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Hemoglobin is present as a canonical $\alpha_2\beta_2$ tetramer in dopaminergic neurons ☆☆

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ABSTRACT

Hemoglobin is the oxygen carrier in blood erythrocytes. Oxygen coordination is mediated by $\alpha_2\beta_2$ tetrameric structure via binding of the ligand to the heme iron atom. This structure is essential for hemoglobin function in the blood. In the last few years, expression of hemoglobin has been found in atypical sites, including the brain. Transcripts for α and β chains of hemoglobin as well as hemoglobin immunoreactivity have been shown in mesencephalic A9 dopaminergic neurons, whose selective degeneration leads to Parkinson's disease. To gain further insights into the roles of hemoglobin in the brain, we examined its quaternary structure in dopaminergic neurons *in vitro* and *in vivo*. Our results indicate that (*i*) in mouse dopaminergic cell line stably over-expressing α and β chains, hemoglobin exists as an $\alpha_2\beta_2$ tetramer; (ii) similarly to the over-expressed protein, endogenous hemoglobin forms a tetramer of 64 kDa; (iii) hemoglobin also forms high molecular weight insoluble aggregates; and (iv) endogenous hemoglobin retains its tetrameric structure in mouse mesencephalon *in vivo*. In conclusion, these results suggest that neuronal hemoglobin may be endowed with some of the biochemical activities and biological function associated to its role in erythroid cells. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Hemoglobin (Hb) is the major heme-containing protein of erythrocytes, where it mediates the transport of oxygen and carbon dioxide in the blood. Hb is an assembly of four globular protein subunits each containing a heme moiety. In adult humans the most common type of Hb is a hetero-tetramer consisting of two α and two β subunits held together by non-covalent interactions ($\alpha_2\beta_2$). The expression of α and β chains and their post-translational assembly into $\alpha_2\beta_2$ tetramers is fundamental for Hb to function. As a consequence, strong evolutionary pressure has favored the expression of equal amounts of α and β chains, supporting the evidence that all the functional properties displayed by Hb, as cooperativity, pH sensitivity, and anionic regulation, occur only in the $\alpha_2\beta_2$ tetramers [1].

It has long been thought that Hb expression is restricted to erythrocytes and precursor cells of the erythroid lineage. This notion has been extensively revised in recent years. Hb α and β chains have been found co-expressed in alveolar cells [2], mesangial cells of the kidney [3], retinal ganglion cells [4], hepatocytes [5], and neurons [6–8]. Endothelial and peripheral catecholaminergic cells express exclusively the α chain [9] while macrophages present the β chain only [10]. Importantly, Hb expression has been associated to various types of cancer [11–13].

In the brain, Hb can be detected in mesencephalic A9 dopaminergic neurons, whose selective degeneration is the primary cause of Parkinson's disease [14], in cortical and hippocampal astrocytes as well as in virtually all mature oligodentrocytes [6]. Similar pattern of Hb staining is present in mouse, rat and human central nervous systems [6,7]. Importantly, altered Hb levels have been observed in neurodegenerative diseases *post-mortem* brains. Hb expression is increased in pyramidal neurons in the cortex of multiple sclerosis's patients [15], whereas it is decreased in neurons of Alzheimer's and Parkinson disease brains [16]. Elevated levels of Hb can be found in extracellular amyloid plaques and aged brains, possibly due to compromised brain-blood barrier [17].

The specific function of Hb in neurons remains unclear. We have previously showed that Hb regulates genes involved in oxygen homeostasis and oxidative phosphorylation, linking Hb expression to mitochondrial activity [6]. This is particularly relevant in the context of mesencephalic A9 dopaminergic neurons, since these cells specifically express genes involved in energy metabolism and

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mitochondrial function and present a high metabolic rate and oxidative stress [18,19]. These features are believed to be important for cells' selective vulnerability in Parkinson disease.

In red blood cells Hb functions are achieved through tetrameric structure, but so far no evidence is available for neuronal Hb.

To gain further insights into the biochemical activities and biological functions of Hb in the brain, we have examined the quaternary structure of Hb in dopaminergic neurons *in vitro* and *in vivo*. In this study, we demonstrate for the first time that Hb exhibits a $\alpha_2\beta_2$ hetero-tetrameric state in mouse and human neuronal cell lines as well as in mouse mesencephalon. These results may have important implications in the physiology and pathology of the brain given the many defined roles inherent to the structure of Hb including gas exchange, NO metabolism, and protection against oxidative and nitrosative stress.

2. Materials and methods

2.1. Cell culture

Mouse dopaminergic neuroblastoma iMN9D cells were maintained in culture using Dulbecco's Modified Eagle Medium (DMEM)/ F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 μg/mL penicillin and 100 μg/mL streptomycin. Neuro2a mouse neuroblastoma cells were cultured in Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum (FBS), 1% GlutaMAX, 1% non-essential amino acids (NEAA) and antibiotics.

2.2. Cell lysis, protein extraction and protein assay

Cells were harvested and lysed by osmotic shock in Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5 and 0.5% NP-40) with the addition of protease inhibitor mixture (Roche). Lysates were then cleared by centrifugation (14,000 ×*g*, 30 min, 4 °C) and supernatants were transferred in new tubes. The total amount of solubilized protein was estimated with the Bio-Rad protein assay kit, according to the manufacturer's instructions. Insoluble proteins were also collected upon resuspending the pellets in sample buffer. Soluble and insoluble protein extracts were used for Western blot analysis. For competition assay, the anti-Hb (1:1000, Cappel) antibody was incubated with purified human Hb (HbA) (Sigma-Aldrich) at 1 mg/mL for 2 h at 4 °C. Nitrocellulose membranes were then incubated for 2 h at room temperature with the appropriate antibody–protein solutions, and subjected to Western blot detection as already described.

2.3. Native PAGE Electrophoresis

Native PAGE was performed on gradient polyacrylamide gel electrophoresis (10–12–17% of acrylamide) for approximately 3 h. Standard proteins were loaded using Native Mark Unstained Protein Standard (Sigma-Aldrich). The proteins supplied in the kit have a molecular mass range of 14.2–545 kDa.

For Western Blot analysis, proteins were transferred to nitrocellulose membrane (GE Healthcare). Membrane was blocked with 5% non-fat milk in Tris buffered saline (TBS) solution (TBS + 0.1% Tween-20), then incubated with primary antibodies overnight at 4 °C or at room temperature for 2 h. Proteins were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence reagents (ECL) (GE Healthcare).

2.4. Primary antibodies

The following antibodies were used in this study for Western blot: anti-FLAG (1:1000, Sigma-Aldrich), anti-MYC (1:2000, Cell Signaling), anti-Hb (1:1000, Cappel), anti-β-actin (1:5000, Sigma). For detection, anti-mouse-HRP or anti-rabbit-HRP (Dako) in combination with ECL (GE Healthcare) was used.

2.5. In-cell cross-linking

Cells were extensively washed with ice-cold phosphate-buffered saline (PBS) (pH 8.0) to remove amine-containing culture media. Cells were incubated with 5 mM of the homobifunctional amine-reactive crosslinking reagent disuccinimidyl suberate (DSS) (Pierce Biotechnology) for 30, 60 or 360 min at room temperature. The reaction was quenched by incubation with 50 mM Tris-HCl, pH 7.5, for 15 min at room temperature. Samples were diluted in loading buffer and separated on homogenous 10–12–17% SDS–PAGE gels. The electrophoresis and Western blot were conducted as previously described for native PAGE and analyzed with an anti-Hb antibody.

2.6. Animals

All animal experiments were performed in accordance with European guidelines for animal care and following SISSA Ethical Committee permissions and SISSA veterinary service. Mice were housed and bred in SISSA non-specific pathogen free (SPF) animal facility, with 12 h dark/light cycles and controlled temperature and humidity. Mice had *ad libitum* access to food and water.

2.7. Extraction of native Hb from mouse midbrain

C57BL6 mice ($n = 4$), 12 months-old, were deeply anesthetized with a single intra-peritoneal injection of urethane (ethyl carbamate, Sigma, Cat. No. U2500) at the dose of 2 g/kg. Prior to brain sample collection, mice were extensively perfused with saline buffer through the circulatory system. Briefly, a cannula tip was inserted through the left ventricle and connected to a perfusion apparatus that utilizes a pump with fixed pressure flow to quickly reach every organ using the natural vascular network. The perfusion pump was set to match physiological pressure. After perfusion, mice were sacrificed by cervical dislocation and the ventral midbrain was dissected and immediately frozen in liquid nitrogen. Tissues were homogenized briefly with the aid of a mechanical homogenizer in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5 and 0.5% NP-40) and complete protease inhibitor cocktail (Roche). Extraction volume was 1.5 mL. All steps were performed at 4 °C, in the absence of any detergent or denaturant. Following homogenization, the samples were subjected to centrifugation at 350 ×*g* for 20 min at 4 °C, and the supernatants and pellets were collected. Protein concentration was determined in the total lysate with the Bio-Rad protein assay kit, according to the manufacturer's instructions. The samples were maintained on ice at all times during preparation, avoiding freezing and thawing steps. Total lysate (TL), pellet (P) and supernatants (S) were loaded on SDS–PAGE and native Page as previously described.

3. Results and discussion

3.1. Hemoglobin is an $\alpha_2\beta_2$ tetramer in murine dopaminergic iMN9D cells

In the blood, functional Hb folds as a tetramer composed by two α and two β chains. To study the structure of Hb in neurons, we first determined the assembly of α and β chains in mouse dopaminergic iMN9D cells, a well-accepted *in vitro* model to study dopaminergic cell physiology and dysfunctions [20]. In a previous study we generated iMN9D cells stably expressing tagged version of α and β chains of mouse Hb [6]. Since most antibodies raised against mouse Hb do not discriminate between the two chains, we used anti-FLAG and anti-MYC monoclonals to determine the presence of each chain within Hb quaternary structure. In contrast to SDS–PAGE, native PAGE is performed in non-denaturing conditions and thus permits separation of intact non-covalent protein complexes [21,22]. We used this technique to determine the native molecular mass of Hb extracted from iMN9D cells expressing FLAG-α and MYC-β chains. As control, we included protein extracts prepared from

iMN9D cells stably transfected with an empty vector. Both anti-FLAG and anti-MYC antibodies detected a single band with an estimated mass of ~64 kDa, based on its mobility relative to native molecular weight markers (Fig. 1). As expected, no bands were observed in cells expressing an empty control vector. These data indicate that over-expressed Hb folds as a tetramer that contains two α and β chains. Interestingly, high molecular weight (HMW) complexes have been also detected. The same result was obtained when the lysates were probed with an anti-Hb specific antibody, thus confirming that anti-tag monoclonals detected Hb-containing bands.

Altogether these data demonstrate that Hb, when overexpressed in mouse dopaminergic cell line, is present as $\alpha_2\,\beta_2$ tetramer.

3.2. In-cell cross-linking assay

Since the migration of a protein on native PAGE does not depend solely on its mass but also on its conformation and charge, we stabilized α and β subunits within Hb with the bifunctional cross-linking reagent disuccinimidyl suberate (DSS). Protein chains that are in quaternary structure become covalently linked and their molecular mass can be determined by denaturing SDS–PAGE. We first examined DSS-mediated cross-linking on freshly prepared protein lysates, using a standard protocol [23]. Under these conditions, we noticed that the efficiency of chemical ligation was extremely low and only dimers could be observed (data not shown). The presence of α/β heterodimers upon *in vitro* cross-linking nonetheless confirms previous data showing physical interaction between the two chains by co-immunoprecipitation experiments [6].

To improve the efficacy of DSS ligation, we resorted to in-cell cross-linking. DSS is a membrane permeable molecule, and therefore can be added directly to the cell culture medium. Under these conditions, the proximity-induced cross-linking took place in the protein own cellular milieu. We applied in-cell cross-linking to iMN9D cells stably expressing tagged α and β chains of Hb or to empty vector-expressing cells as control. After protein extraction, lysates were subjected to SDS–PAGE followed by immuno-blot analysis. Anti-FLAG and anti-MYC monoclonals detected multiple unspecific bands and high background in denaturing conditions (data not shown), so anti-Hb antibody was used for detection. As shown in Fig. 2A, a protein band could be observed at about 32 kDa, compatible with the molecular size of covalently linked globin dimers thus confirming data obtained with previous protocols. We also observed SDS-stable Hb bands migrating at the expected positions of a trimer (~48 kDa) and tetramer (~64 kDa). Relative ratio between trimer and tetramer bands was strongly in favor of a trimeric structure, probably as a consequence of facilitated cross-linking for less complex structures as observed with α/β heterodimers. This conclusion is also supported by titration assays, showing an increasing proportion of oligomers (trimer and tetramers) with decreasing levels of monomers along with longer incubation times (Fig. 2B).

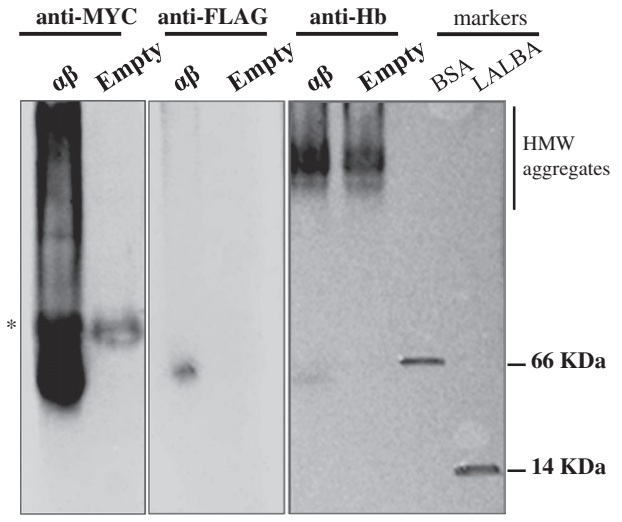

Fig. 1. Hb overexpressed in iMN9D dopaminergic cells is present as $\alpha_2\beta_2$. Native PAGE of dopaminergic iMN9D cells over-expressing FLAG-tagged α and MYC-tagged β chains. Lysates from iMN9D cells expressing empty vector were included as control. Purified proteins were used as markers, as indicated (BSA, bovine serum albumin; LALBA, α-lactalbumin). Relative molecular weights are indicated on the right (kDa). Anti-MYC, anti-FLAG and anti-Hb antibodies were used for immune-staining, as indicated. The asterisk indicates non-specific band. Images are representative of four independent experiments.

It has to be noted that bands of lower intensities but same molecular weight could be observed in empty control cells (Fig. 2A). Since these are revealed with an anti-Hb antibody, these data indicate that endogenous Hb protein is expressed in dopaminergic neuronal iMN9D cells. Importantly, chemical cross-linking indicates that the endogenous Hb can be detected as trimer and tetramer.

Altogether, these experiments further prove that overexpressed Hb has an $\alpha_2\beta_2$ fold and support the existence of native tetramers in dopaminergic neuronal cells.

3.3. Endogenous hemoglobin is a tetramer in neuronal cell lines

To further study the status of endogenous Hb, we used native PAGE to examine the protein expressed in dopaminergic (iMN9D) and in a non-dopaminergic (Neuro2a) neuronal cell lines. We choose Neuro2a since we have previously detected the expression of globin α and β chains mRNAs by RT-PCR (data not shown). Soluble extracts were separated from detergent-insoluble proteins to determine the distribution of Hb and its folding within biochemically-defined sub-cellular fractions. A clear band at ~64 kDa, corresponding to Hb tetramer, was observed in the soluble fractions of iMN9D as well as Neuro2a cells (Fig. 3A). Signal specificity was verified by competition assay, using purified HbA to compete for binding (Fig. 3A). Interestingly, both cell lines presented HMW complexes where multiple species could be detected.

To gain further insights into the existence of HMW aggregates of endogenous Hb in neuronal cells, we prepared detergent-insoluble fractions from iMN9D and Neuro2a cells. As shown in Fig. 3B, when equal amounts of total proteins were loaded, insoluble fractions revealed a much fainter signal at ~64 kDa, as expected for a globular soluble protein. The band corresponding to tetrameric Hb was almost undetectable. Instead, a clear smear in the region of HMW could be measured, thus indicating that Hb exists as part of large insoluble aggregates in neuronal cells *in vitro*.

Insoluble aggregates play an important role in neurodegenerative diseases, albeit their exact contribution to neuronal cell death is always under intense debate. Endogenous Hb has been detected in proteinaceous aggregates in human post-mortem brains from Alzheimer's disease [24], Parkinson's disease and Dementia with Lewy Body disease [16] patients as well as in neurofibrillary tangles in mouse models of familial Alzheimer's disease [17]. It will be interesting in the future to assess whether Hb aggregates are neuroprotective or neurotoxic and what are the molecular mechanisms underlying their formation.

3.4. Endogenous hemoglobin is expressed as a tetramer in vivo in the mouse brain

We then sought to assess the quaternary structure of endogenous Hb expressed in the mouse ventral midbrain, the region that is enriched of dopaminergic neurons and whose selective degeneration leads to Parkinson's disease. The ventral midbrain region was dissected from 12-months old C57BL6 mice. To preserve the native conformation of the proteins and to prevent breakdown of potential higher protein assemblies, the lysis buffer used to homogenize the tissues was free of detergents and denaturing agents. Total lysate (TL) was then separated by centrifugation into detergent-insoluble pellet (P) and supernatant

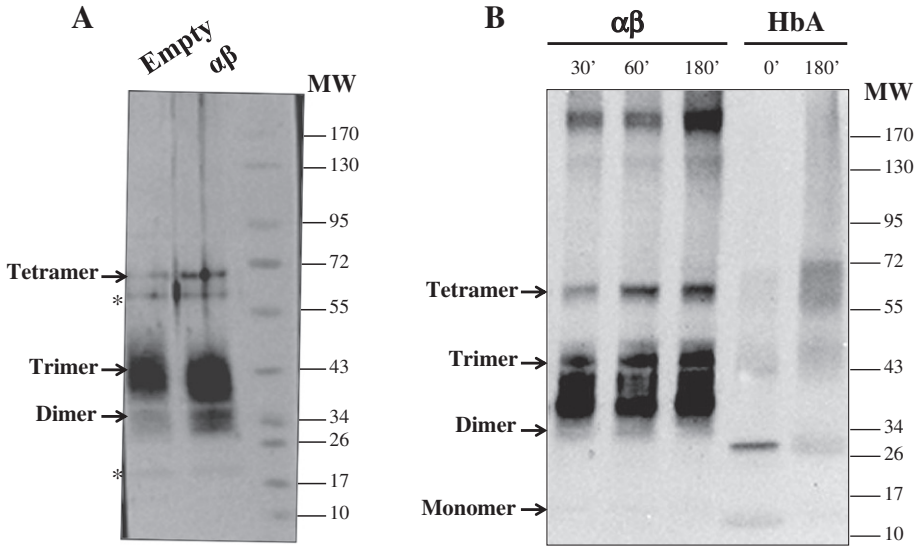

Fig. 2. In-cell cross-linking analysis of Hb structure. A) iMN9D cells overexpressing tagged α and β chains or empty control cells were treated with chemical cross-linker DSS while in culture. Equal amounts of total proteins were loaded on SDS–PAGE and analyzed by Western blotting using anti-Hb antibody. Hb staining is visible in empty cells, indicating the endogenous protein. Asterisks indicate non-specific bands. Images are representative of three independent experiments. B) iMN9D cells stably expressing α and β chains were incubated with chemical cross-linker DSS for the indicated time. After cell lysis, equal amounts of proteins were processed as in A. Purified HbA was included as control. Molecular weight markers are indicated for each gel (kDa). Monomeric, dimeric, trimeric and tetrameric species are indicated by arrows.

detergent-soluble (S) fractions. TL, S and P were loaded on native PAGE along with protein standards. Purified recombinant HbA was also included as control. Immunoblotting with Hb-specific antibody revealed that *in vivo* endogenous Hb from mouse ventral midbrain migrates with an apparent molecular weight of 64 kDa similarly to the purified recombinant protein (Fig. 4). Tetrameric Hb could be observed at similar levels in TL and S fractions. Interestingly, a smaller but detectable amount of Hb was associated to soluble HMW aggregates. Competition experiment verified that bands corresponding to tetrameric and HMW-associated Hb were indeed specific. Surprisingly, fraction P did not contain any Hb neither as tetramer or HMW aggregate (data not shown). All samples contained monomeric and dimeric species under denaturing conditions (data not shown).

To our knowledge this is the first time that native Hb from mouse brain is shown to exist as a tetramer *in vivo*. Recent studies indicate that upon aging, Hb levels are increased in neurons and the protein co-localizes with proteinaceous aggregates [16,24]. We could not find HMW insoluble aggregates of Hb *in vivo*, albeit these forms could be easily detectable in soluble fraction in the brain as well as in the insoluble fractions in neuronal cell lines. It remains to be assessed whether Hb retains its tetrameric structure and/or form intracellular insoluble aggregates when pathological conditions occur.

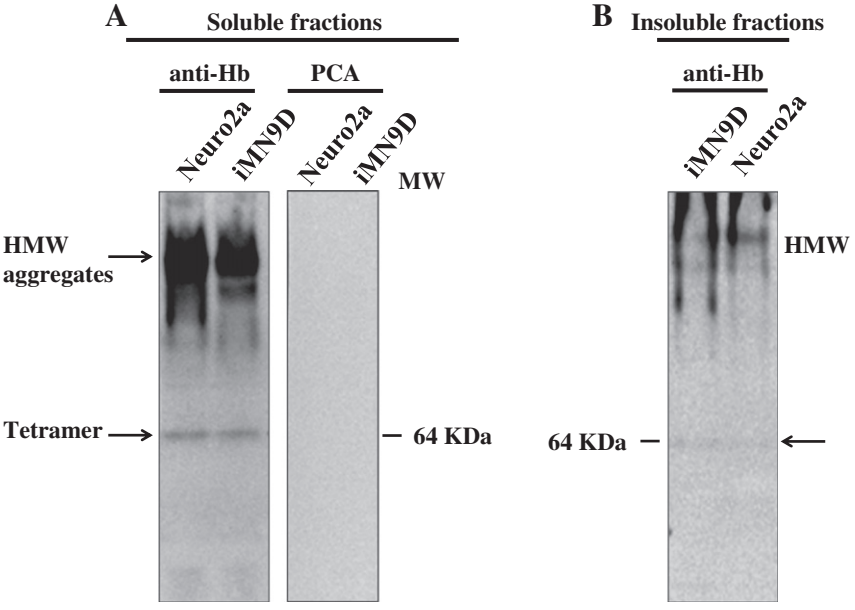

Fig. 3. Biochemical analysis of native endogenous hemoglobin in neuronal cell lines. A) Endogenous Hb is a tetramer in neuroblastoma cells. Dopaminergic (iMN9D) and non-dopaminergic (Neuro2a) neuronal cells were lysed and detergent-soluble proteins were recovered after centrifugation. Total protein content was determined with the Bio-Rad protein assay kit. Equal quantities were loaded on native PAGE and endogenous Hb was visualized by Western blot with anti-Hb antibody. Purified recombinant human Hb was used for protein competition assay (PCA). Relative molecular weight (MW) is indicated on the right (kDa). Tetramers and high molecular weight (HMW) aggregates are shown by arrows. Images are representative of three independent experiments. B) HMW aggregates containing Hb are present in the detergent-insoluble fraction of neuronal cells lines. iMN9D and Neuro2a cells were lysated as in A. After centrifugation, pellet was recovered and analyzed by native PAGE. Endogenous Hb was detected using anti-Hb antibody.

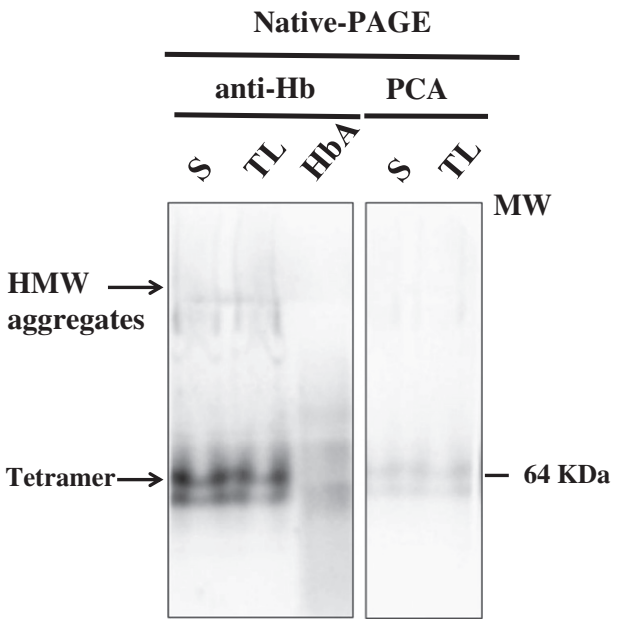

Fig. 4. Endogenous Hb is a tetramer *in vivo* in the mouse brain. Native Hb was extracted from the ventral midbrain of 12-months old C57BL6 mice. Tissue homogenization was performed under non-reducing and non-denaturing conditions. Total lysate (TL), detergent soluble (S) and insoluble (P) fractions were separated. Equal quantities of proteins were loaded on native PAGE. Purified recombinant HbA was included as control. Specificity of bands was assessed by competition assay (PCA). Relative molecular weight (MW) is indicated on the right (kDa). Hb tetramer is indicated by an arrow. The image is representative of two independent tissue preparations from four C57BL6 mice.

4. Conclusions

Hb is the main component of erythrocytes in the blood, where it serves as a molecular cargo of oxygen and carbon dioxide to and from tissues. The assembly of $\alpha_2\beta_2$ tetramer is essential for Hb to function as oxygen carrier in the blood. In recent years, expression of Hb has been observed in cell types other than erythrocytes, including dopaminergic neurons in the mouse [6] and human brain [16]. The exact function and structure of Hb in neurons is presently unknown.

Using native PAGE and in-cell cross-linking, we demonstrate that Hb is present as $\alpha_2\beta_2$ tetramer when overexpressed in dopaminergic iMN9D cell line. The canonical $\alpha_2\beta_2$ structure can also be observed for endogenous Hb expressed by dopaminergic and non-dopaminergic neuronal cell lines. Importantly, native Hb exists as a 64 kDa tetramer in mouse ventral midbrain *in vivo*.

This work suggests that neuronal Hb may retain some of the biochemical activities and biological functions of Hb of erythroid lineage laying down the foundation for a better understanding of its role in brain physiology and in neurodegenerative diseases.

Abbreviations

Hb	hemoglobin
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
kDa	kilo Dalton
HMW	high molecular weight
DSS	disuccinimidyl suberate
DMEM	Dulbecco's modified Eagle's medium
EMEM	Eagle's minimal essential medium

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