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Quantitative Immunoblotting Analyses Reveal that the Abundance of Actin, Tubulin, Synaptophysin and EEA1 Proteins is Altered in the Brains of Aged Mice

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Abstract—Optimal synaptic activity is essential for cognitive function, including memory and learning. Evidence indicates that cognitive decline in elderly individuals is associated with altered synaptic function. However, the impact of aging on the expression of neurotransmitter receptors and accessory proteins in brain synapses remains unclear. To fill this knowledge gap, we investigated the effect of aging on the mouse brain by utilizing a subcellular brain tissue fractionation procedure to measure protein abundance using quantitative Western Blotting. Comparing 7-month- (control) and 22-month- (aged) old mouse tissue, no significant differences were identified in the levels of AMPA receptor subunits between the experimental groups. The abundance of GluN2B NMDA receptor subunits decreased in aged mice, whereas the levels of GluN2A did not change. The analysis of cytoskeletal proteins showed an altered level of actin and tubulin in aged mice while PSD-95 protein did not change. Vesicle protein analysis revealed that synaptophysin abundance is decreased in older brains whereas EEA1 was significantly increased. Thus, our results suggest that physiological aging profoundly impacts the abundance of molecules associated with neurotransmitter release and vesicle cycling, proteins implicated in cognitive function.

Key words: aging, brain, cortex, synapse, glutamate receptor, vesicle.

INTRODUCTION

The central nervous system (CNS) controls cognitive abilities – learning, memory, planning and organization - that are crucial for peoples' lives. The CNS not only integrates sensory information from the external environment but also plays a critical role in regulating important physiological processes, such as growth, metabolism and reproduction (Alcedo et al., 2013). While the CNS displays aging-related positive cognitive changes, including increased knowledge from experiences over a lifetime, it is particularly vulnerable to aging (Mattson and Magnus, 2006). Typically, age-related cognitive impairment is associated with negative aspects, such as exhibiting difficulties during multi-tasking, in finding words or when trying to recall a name or a phone number. Although it is extremely difficult to dissociate the effects of aging from the effects of undiagnosed diseases in an aged population, uncovering changes that are not related to dementia in the brain are arguably as important as understanding disorders, such as Alzheimer's and other neurological diseases (Fjell et al., 2014).

During aging, the brain displays various biological and functional changes that entail alterations at all levels from gross morphology and vasculature to molecules, including hormones and neurotransmitters (Peters, 2006). For example, recent magnetic resonance imaging studies have shown that the prefrontal cortex and the hippocampus shrink at a rate of up to 1% per year during

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionique acid; AMPAR, AMPA receptor; CNS, central nervous system; ECL, enhanced chemiluminescence; EEA1, early endosome Antigen 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluA, AMPAR subunit; GluN, NMDAR subunit; iGluRs, ionotropic glutamate-gated receptors; LP1, synaptosomal membrane; LS1, supernatant of LP1 fraction containing synaptic vesicles and other cytosolic organelles; NMDA, N-methyl-D-aspartic acid; NMDAR, NMDA receptor; NSF, N-ethymaleimide-sensitive factor; P2, crude synaptosome; PSD, postsynaptic density; PVDF, polyvinylidene difluoride; RRID, research resource identifier; RQRV, Réseau québécois de recherche sur le vieillissement; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SSM, soluble synaptosomal membrane.

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healthy aging (Raz et al., 2005; Raz and Rodrigue, 2006). In healthy individuals, the prefrontal cortex mediates complex tasks, such as working memory and goal-directed behaviour, and the hippocampus plays a central role in spatial and navigation memory and in processes that code and store memories (Funahashi and Takeda, 2002; Watanabe and Sakagami, 2007; Chudasama, 2011). In the aging brain, alterations in neuronal circuitry and in the morphology of neurons, decreased activity of enzymes or chemical messengers and alterations in gene expression have been reported (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Hof and Morrison, 2004; Burke and Barnes, 2006; Morrison and Baxter, 2012). These alterations surely lead to impaired synaptic function and, therefore, contribute to the aging-related decline in complex brain function. Importantly, glutamatergic pyramidal neurons typically constitute the age-vulnerable neural circuits for cortico-cortical connections associating the cortices along with the hippocampal networks (Morrison and Hof, 1997; Morrison and Baxter, 2012; Pereira et al., 2014). Furthermore, evidence suggests that normal age-related decline in the prefrontal cortex and in hippocampal functions is due to subtle alteration in the content and function of glutamatergic synapses rather than in reduced synapse number or in neuronal loss (Hsia et al., 1999; Kamenetz et al., 2003; Hsieh et al., 2006; Rui et al., 2010). Since synapses are the functional units of the brain where synaptic plasticity occurs to generate correlates for learning and memory, it is of prime importance to better understand the molecular elements that are vulnerable to the physiological aging process.

Synaptic excitatory neurotransmission is achieved via the release of glutamate into the synaptic cleft where it diffuses and binds to the extracellular regions of postsynaptic ionotropic glutamate-gated receptors (iGluRs). Excitatory neurotransmission in pyramidal neurons is mainly achieved by two distinct subfamilies of iGluRs: the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) and the N-methyl-D-aspartate receptor (NMDAR) (Malinow and Malenka, 2002; Shepherd and Huganir, 2007; Huganir and Nicoll, 2013). The subunit composition of iGluR tetrameric channels varies in different brain regions. AMPAR complexes are formed by the assembly of homologous subunits (GluA1-4), whereas NMDARs are made of two dimers composed of one obligatory GluN1 subunit that must assemble with either GluN2A-D or GluN3A-B. The activation of NMDARs induces most synaptic plasticity processes by altering the content of dendritic spines and the amount of synaptic AMPARs (Malinow and Malenka, 2002; Shepherd and Huganir, 2007; Huganir and Nicoll, 2013). Importantly, scaffold proteins play important roles in neuronal structure as well as for the postsynaptic density (PSD) architecture. For instance, it is well documented that PSD-95, a major postsynaptic scaffolding protein at excitatory synapses, localizes, anchors and clusters glutamate receptors at the PSD (Sheng and Pak, 1999). In fact, PSD-95 knockout mice demonstrate a lack of sociability and deficits in learning and working memory (Coley and Gao, 2019).

Mature and functional synapses are composed of presynaptic neurotransmitter-releasing components and of postsynaptic elements required to receive and process neurotransmitter signals. Since excitatory neurotransmission is achieved via the release of glutamate into the synaptic cleft, glutamate can either diffuse to bind the extracellular regions of postsynaptic iGluRs, be recaptured by the action of specific carriers (Shigeri et al., 2004) or be recycled by the formation of new synaptic vesicles by endocytosis (Sudhof, 2004). Specifically, recent evidence demonstrates that synaptophysin, a membrane-spanning glycoprotein that accounts for up to 10% of total synaptic vesicle proteins (Takamori et al., 2006), is implicated during the endocytosis and recycling of neurotransmitter vesicles (Kwon and Chapman, 2011). Furthermore, early endosome antigen 1 (EEA1), a peripheral membrane protein required for fusion and maturation of early endosomes (Ramanathan et al., 2013), is needed for the recycling of glutamate receptors (Gurtler et al., 2013). Indeed, inhibition of EEA1 leads to neurological deficits and affects excitatory synaptic transmission (Gurtler et al., 2013). Accordingly, many cellular mechanisms ensure that the remodeling of synaptic content adjusts the efficacy of glutamatergic neurotransmission. For example, the insertion and removal of synaptic AMPARs are thought to maintain stable excitability of synapses through slow homeostatic plasticity (i.e. synaptic scaling) and to underlie the rapid Hebbian forms of plasticity (i.e. long-term potentiation and long-term depression) (Shepherd and Huganir, 2007; Huganir and Nicoll, 2013). Dendritic spines are the principal postsynaptic sites for iGluRs and are important in synaptogenesis, synaptic regulation and cognition (Jia et al., 2009). Spines are dynamic and change their shape and size depending on the strength of excitatory synaptic connections, a process that relies on remodeling of the actin cytoskeleton (Hotulainen and Hoogenraad, 2010). For example, LTP can occur when the actin cytoskeleton grows and, thus, enlarges the dendritic spine (Borovac et al., 2018). Independently of time, these forms of plasticity share common effectors at synapses (Vitureira and Goda, 2013).

There is a significant body of literature demonstrating that abnormal subcellular localization of receptors and other signaling proteins might be associated with neurological disorders (Torres et al., 2017; Bereczki et al., 2018, 2016; Nishiyama, 2019). Accordingly, powerful tools are needed to understand the spatial and temporal impacts of aging on proteome function in the brain. Over the years, the rapid and reliable separation of proteins localized in discrete subcellular compartments and organelles from rodents' brains has proven its usefulness when combined with analytic methods, such as quantitative immunoblotting. Immunoblotting was first described in 1979 (Towbin et al., 1979) and is still a very important tool used in research laboratories around the world. This biochemical technique was not initially intended for providing quantitative data about protein abundance in a specific sample. However, it is now common to do exactly that where the abundance of a protein-of-interest is normalized against housekeeping proteins, typically either

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ß-actin or ß-tubulin, as a way to correct for protein loading variability and other factors, such as total protein quantitation and transfer efficiency (Taylor et al., 2013; Ghosh et al., 2014). However, the lack of changes in the abundance of those housekeeping proteins is typically assumed rather than being established. This represents a major caveat knowing that the content of most housekeeping proteins is actually altered in various experimental conditions and tissues (Ferguson et al., 2005; Rodriguez-Mulero and Montanya, 2005; Ruan and Lai, 2007). For instance, GAPDH abundance varies across skeletal muscle fiber types (Vigelso et al., 2015), and the levels of the cytoskeletal proteins ß-actin or ß-tubulin are altered during neuronal cell differentiation (Castaño and Kypta, 2008). These examples highlight the important caveats associated with using housekeeping proteins as loading controls. As a result, this method has potentially led to major discrepancies in the literature, partially explained by irregularities in execution and in the interpretation of results obtained via biased quantitation. Therefore, a more rigorous method for standardizing immunoblotting approaches is needed. To this end, the Stain-free technology allows for quantitative evaluation of the SDS-PAGE protein separation and the subsequent protein transfer onto membranes before beginning the immunoblotting procedure (Taylor et al., 2013; Ghosh et al., 2014). Compared to traditional polyacrylamide gels, Stain-free gels contain a trihalogenic chemical compound that covalently bonds with tryptophan residues after UV light exposure (Ladner et al., 2004). After bonding, UV light excitation of the covalent bond emits fluorescence that can be captured by a digital camera. Importantly, the trihalogenic-modified proteins can be transferred to membranes and, thus, serve as reference for quantitative immunoassays.

The present study assesses the impact of physiological aging on the expression of proteins involved in excitatory neurotransmission. We used a brain fractionation protocol (Hallett et al., 2008) to isolate distinct cortical subcellular compartments from 7-month- and 22-month old mice. We then methodically quantified various proteins in each compartment with the Stain-free technology. Although the abundance of many proteins, such as the AMPARs, were not affected by age, our study demonstrates that the levels of actin as well as the synaptic vesicle protein, synaptophysin, are significantly lower in the brains of aged mice while the abundance of EEA1 was increased.

EXPERIMENTAL PROCEDURES

Animal use and brain tissue collection

The use and care of animals was approved by the *Comité Institutionnel de Protection des Animaux* de l'Université du Québec à Montréal (protocol #883) in compliance with the Canadian Council of Animal Care guideline. We purchased 3-month old C57BL/6J male mice from the Jackson Laboratory (RRID:IMSR_JAX:000664, in 2016) and obtained 18-month old C57BL/6J male mice from the colony maintained by the *Réseau québécois de recherche sur le vieillissement (RQRV)*. The RQRV colony is maintained by acquiring directly from the Jackson Laboratory new young C57BL/6J male mice every 6 months. These animals are then allowed to age within the RQRV facility. Since the RQRV does not perform breeding, and because the Jackson Laboratory initiated its patented Genetic Stability Program for the C57BL/6J strain in 2003, it is highly unlikely that the young and old mice used in our study are distinct substrains. For the study, a total of eight mice were obtained for each group, and no predetermination of sample size calculation was performed. Four mice were housed per cage (at 24 ± 1 °C, 50–60% relative humidity) under standard 12 h light–dark cycles and fed *ad-libitum* using NIH-31 rodent chow with an unrestricted access to water. To minimize stress to the mice, only standard animal husbandry was performed while mice were housed four months in our animal facility. After this 4-month period, which represents the primary endpoint for our study and in which no animals died, the mice were anaesthetized between 11am and 1 pm with 4–5% isoflurane (Canadian Council of Animal Care primary choice of anaesthetic) in 100% medical-grade oxygen for ~4 min in a Plexiglas container before being subsequently decapitated. Brain tissues were extracted from the skull and the cortices were rapidly dissected out at room temperature before being frozen on dry ice and later stored at −80 °C until further use. At term, $n = 3$ mice of each group (7- or 22-month-old mice) were used to establish the subcellular fractionation efficiency (supplemental data) and $n = 5$ mice in each group for main study (7- or 22-month-old mice). No randomization was performed to allocate subjects in each group. Experiments were performed without blinding. Sacrifice and protein extraction were performed by alternating subject between each group.

Isolation of post-synaptic density from mouse brain

In order to purify the post-synaptic density from mouse brain, all operations were performed at 4 °C to minimize proteolysis. All buffers used contained 1× complete protease inhibitor cocktail tablet (Roche, Cat# 06 538 282 001) and 1× phosphatase inhibitor cocktail (Bimake.com, Cat# B15001). Using a Teflon pestle in a glass tube (Thermo Scientific, Cat# 10630743, 2016), mouse brain cortices (0.16–0.20 g) were homogenized in ice-cold TEVP buffer (10 mM Tris-HCl, 1 mM EDTA, 320 mM sucrose, pH 7.5) using a ratio of 10 ml TEVP buffer per gram of tissue. The homogenate was centrifuged (10 min, 800×*g*, 4 °C) to generate a pellet that was discarded, and a supernatant (PNS) was further centrifuged (15 min; 9200×*g*; 4 °C). This operation generated a pellet (crude synaptosomes, P2) and a supernatant that was discarded. The P2 pellet fraction was suspended in 100 µl of hypotonic TEVP buffer (10 mM Tris-HCl, 1 mM EDTA, 35.6 mM sucrose, pH 7.5) and incubated on ice for 15 min. This resuspended pellet sample was then centrifuged at 30,000×*g* for 20 min at 4 °C to yield a supernatant that contains synaptic vesicles and other cytoplasmic organelles (LS1) and a pellet that contains

synaptosomal membranes (LP1). The LP1 fraction pellet was suspended in 400 μl hypotonic TEVP buffer supplemented with 1% Triton X-100. The triton-treated fraction sample was incubated with head-over-heels rotation for 15 min at 4 °C. This sample was then centrifuged at 150,000×*g* for 20 min at 4 °C, generating a supernatant that contains the triton-soluble synaptosomal membranes (SSM) and a pellet defined as the postsynaptic density (PSD) fraction that is re-suspended in 300 μl of hypotonic TEVP buffer. Protein concentration of each sample was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Cat# 23227) before aliquots of each fraction were stored at −80 °C until use.

Sample separation, stain-free gel imaging and transfer

Samples were separated on hand-casted Mini-PROTEAN TGX Stain-free gels (Bio-Rad Cat# 1610181, 1610183 and 1610185). After electrophoresis, the stain-free gel was activated for 1 min and imaged using the ChemiDoc MP system (Bio-Rad, Cat# 1708280) and Bio-Rad Image Lab software version 5.2.1 (RRID: SCR_014210). Once activated, the proteins in the gel were transferred to 0.45 μm Immun-Blot low-fluorescence polyvinylidene difluoride (PVDF) membrane from the Trans-Blot Turbo RTA Midi LF Transfer kit (Bio-Rad, Cat# 1704274) using the Trans-Blot Turbo Transfer system (Bio-Rad, Cat# 704150) for 10 min, 25 V and 2.5 A. Stain-free signal from proteins on membranes after transfer were imaged with ChemiDoc MP System and its Image Lab software for use in subsequent analysis.

Western blotting

Immunostaining of PVDF membranes were performed as follows: after transfer, membranes were incubated for 1 h at room temperature with TBST buffer (0.1% Tween 20 and 150 mM NaCl in 10 mM Tris–HCL, pH 7.4) containing 5% non-fat dry milk. Blocked membrane was then incubated for 1 h at room temperature with primary monoclonal antibody (mAb) or polyclonal antibody (pAb) diluted in TBST buffer. Purchases for the following antibodies were made in 2016–2018. Anti-GluR1 mAb (Cell Signaling Technology, Cat# 13185, RRID: AB_2732897, 1:1000), anti-GluR2 mAb (Cell Signaling Technology, Cat# 5306, RRID:AB_10622024, 1:1000), anti-GluR3 mAb (Cell Signaling Technology, Cat# 4676, RRID:AB_10547136, 1:1000), anti-GluR4 mAb (Cell Signaling Technology, Cat# 8070, RRID:AB_10829469, 1:1000), anti-NMDAR1 mAb (Cell Signaling Technology, Cat# 5704, RRID:AB_1904067, 1:1000), anti-NMDAR2A pAb (Cell Signaling Technology, Cat# 4205, RRID: AB_2112295, 1:1000), anti-synaptophysin mAb (Cell Signaling Technology, Cat# 5461, RRID:AB_10698743, 1:1000), anti-NMDAR2B mAb (UC Davis/NIH NeuroMab Facility, Cat# 75-097, RRID:AB_10673405, 1:1000), anti-EEA1 (Cell Signaling Technology, Cat# 3288, RRID:AB_2096811, 1:1000) anti-PSD-95 (Cell Signaling Technology, Cat# 3450, RRID:AB_2292883, 1:1000), anti-beta-actin mAb (Applied Biological Materials, Cat# G043, RRID:AB_2631287, 1:10000), anti-alpha-tubulin (Sigma-Aldrich, Cat# T6199, RRID:AB_477583, 1:50000). After incubation with primary antibodies, the blotting membrane was washed 3× 5 min with TBST. Horseradish peroxidase-conjugated secondary antibodies, either goat anti-rabbit IgG (Cell Signaling Technology, Cat# 7074, RRID:AB_2099233) or horse anti-mouse IgG (Cell Signaling Technology, Cat# 7076, RRID:AB_330924) were diluted 1:10,000 in TBST and incubated with the membranes for 1 h at room temperature. Following 3x 5 min washes in TBST, membranes were incubated with the Clarity Western Enhanced Chemiluminescence (ECL) Substrate (Bio-Rad, Cat# 1705060) and the emitted signal was captured using Bio-Rad ChemiDoc MP.

Data and statistical analyses

Data analysis was performed as previously published (Taylor et al., 2013). Briefly, the ChemiDoc MP detected ECL-emitted signal intensity from a protein of interest localized in a single sample lane was normalized to the Stain-free fluorescence signal detected from total protein transferred to the PVDF membrane in its corresponding lane. Our analyses used the "Lane and Bands" tool of ImageLab software, and the intensities for each individual sample were automatically normalized to the signal captured from a mixed-samples pool on the blots. These normalized datasets were used to evaluate the statistical significance using One-way ANOVA with post-hoc Dunnett pairwise multiple comparison test (where the normality assumption is reasonable) in GraphPad Prism 7 Software (GraphPad Prism, RRID:SCR_002798). Statistical value of $p < 0.05$ was considered significantly different. When statistically different, individual p values are reported in the result section associated with a particular figure. Data are reported as the mean ± standard error of the mean (SEM) with individual data points shown, and no exclusion criteria were used.

RESULTS

Extent of linear dynamic range for quantification

Significant changes in the postsynaptic receptors and scaffolding proteins related to chemical synapses have been previously reported (Majdi et al., 2007; Majdi et al., 2009; Yu et al., 2011). Considering that the consequences of biochemical changes in aged brain remain poorly understood, we used quantitative immunoblots to identify which proteins associated with excitatory neurotransmission demonstrate an altered expression level due to normal aging. Specifically, in order to examine the abundance of protein in the mouse brain during aging, we first focused on methodically analyzing the amount of proteins, extracted using a brain fractionation protocol (Hallett et al., 2008), that is required to obtain a linear dynamic signal and, thus, generate real quantitative data. By using our quantitative immunoblot approach that relies on using the Stain-free technology for more reliable quantifications rather than using standard loading controls

(Rivero-Gutierrez et al., 2014), we first defined the linear dynamic range of detection and the limits beyond which Stain-free quantitation cannot be performed (Fig. S1). This is of primary importance since defining the boundaries of the approach employed is a prerequisite to obtain reliable, accurate and reproducible quantitative data from Western Blot. Also, because the expression level of each protein of interest usually varies, we cannot assume a linear dynamic range against a sample until this range is determined for a validated antibody. Accordingly, as shown in Fig. S2, the linear detection limit for each antibody used in the current study was carefully evaluated. A protein sample dilution curve was generated and then probed with each antibody used. The linear dynamic range for quantitative detection was determined, and our analyses show that quantities less than 10 μg of total protein result, for the tested conditions and for a specific antibody, in an appropriate linear dynamic range essential for performing a reliable and quantifiable immunoblot.

Subcellular fractionation of mouse cortex

Neurons possess highly specialized and compartmentalized cellular domains, including dendrites, spines and synapses. Various conditions, such as neuronal activity and age, may impact the abundance of proteins in a specific compartment that total protein immunoblot analysis will not identify. Therefore, to determine whether age of mice influences protein composition in an isolated compartment within the brain, we used 7-month- and 22-month- C57BL/6J mice and performed the subcellular cortex fractionation method shown in Fig. 1A. After subcellular fractionation, 5 μg of total protein from each fraction obtained were probed with the indicated antibodies (Fig. 1B). Our results demonstrate that the postsynaptic density (PSD) fraction, which is defined as the detergent insoluble fraction of the synaptosome membrane, contains the scaffolding protein, PSD-95, glutamate receptors, actin and tubulin. In addition, our results show that AMPAR subunits GluA1-4, NMDAR subunits GluN1 and GluN2A/B, and PSD-95 are enriched in the PSD fraction (Fig. 1B). Furthermore, immunoblots against presynaptic vesicle proteins, such as synaptophysin and early endosome antigen 1 (EEA1), show that they were present in the detergent soluble fraction (SSM) and in the fraction containing cytoplasmic organelles and synaptic vesicles (LS1) but were absent from the PSD fraction (Fig. 1B). In addition, we confirmed the amount of protein used was suitable throughout our analyses by performing a two-fold serial dilution of various fractions. As shown in Fig. S3, our results demonstrate that analyzing 5 μg of protein is appropriate for the P2, LP1, LS1 and SSM fractions while 2.5 μg represents a better choice for the PSD fraction. Taken together, our data shows that the procedure isolates known synaptic proteins and that the fractions obtained can be used for measuring the levels of proteins expressed in distinct subcellular compartments. Furthermore, our data demonstrate that this procedure and PSD isolation is not affected in aged samples.

Abundance of cytoskeleton proteins: actin and tubulin

Cytoskeletal components, for example actin and tubulin, are major components of dendritic organization and are critical for maintaining dendritic spine morphology, organizing the PSD and anchoring and stabilizing postsynaptic receptors (Sheng and Hoogenraad, 2007; Newpher and Ehlers, 2009; Hotulainen and Hoogenraad, 2010; Bellot et al., 2014; Kapitein and Hoogenraad, 2015). Also, actin and tubulin are very often used as housekeeping reference proteins in immunoblotting experiments. Thus, to establish whether aging affects the protein levels of actin and tubulin and if the abundance of those housekeeping proteins is altered by our experimental conditions, quantitative immunoblot analyses were performed. The protein abundance of actin in aged mice remains unchanged in crude synaptosomes (Fig. 2A), which is distinct from the significantly reduced abundance of actin in synaptosomal membranes (Fig. 2A, 1.00 ± 0.04 for 7-months and 0.84 ± 0.03 for 22-months, $p = 0.0037$) and at the PSD (Fig. 2A, 1.00 ± 0.05 for 7-months and 0.76 ± 0.05 for 22-months, $p = 0.0022$). In contrast with the reduced actin level in synaptosomal membranes (Fig. 1A), the quantitative immunoblot analyses of tubulin, a microtubule-forming protein that acts as another structural element of neurons, showed a significant increase in aged synaptosomal membranes (Fig. 2B, 1.00 ± 0.02 for 7-months and 1.27 ± 0.077 for 22-months, $p = 0.0031$). Tubulin expression remains unchanged, however, in crude synaptosomes and at PSD compartments (Fig. 2B). Together, these results illustrate that normal aging differentially modifies the expression of dynamic molecular entities, such as actin and tubulin, that form structural elements of neurons.

Abundance of AMPAR subunits

AMPARs are highly regulated at the synapse, their dynamics being behind the molecular mechanism for memory and learning (Henley and Wilkinson, 2013). Studies have reported various effects of aging on AMPAR expression. Indeed, it was reported that age does not modulate the total expression of the GluA1 subunit (Yu et al., 2011; Yang et al., 2015), whereas a significant reduction of GluA2 expression was also reported (Majdi et al., 2007, 2009; Yu et al., 2011). Importantly, these studies did not examine whether aging modified their intracellular distribution. In this context, we used our subcellular approach, combined with quantitative immunoblotting, to determine whether aging affects the expression of AMPARs in specific subcellular neuronal compartments. Specific and quantitative immunoblotting of AMPAR subunits (GluA1-4) reveals that aging does not significantly alter AMPAR subunit expression within crude synaptosomes and at the PSD (Fig. 3). Consequently, our analysis suggests that normal aging does not alter the expression of AMPAR subunits under the conditions tested.

Abundance of NMDAR subunits

NMDARs are known to regulate synaptic function and plasticity. In addition, alteration in the function of

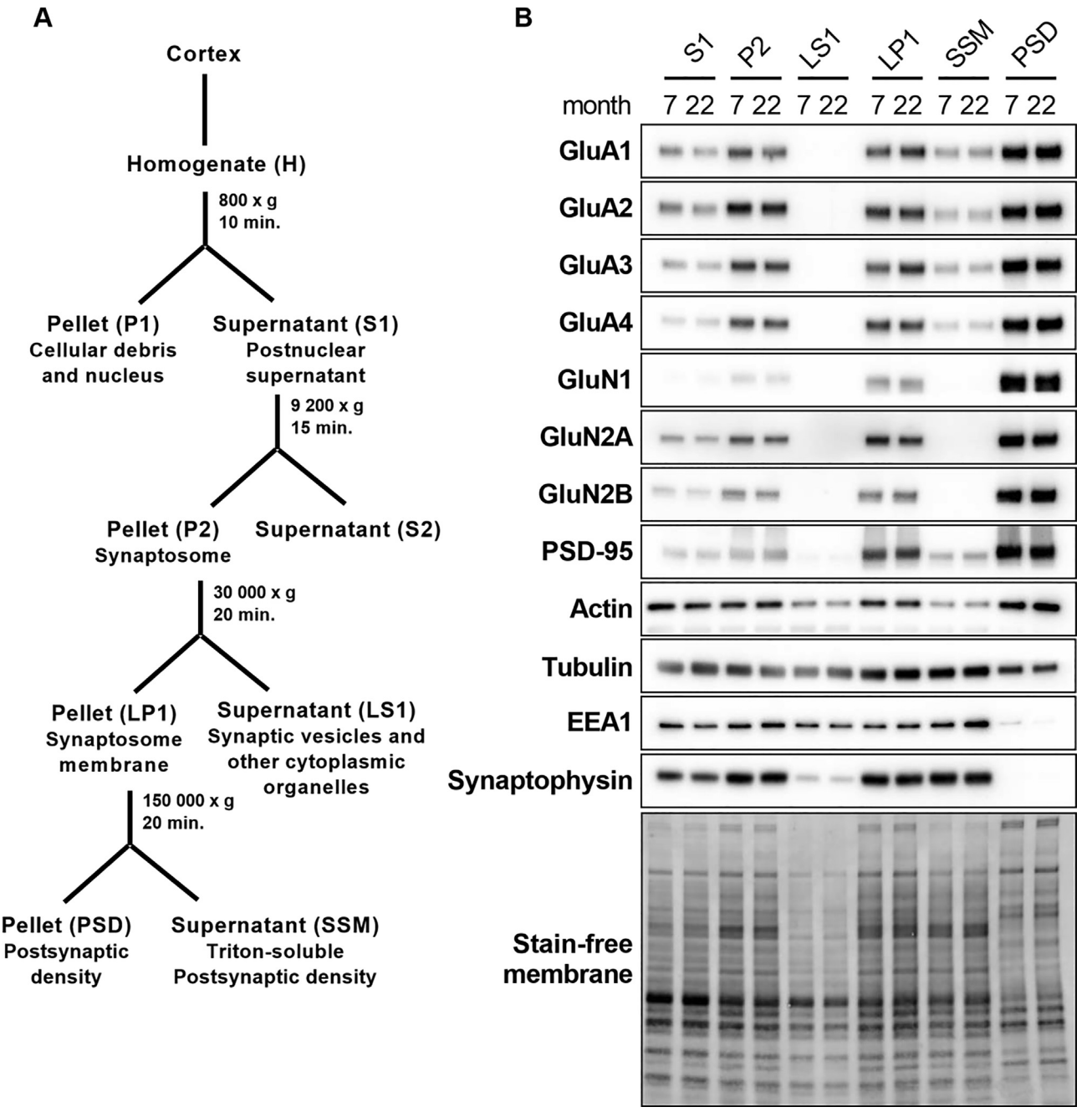

Fig. 1. Immunoblot analyses of mouse brain subcellular compartments confirm the quality of the preparation. **(A)** Schematic representation of the subcellular fractionation procedure. **(B)** Sample (5 μg) from each isolated fraction was separated using Stain-free TGX SDS-PAGE and transferred to low-fluorescence PVDF membrane. Blots were probed with the indicated primary antibodies and then with HRP-conjugated secondary antibodies. Chemiluminescence signal was detected using the ChemiDoc MP imaging system and visualized with Image Lab Software. Three independent experiments were performed, and a typical experiment is shown.

NMDARs has been linked to brain disorders, and there is evidence for changes in NMDAR subunit protein levels across ages in many species (Magnusson et al., 2010). For instance, it was reported that the expression of GluN1 and GluN2B decreases with age, whereas the GluN2A subunit is unaffected (Clayton and Browning, 2001). In sharp contrast, other studies show that GluN2A expression decreases during aging (Kuehl-Kovarik et al., 2000; Magnusson, 2000; Wenk and Barnes, 2000; Clayton and Browning, 2001; Clayton et al., 2002). Therefore, to elucidate whether aging affects the protein level of NMDAR subunits, a quantitative immunoblotting approach against NMDAR subunits (GluN1, GluN2A and GluN2B) was applied to specific subcellular compartments. Our results showed that there was no change in the expression levels of GluN1 and GluN2A in synaptosomes, but there was a significant decrease in GluN2B expression in aged synaptosomes (Fig. 4A, GluN2B:

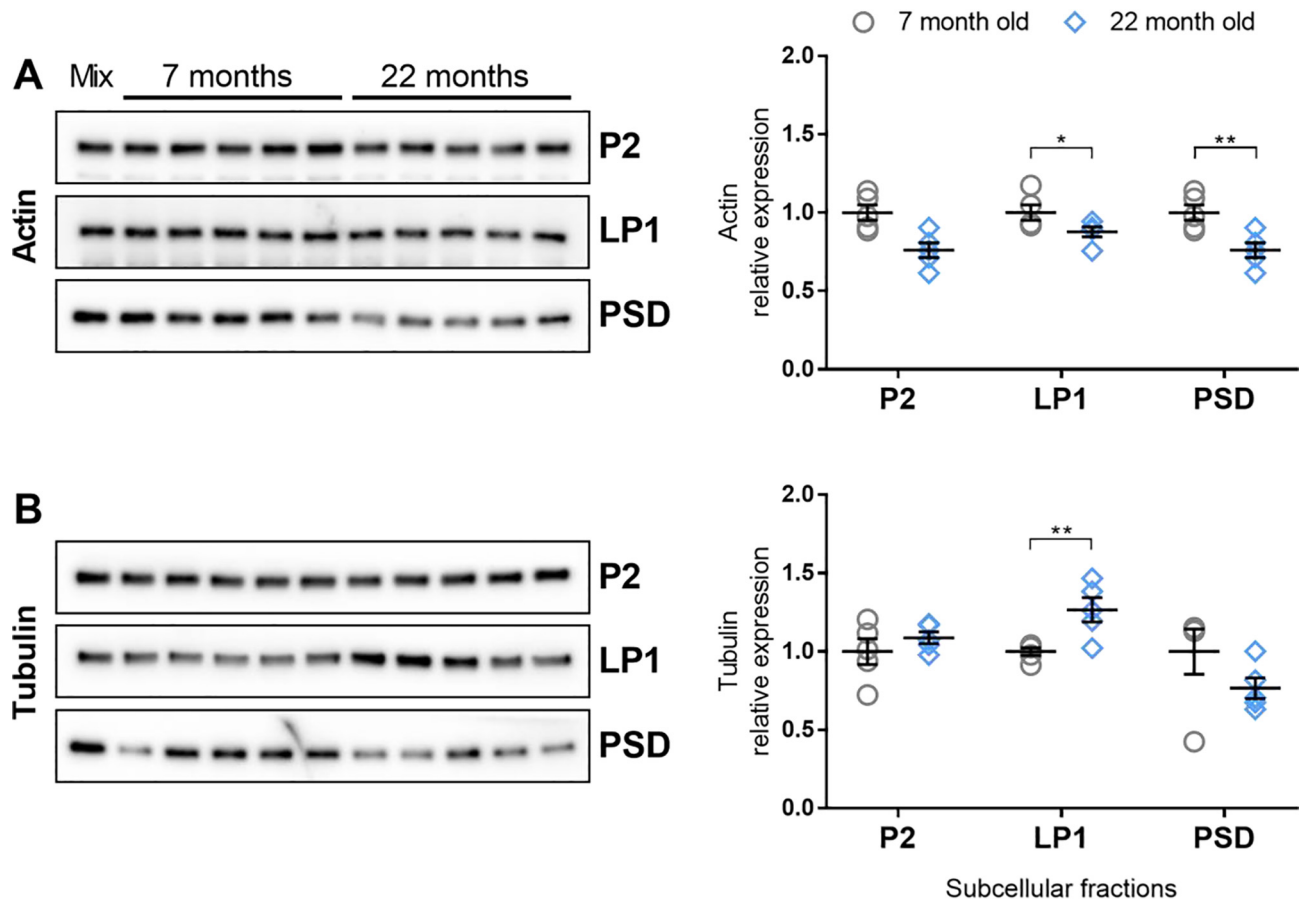

Fig. 2. The levels of actin and tubulin are altered in aged mice. Chemiluminescent Western Blot analysis of actin **(A)** or tubulin **(B)** level in P2 crude synaptosomes, LP1 synaptosomal membrane and PSD fractions from 7- and 22-month-old mouse brain (5 mice in each group). Band densities from **(A)** or **(B)** were analysed and normalized to the band density of the MIX lane using ImageLab software. Data are presented as mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$.

1.00 ± 0.05 for 7-months, 0.72 ± 0.06 for 22 months, $p = 0.0019$). Finally, although there is a trend towards a reduced expression of GluN1 in aged mice, there is no significant aging-related alteration in the expression of NMDAR subunits at the PSD (Fig. 3B). Taken together, our results show that the expression of NMDAR subunits can be reduced in the brain of aged mice, but this finding is restricted to specific neuronal subcellular compartments and specific NMDAR subunits.

Abundance of scaffold protein: PSD-95

Given that normal aging does not modify the localization of AMPAR or NMDAR subunits at the PSD (Figs. 3 and 4), we assessed the effect of aging on the level of PSD-95, a key scaffolding protein that is highly enriched at the PSD and that is involved in localizing, anchoring and clustering glutamate receptors (Fig. 5). Studies have shown that increasing expression of PSD-95 can increase the amplitude and frequency of miniature excitatory postsynaptic currents (Schnell et al., 2002; Stein et al., 2003). Quantitative immunoblotting of brain fractions corresponding to synaptosomes and to the PSD shows no significant change in expression of PSD-95 (Fig. 5A, B). Our results demonstrate that the level of PSD-95 is not significantly altered with aging.

Abundance of organelle proteins: synaptophysin and EEA1

Up to now, our study focused on the abundance of cytoskeleton and scaffolding proteins as well as postsynaptic ionotropic glutamate receptors. However, it is known that neurotransmission also critically relies on the functional integrity of synaptic signaling. Accordingly, synaptophysin is one of the proteins that play a critical role in synaptic plasticity by targeting neurotransmitter vesicles to the active area of the presynaptic membrane and, thus, controlling neurotransmitter release (Kwon and Chapman, 2011). In order to determine if synaptophysin abundance is affected by normal aging of the brain, we performed quantitative immunoblotting on samples obtained from our brain fractionation procedure. The results demonstrate a significantly lower abundance of synaptophysin in all subcellular fractions isolated from aged animals (Fig. 6A, P2 fraction: 1.00 ± 0.10 for 7-months and 0.69 ± 0.09 for 22-months, $p = 0.023$; LP1 fraction: 1.00 ± 0.06 for 7-months and 0.60 ± 0.11 for 22-months, $p = 0.003$; LS1 fraction: 1.00 ± 0.04 for

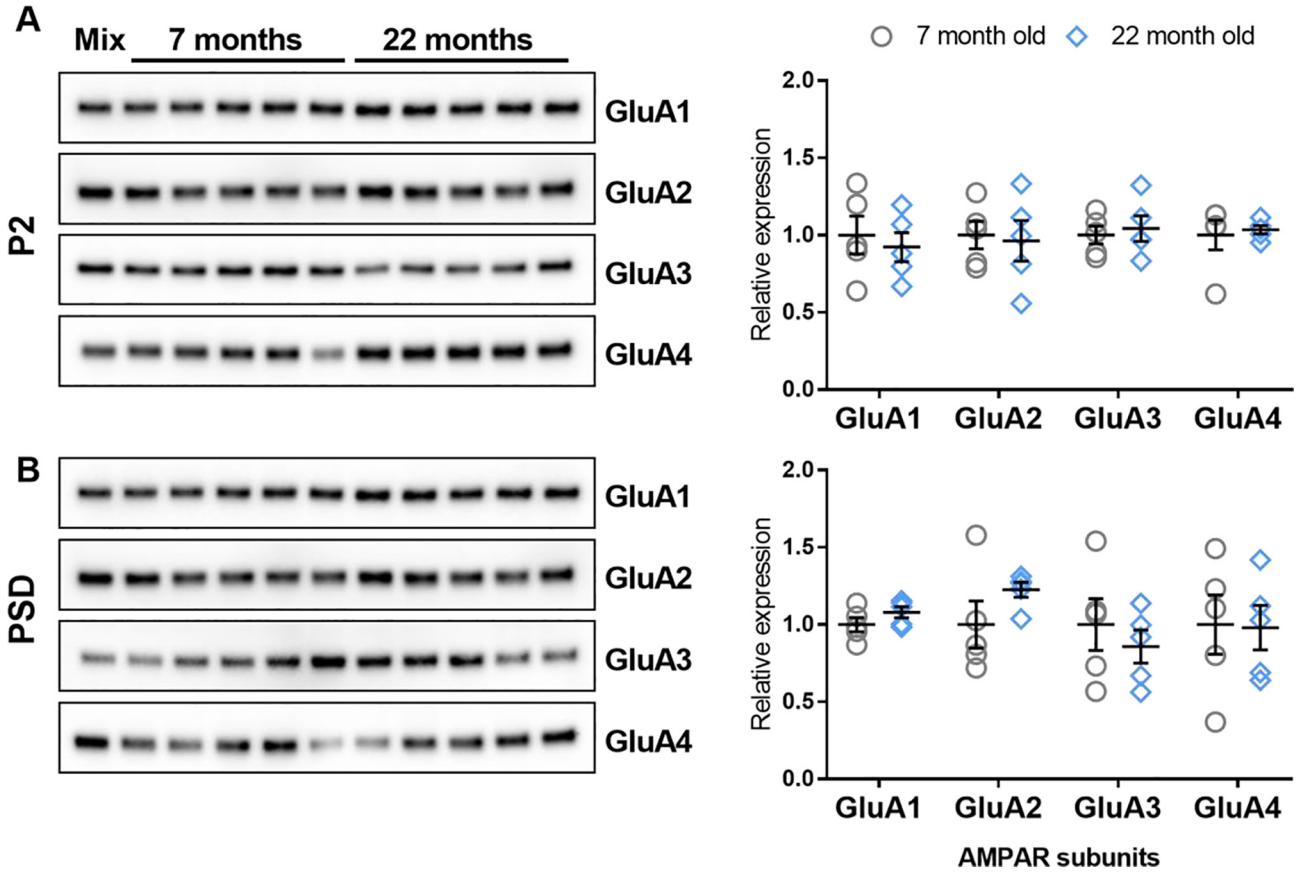

Fig. 3. Aging does not alter the abundance of AMPAR subunits. Selected subcellular fractions from 7- and 22-month-old mice (5 mice in each group) were analysed using immunobloting (left panel) for GluA1-4 subunits. Band densities for each AMPAR subunit expressed in **(A)** P2 fraction and in **(B)** PSD postsynaptic compartment were quantified and normalized to the band density of the MIX lane. Data are presented as mean ± SEM (right panels).

7-months and 0.85 ± 0.03 for 22-months, $p = 0.011$; SSM fraction: 1.00 ± 0.06 for 7-months and 0.57 ± 0.06 for 22-months, $p < 0.0001$). We next examined whether the abundance of other proteins involved in vesicle trafficking might also be affected in the aged brain. As shown in Fig. 6B, the protein level of EEA1 significantly increased in synaptosomal membranes (LP1: 1.00 ± 0.09 for 7-months and 1.79 ± 0.06 for 22-months, $p < 0.0001$) and in the fraction corresponding to cytoplasmic organelles and synaptic vesicles (LS1: 1.00 ± 0.09 for 7-months and 1.35 ± 0.09 for 22-months, $p = 0.01$) isolated from aged animals, suggesting that a cellular mechanism involving EEA1 may be compensating for the reduced abundance of synaptophysin in the brain of 22-month-old mice.

DISCUSSION

The current worldwide demographic change of our population is accompanied by colossal societal impacts on the economy and public health. Indeed, the worldwide proportion of individuals over 60 years old is predicted to reach more than 20% of the population by 2050 (Sander et al., 2015). Aging affects all tissues of the human body and particularly the brain. Importantly, alteration to synaptic connections during brain aging is a physiological process that can lead to memory deficits even in the absence of neurological disorders. Thus, it is surprising that most studies on brain aging focus on the evolution of neurological disorders rather than changes that occur in the absence of pathological conditions, including those that modify synaptic connections and cognitive functions (Harada et al., 2013; Murman, 2015).

The present study expands the current knowledge of the biology/physiology of aging in healthy individuals. Mature adult controls (7 month-old) and aged (22 month-old) mice were used to elucidate the impact of normal brain aging on protein expression at synapses. Using a biochemical approach that combined subcellular fractionation of mouse brain cortices with quantitative immunoblotting, the results show that normal aging is associated with alterations in the expression of many proteins fundamental to glutamatergic neurotransmission. Specifically, the expression of the NMDAR subunit GluN2B, the cytoskeleton proteins actin and tubulin, the synaptic vesicle protein, synaptophysin, and EEA1 are all altered in the cortex of aged mice.

It is well documented that synaptic structures and neuronal proteins are vulnerable to various factors, such as age and neurodevelopmental or neurodegenerative

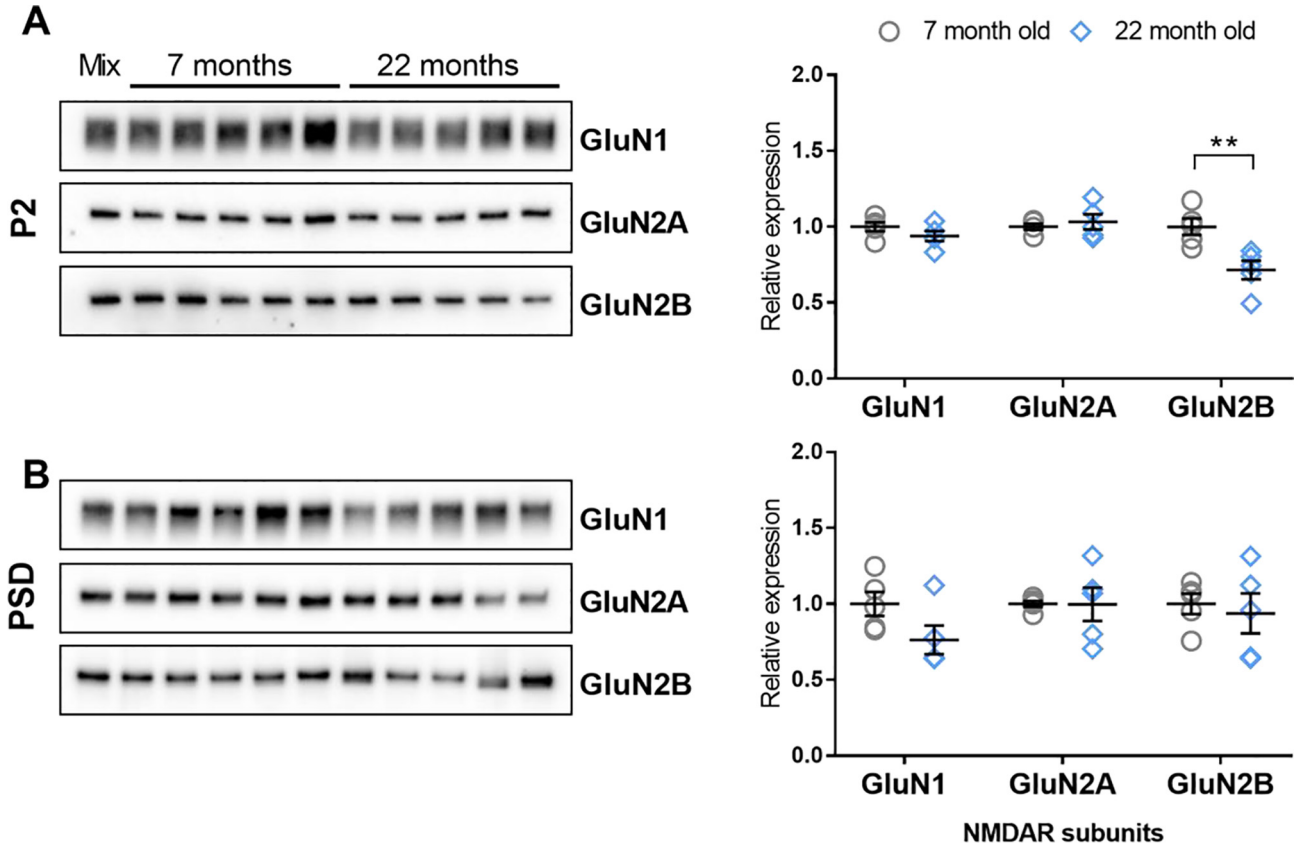

Fig. 4. Protein level of GluN2B is significantly reduced in synaptosomes isolated from aged mice. Abundance of GluN1, GluN2A and GluN2B proteins in P2 **(A)** and PSD fractions **(B)** isolated from cortices of mice either 7- or 22-month-old (5 mice in each group) were analysed using specific immunoblotting (left panel). Band densities were analysed and normalized to the band density of the MIX lane. The relative protein level of NMDAR subunits is presented as mean ± SEM, $*p < 0.05$ (right panels).

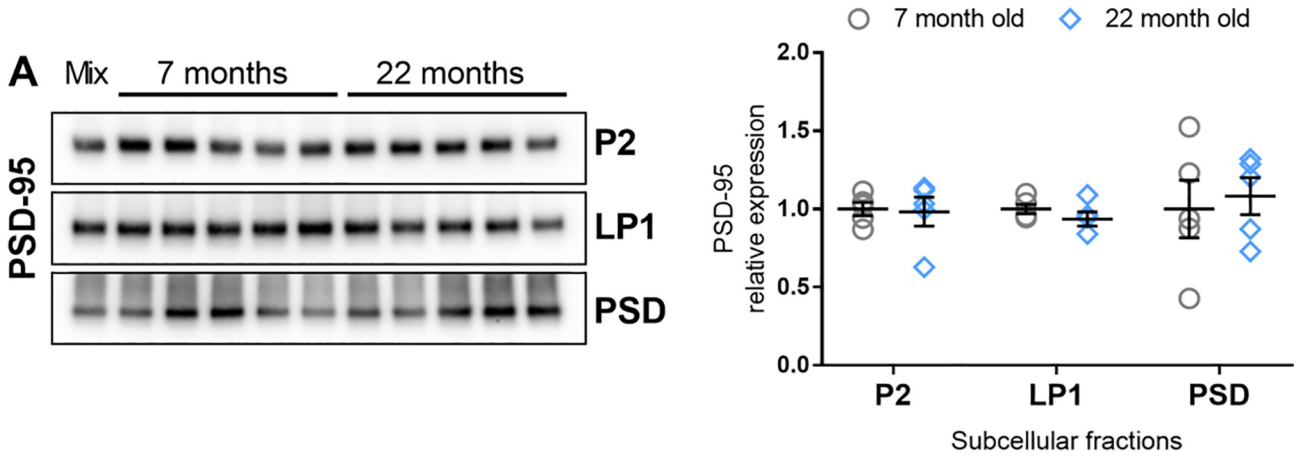

Fig. 5. PSD-95 protein level is not affected in aged mouse brain. **(A)** Abundance of PSD95 and P2 crude synaptosome, LP1 synaptosomal membrane and in PSD postsynaptic fraction from 7- and 22-month-old mice (5 mice in each group) was analysed by immunoblotting. **(B)** Band densities from immunoblots **(A)** were analysed and normalized to the band density of the MIX lane using ImageLab software. Data are expressed as mean ± SEM.

disorders (Fourie et al., 2014; Vyas and Montgomery, 2016). Various studies have shown that mRNA and protein levels of GluN2B are modified from birth to adulthood (Magnusson, 2000; Ontl et al., 2004; Sanz-Clemente et al., 2010). Our data demonstrate that the protein level of GluN2B significantly declines in synaptosomes as a function of age whereas GluN2B abundance at the PSD is similar between 7- and 22-month-old mice. Since NMDARs are composed of one obligatory GluN1 dimer that must assemble with another subunit dimer, the

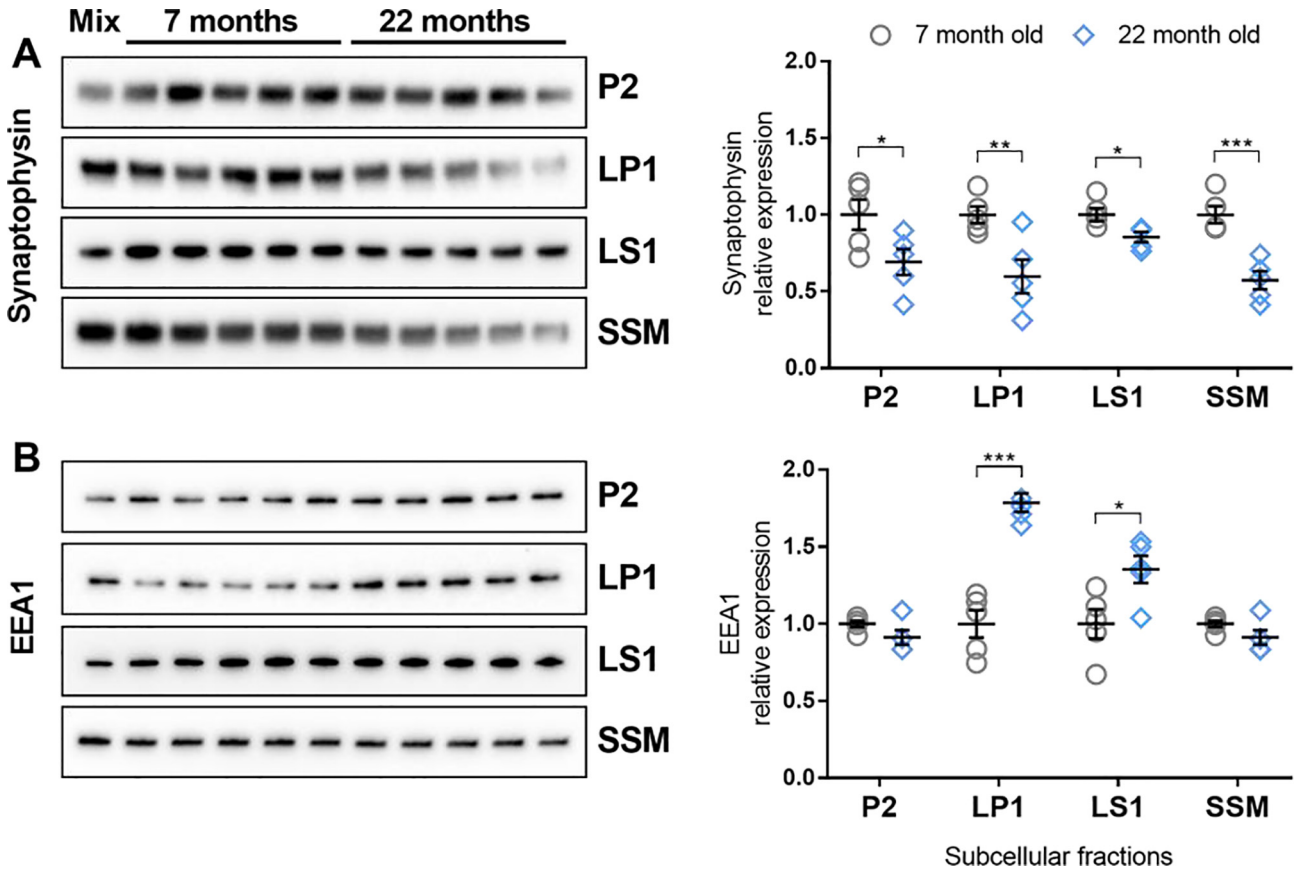

Fig. 6. Synaptophysin and EEA1 protein levels are significantly altered in aged mice. Quantitative immunoblots were completed to determine synaptophysin **(A)** and EEA1 **(B)** protein levels in crude synaptosome (P2), in synaptosomal membranes (LP1), in cytosolic organelles (LS1) and in soluble synaptosomal membrane (SSM) fractions isolated from the cortex of 7- and 22-month-old mice (5 in each group) (left panel). Band densities were analysed and normalized to the band density of the MIX lane using ImageLab software. Data present relative expression as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ (right panel).

decreased level in GluN2B might indicate that another subunit is upregulated to take its place. In contrast, the abundance of GluN2A, as well as the AMPAR subunits and PSD-95 are unaltered by age in the subcellular fractions we analyzed.

Because cognitive impairments in aged rats have been shown to reduce the abundance of GluA2 and PSD-95 (Majdi et al., 2007, 2009; Yu et al., 2011), our results suggest that the cognitive functions of these mice were unaltered. The effect of normal aging on dendritic spines has led over the years to a plethora of conflicting and divergent results (Davidson et al., 2019), and the structure of the PSD as well as its protein content is known to change on many levels across the lifespan. Interestingly, our results demonstrate only a decrease in actin levels in the PSD fraction. Actin plays an essential role in the formation, maintenance and modulation of dendritic spines and, thus, in learning behavior (Borovac et al., 2018). Our results might suggest a decreased ability to undergo long-term potentiation. Furthermore, as recently reviewed by Mack et al. (2016), age-dependent alteration to the abundance and function of the actin cytoskeleton in dendritic spines should not be seen as a surprise but as an element for predicting the severity of synapse deterioration.

Our data demonstrates that the synaptic vesicle glycoprotein, synaptophysin, is significantly reduced in each subcellular compartment isolated from aged mice and not exclusively in the LS1 fraction corresponding to synaptic vesicles and other cytoplasmic organelles. In humans, synaptophysin expression is reportedly reduced during aging (Head et al., 2009). Importantly, our results are in line with a study showing an age-dependent decrease in synaptophysin level, a reduction that was not correlated with cognitive decline in aged animals (VanGuilder et al., 2011, 2010). In addition, abolition of synaptophysin gene function in mice does not phenotypically change the brain anatomic structure or alter synaptic transmission and long-term synaptic plasticity in mice (McMahon et al., 1996). While we did not assess whether cognitive functions were affected in our 22-month-old mice, our own data support an age-dependent change in synaptic structure and function that suggests a compensatory mechanism takes place during normal aging. While neurotransmitter-filled vesicles fuse with the plasma membrane at the active zone, synaptic vesicles must be regenerated via various endocytic mechanisms, including membrane retrieval and vesicle refilling, before being transported back to the exocytosis zone (Hosoi et al., 2009; Hua et al., 2013; Gan and

Watanabe, 2018). In this context, the significantly higher EEA1 protein level observed in the aged crude synaptosomal membrane and LS1 fractions (synaptic vesicles and other cytoplasmic organelles) may be participating in a mechanism by which EEA1 compensates for the reduced abundance of synaptophysin and neurotransmitter release. Although we cannot completely exclude that the EEA1 putative compensatory mechanism may occur in the presynaptic compartment, we would argue that this is unlikely since the abundance of axonal EEA1 was significantly reduced when Piccolo expression was abrogated in cultured hippocampal rat neurons (Ackermann et al., 2019). In this model, the authors showed that disrupted Piccolo expression reduced axonal EEA1 expression but increased the abundance of EEA1 in MAP2-positive dendrites. However, EEA1 protein level in the soma was unaltered (Ackermann et al., 2019). The increased EEA1 measured in our study could indeed emanate from the postsynaptic compartment, which is the previously demonstrated localization for EEA1 (Wilson et al., 2000; Selak et al., 2006; Lomash et al., 2015). The protein encoded by the EEA1 gene is known to interact with Rab5, N-ethymaleimide-sensitive factor (NSF) and Syntaxin 13 in order to regulate vesicle fusion at the early endosome after endocytosis (McBride et al., 1999; Lawe et al., 2002; Selak et al., 2006). In this context and with our results, the proteins EEA1, NSF, Syntaxin 13, and other important molecular entities forming the endosomal machinery should be targeted in futures studies in order to uncover the extent of the putative age-related compensatory mechanism observed from our analyses. In addition, since NMDARs and AMPARs are subjected to controlled trafficking to EEA1-labeled endosomes (Collingridge et al., 2004), the postsynaptic implication of EEA1 should not be excluded from the list of molecules that contribute to a stable pool of postsynaptic proteins in an aged brain.

Modern studies focused on brain circuitry and synapse functions must rely on a variety of assays, such as electrophysiology. Accordingly, patch clamping on rodent brain slices has historically been the gold-standard approach for uncovering cellular and molecular determinants essential to neuronal synapse function. However, whole-cell patch clamp analyses remain commonly conducted with juvenile and adolescent animals or sometimes sporadically with mature adult animal such as 3–6 month-old mice (Ting et al., 2014). Because the patch clamp methodology remains prohibitively challenging in middle-aged to senescent animals (Ting et al., 2014), researchers typically use various imaging and biochemical techniques to better understand the molecular determinants of synaptic dysfunction and memory deficits associated with the aged brain. Among the biochemical approaches available to quantify the expression level of proteins, the present study solely employed immunoblotting (i.e. Western Blot) since it remains an important technique used in modern neuroscience. To ensure the reliability of quantifiable results when performing immunoblotting, many important experimental controls were performed for the present study. For example, it is reported that excessive protein loading on the gel does not improve signals in Western Blotting and in fact lead to saturated signals (Taylor et al., 2013). Consequently, the present study subjected an appropriate amount of total protein to SDS-PAGE electrophoresis for performing quantitative immunoblotting analysis (Supplemental data). In addition, a charge-coupled device (CCD) – based camera method was utilized so that the captured signals from chemiluminescent immunoblots were acquired within the linear range without hitting the limit of detection. This contrasts with the widely used radiographic films that are easily saturated by chemiluminescent signals, which in turn limit the accurate quantification obtained from the blot (Taylor et al., 2013). Also, the trihalogenic compound used in our study allows monitoring of the quality of protein separation on the gel and after transfer efficiency onto the membrane and offers good sensitivity with a wide linear dynamic range that enables performance of total protein normalization (Gurtler et al., 2013). Notably, many studies previously reported that the protein level of actin and tubulin, standard housekeeping proteins used as internal normalization standards in Western Blot, varies across different tissues and experimental conditions (Ferguson et al., 2005; Vegh et al., 2012). Taken together, our results not only demonstrate that the protein levels of actin are affected by age but also highlights the importance of selecting an appropriate analytical approach while demonstrating that the housekeeping protein actin is not suitable as a loading control for immunoblot studies when comparing young and aged brain.

In summary, the present study highlights the importance of an appropriate quantitative immunoblot approach for data confidence when criteria, such as the amount of proteins loaded in each lane on a gel, the antibody used and its dilution, are carefully taken into consideration. Importantly, our work shows that normal brain aging is associated with alterations in the abundance of many proteins implicated in neuronal signaling. By using subcellular fractionation, our study shows that Western Blot can generate critical data to uncover how aging affects protein expression in a specific subcellular compartment. However, it is clear that one pitfall of our study is the inability to distinguish whether the measured differences reflect changes affecting most of the cortical cells or only a subset of cells that have a greater predisposition to age-related alteration within the cortex. Further studies will be required to clarify this issue.

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AUTHOR CONTRIBUTIONS

MPL conceived and supervised the study. HV and MPL designed the experiments. HV performed the experiments. HV and MPL collected, analyzed and interpreted data. HV, VCC, GG and MPL wrote the manuscript. HV, VCC, GG and MPL revised the manuscript.

PERMISSION NOTE

This study is part of Hou Ve's MSc graduate studies in the program of biochemistry at UQAM.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2020.06.044.

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