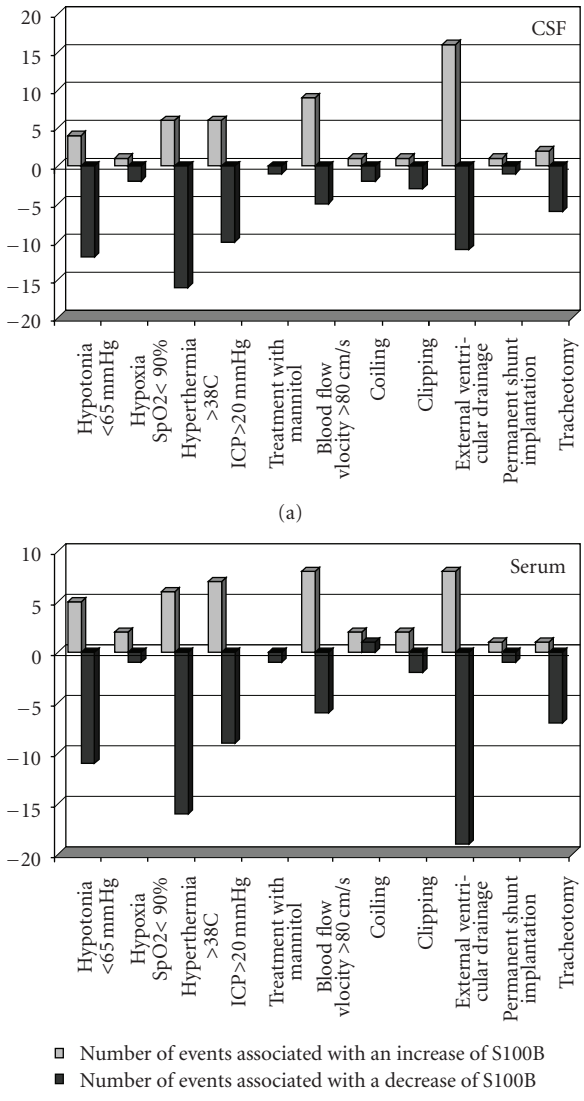


Figure 3: Events potentially interfering with the blood-brain barrier (BBB) and the effect on the S100B concentration in CSF and serum following subarachnoid hemorrhage (SAH) or traumatic brain injury (TBI). The chi-test did not reveal any impact of events on S100B levels.

the blood-CSF barrier dysfunction [19, 20], several authors calculated indices using the Q_A as a measure of "BBB" permeability following TBI, such for the intercellular adhesion molecule-1 (ICAM-1) [34], for the antiinflammatory transforming growth factor-beta (TGF-beta) [35], or for the complement-derived soluble membrane attack complex (sC5b-9) [36], and they reported the respective CSF levels paralleling the "BBB" function as assessed by the Q_A. However, other authors could not confirm such a correlation, for example, assessing the cerebral production of interleukin (IL)-10 [37] or IL-6, IL-8 and IL-10 [38].

The lessons we learned from these controversies are twofold. First, any ratio $CSF_{SUBSTANCE}/serum_{SUBSTANCE}$ does reflect the passage of the respective protein through the blood-CSF barrier that is clearly to distinguish from the BBB and is a measure of an altered CSF flow [22]. Second, the $albumin_{CSF}/albumin_{serum}$ quotient Q_A allows inference on proteins around 66 KD, while the dimeric 22 KD protein S100B may comply different dynamics.

4.3. Passage of S100B from Brain to Blood. Following a preliminary study including few patients and assessing the release and wash-out pattern of S100B in serum and CSF, the importance to know the underlying pathology and timing in interpreting S100B levels has been highlighted [39]. We analyzed the impact of several pathophysiological dysregulations like hypotonia, hypoxia, hyperthermia, increased intracranial pressure, vasospasm, and craniotomy known to affect the BBB. Furthermore, the BBB has been reported to be opened osmotically [40]. In our study, those events likely to interfere with the BBB and treatment with mannitol did not affect the passage of S100B from the CSF to blood.

Opposite to the BBB, the compartmental barriers between the CSF and blood are leaky capillaries of the choroid plexus allowing protein secretion into the CSF. Little is known whether this secretion is unidirectional or may allow the reabsorption of proteins from the CSF into blood. Furthermore, the relevance of the blood-CSF barrier in acute brain damage remains unclear. Ultrastructural examinations of the choroidal epithelial cells forming the CSF-blood barrier following experimental injury demonstrate pronounced changes lasting up to 4 weeks postinjury [41]. Accordingly, the reduced ratio of S100B serum/CSF found in our patients may result from damaged choroidal epithelial cells hampering with the S100B passage from CSF to blood [31]. However, considerable evidence indicates that S100B is not only a biomarker of brain damage but also represents ongoing repair or neuroregeneration [10]. Thus, the reduced passage of the neurotrophic protein S00B from CSF to blood may result from an increased demand in injured tissue.

5. Conclusion

Although there is a reasonable desire for a reliable indicator to accurately determine the extent of brain injury and to monitor therapeutic interventions, advocating S100B in this role remains problematic. While a substantial body of evidence demonstrates an association between S100B and bad outcome after brain injury, it is important to be aware that proof of an association is not proof of causation in science. In the present large cohort of patients, the concurrent measurement of S100B in serum and CSF, we found some association of high S100B levels and worse outcome, but far from any prerequisite of unequivocal outcome prediction. Opposite to common reasoning, we found the passage of S100B from CSF to serum impaired following acute brain injury. We could not confirm any contribution of a compromised BBB or blood-CSF barrier to S100B serum levels.

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