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1 **The cellular prion protein interacts with the tissue non-specific alkaline phosphatase in**  
2 **membrane microdomains of bioaminergic neuronal cells**

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1

## 2 **Abstract**

3 **Background** : The cellular prion protein, PrP<sup>C</sup>, is GPI anchored and abundant in lipid rafts.  
4 The absolute requirement of PrP<sup>C</sup> in neurodegeneration associated to prion diseases is well  
5 established. However, the function of this ubiquitous protein is still puzzling. Our previous  
6 work using the 1C11 neuronal model, provided evidence that PrP<sup>C</sup> acts as a cell surface  
7 receptor. Besides a ubiquitous signaling function of PrP<sup>C</sup>, we have described a neuronal  
8 specificity pointing to a role of PrP<sup>C</sup> in neuronal homeostasis. 1C11 cells, upon appropriate  
9 induction, engage into neuronal differentiation programs, giving rise either to serotonergic  
10 (1C11<sup>5-HT</sup>) or noradrenergic (1C11<sup>NE</sup>) derivatives.

11 **Methodology/Principal Findings**: The neuronal specificity of PrP<sup>C</sup> signaling prompted us to  
12 search for PrP<sup>C</sup> partners in 1C11-derived bioaminergic neuronal cells. We show here by  
13 immunoprecipitation an association of PrP<sup>C</sup> with an 80 kDa protein identified by mass  
14 spectrometry as the tissue non-specific alkaline phosphatase (TNAP). This interaction occurs  
15 in lipid rafts and is restricted to 1C11-derived neuronal progenies. Our data indicate that  
16 TNAP is implemented during the differentiation programs of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells and  
17 is active at their cell surface. Noteworthy, TNAP may contribute to the regulation of serotonin  
18 or catecholamine synthesis in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> bioaminergic cells by controlling  
19 pyridoxal phosphate levels. Finally, TNAP activity is shown to modulate the phosphorylation  
20 status of laminin and thereby its interaction with PrP.

21 **Conclusion/significance**: The identification of a novel PrP<sup>C</sup> partner in lipid rafts of neuronal  
22 cells favors the idea of a role of PrP in multiple functions. Because PrP<sup>C</sup> and laminin  
23 functionally interact to support neuronal differentiation and memory consolidation, our  
24 findings introduce TNAP as a functional protagonist in the PrP<sup>C</sup>-laminin interplay. The

- 1 partnership between TNAP and PrP<sup>C</sup> in neuronal cells may provide new clues as to the
- 2 neurospecificity of PrP<sup>C</sup> function.
- 3

## 1 **Introduction**

2           The cellular prion protein PrP<sup>C</sup> is a ubiquitous glycoprotein anchored at the plasma  
3 membrane through a glycosylphosphatidylinositol (GPI) lipid moiety. It is abundantly  
4 expressed in neurons of the central nervous system (CNS), which are the main target of  
5 transmissible spongiform encephalopathies (TSE). The conversion of PrP<sup>C</sup> into an abnormal  
6 conformer, PrP<sup>Sc</sup>, prone to aggregation, is a hallmark of prion diseases. In addition to having a  
7 genetic or sporadic origin like other neurodegenerative disorders, prion diseases have the  
8 unique peculiarity to be transmissible, the PrP<sup>Sc</sup> conformer being the main if not the only  
9 component of the pathogenic agent [1].

10           The absolute requirement of PrP<sup>C</sup> for the development of prion diseases is well  
11 established. However, the precise role of this protein is yet to be fully determined. Its  
12 identification should help to understand how the pathogenic isoforms interfere with the  
13 cellular function of normal PrP<sup>C</sup> [2]. Recent data have shown that PrP<sup>C</sup> plays a role in cell  
14 signaling and cell adhesion and may act as a membrane receptor or co-receptor [3-5],  
15 consistent with its extra-cellular orientation. Interestingly, PrP<sup>C</sup> is expressed at the plasma  
16 membrane in sub-domains enriched in cholesterol and sphingolipid [6] described as rafts and  
17 known to play a role in cellular events such as sorting of membrane constituents and signal  
18 transduction [7]. While the location of PrP<sup>C</sup> in lipid rafts is suspected to be required for its  
19 conversion into PrP<sup>Sc</sup> [8,9], it could also have implications as to PrP<sup>C</sup> function.

20           Attempts to identify physiological ligands or partners that could bring light on PrP<sup>C</sup>  
21 function have relied on different approaches (two hybrid techniques, immunoprecipitation of  
22 cellular PrP<sup>C</sup> complexes, complementary hydrophathy analyses...). Only some of the  
23 interactions have been confirmed and/or shown to have functional relevance at a cellular level  
24 [10]. PrP<sup>C</sup> associates with molecular chaperones such as BiP, grp94, protein disulfide  
25 isomerase or calnexin, required for the proper folding of glycoproteins [11]. Another PrP<sup>C</sup>-

1 interacting molecule is the stress inducible protein I (STI-I) chaperone, described as having a  
2 neuroprotective action [12]. PrP<sup>C</sup> partners also include proteins involved in signal  
3 transduction such as synapsin 1, important for synapse formation and neurotransmitter  
4 release, the adaptor Grb2 molecule [13] and the protein casein kinase 2, CK2 [14]. Also,  
5 adhesion molecules such as laminin and the 37/67 kDa laminin receptor have been shown to  
6 interact with PrP<sup>C</sup> [15-17], with heparan sulphated molecules acting as intermediates [18].  
7 Graner et al. have notably reported on the impact of the PrP<sup>C</sup>-laminin interaction on neurite  
8 outgrowth [16]. Chemical cross-linking analyses have identified the neuronal adhesion  
9 molecule, NCAM, as another PrP<sup>C</sup> interacting protein [19]. This interaction appears to sustain  
10 the recruitment of NCAM into lipid rafts, the activation of the Fyn tyrosine kinase and N-  
11 CAM-mediated neurite outgrowth [20]. The latter observation recalls our demonstration that  
12 antibody mediated PrP<sup>C</sup> cross-linking triggers Fyn activation in 1C11-derived neuronal cells  
13 via the lipid raft protein caveolin [3].

14 In order to search for PrP<sup>C</sup> partners, we took advantage of the 1C11 neuronal  
15 differentiation model [21], which previously allowed us to substantiate a role for PrP<sup>C</sup> in  
16 signal transduction. Upon appropriate induction, the 1C11 neuroepithelial cell line engages  
17 into a neuronal differentiation program. Nearly 100% cells acquire the overall functions of  
18 serotonergic (1C11<sup>5-HT</sup>) or noradrenergic (1C11<sup>NE</sup>) neurons, within 4 or 12 days, respectively.  
19 By unraveling some signal transduction events instructed by PrP<sup>C</sup>, our previous work has  
20 pointed to the implication of the cellular prion protein in cell homeostasis [3,22-24]. In 1C11<sup>5-</sup>  
21 <sup>HT</sup> and 1C11<sup>NE</sup> differentiated cells, the implementation of a PrP<sup>C</sup>-caveolin-Fyn platform on  
22 neuritic extensions controls multiple pathways converging to the MAP kinases, ERK1/2.  
23 Furthermore, in addition to its proper signaling activity, PrP<sup>C</sup> modulates the agonist-induced  
24 response of the three serotonin receptors coupled to G-proteins present on 1C11<sup>5-HT</sup> cells,

1 themselves regulating the overall serotonergic functions [25]. Interestingly, this modulatory  
2 role of PrP<sup>C</sup> is also restricted to fully differentiated cells and is caveolin-dependent.

3         The neuronal specificity of PrP<sup>C</sup> signaling function may rely on some of the numerous  
4 isoforms and/or glycoforms of this protein resulting from proteolytic cleavage and  
5 heterogenous glycosylation [26, 27]. It could also depend on PrP<sup>C</sup> partners induced during the  
6 bioaminergic programs and / or recruited into lipid rafts. The purpose of the present study was  
7 to search for PrP<sup>C</sup> partners in lipid microdomains of differentiated neuronal cells. By an  
8 approach combining immunoprecipitation of PrP<sup>C</sup> from lipid rafts and mass spectrometry  
9 analysis, we identify the tissue non-specific alkaline phosphatase (TNAP) as interacting with  
10 PrP<sup>C</sup> in membrane microdomains of both 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. TNAP is a GPI  
11 membrane-bound alkaline phosphatase (AP) expressed as three distinct isoforms found  
12 respectively in liver, kidney and at a high level in bone where it plays an essential role in  
13 osteogenesis [28]. Recent data identified TNAP in different cell types of the brain [29, 30].  
14 While its role is still elusive, it has been proposed to participate to neurotransmission [29].

15         Here, we show that TNAP is induced along either the serotonergic or noradrenergic  
16 differentiation program of 1C11 cells. This ectoenzyme is active under physiological  
17 conditions and may participate in bioamine synthesis. Besides, we provide evidence that the  
18 PrP<sup>C</sup>-interacting protein laminin is a substrate for TNAP in 1C11-derived neuronal cells, and  
19 that, by modulating the phosphorylation level of laminin, TNAP impacts on the interaction  
20 between PrP and laminin.

21

## 1 **Results**

### 2 **PrP<sup>C</sup> partitions in lipid rafts irrespective of the differentiation state of 1C11 cells**

3 The presence of PrP<sup>C</sup> in lipid rafts of 1C11 precursor, 1C11<sup>5-HT</sup> or 1C11<sup>NE</sup> fully  
4 differentiated cells, was assessed using TritonX-100 insoluble glycosphingolipid (GSL) rich  
5 microdomains isolated by flotation on sucrose gradient and solubilized in 6% SDS. As  
6 revealed by Western blot, PrP<sup>C</sup> majorly segregated into the GSL fraction of 1C11 cells (Fig.  
7 1A) and its neuronal progenies (not shown). A similar result was obtained for caveolin 1, a  
8 marker of caveolae which are subtypes of lipid rafts (Fig. 1B).

9 To evaluate the degree of PrP<sup>C</sup> enrichment in lipid rafts, comparative analyses were  
10 performed by Western blot using proteins of raft preparations (1 µg) and total extracts (15 µg)  
11 from 1C11 precursor, 1C11<sup>5-HT</sup> (day 4) and 1C11<sup>NE</sup> (day 12) cells. Irrespective of the  
12 differentiation state, a 100- to 200-fold increase in the amount of PrP<sup>C</sup> was observed in GSL  
13 fractions (Fig. 2A). A similar enrichment was observed for other proteins specific of lipid  
14 rafts such as flotillin (Fig. 2B), caveolin 1 (Fig. 1B and not shown) and the GPI-anchored 120  
15 kDa isoform of NCAM (Fig. 2C), the latter two described as interacting with PrP<sup>C</sup> [3,19].  
16 While NCAM120 was enriched in lipid rafts, it is noteworthy that the 140 kDa  
17 transmembrane form of NCAM was predominant in total extracts.

18 These data indicate that the enrichment of PrP<sup>C</sup> in lipid rafts is independent from the  
19 differentiation state, precursor vs neuronal, of 1C11 cells.

20

### 21 **PrP<sup>C</sup> interacts in microdomains of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> neuronal cells with an 80 kDa** 22 **protein identified as the tissue non-specific alkaline phosphatase, TNAP.**

23 In order to search for potential PrP<sup>C</sup> partners in such specialized microdomains, plasma  
24 membrane proteins of 1C11, 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells were labelled with biotin before raft  
25 preparation. GSL fractions were dissolved in non ionic detergent (1% TritonX-100) to



1 maintain some protein interactions and heated for 1 hour at 37°C to allow extraction of  
2 proteins from membrane cholesterol. Antibodies recognizing either N-ter (SAF34) or C-ter  
3 (Bar221) epitopes of PrP<sup>C</sup> were covalently linked to sepharose beads and used to  
4 immunoprecipitate PrP<sup>C</sup>. The immunoprecipitated complexes were resolved on a 12% SDS-  
5 PAGE (Fig. 3A). The biotinylated full-length mono or bi-glycosylated PrP<sup>C</sup> species were  
6 immunoprecipitated with both antibodies in 1C11<sup>5-HT</sup> differentiated cells as well as in 1C11  
7 precursor cells. The glycoforms corresponding to the N-terminally truncated fragments of  
8 PrP<sup>C</sup> were recovered with the Bar221 antibody only. A few other biotinylated proteins  
9 appeared to be co-precipitating with PrP<sup>C</sup> both in 1C11 precursor cells and in bioaminergic  
10 neuronal cells. These include proteins with an apparent molecular mass between 45-65kDa  
11 (fig 3A and B) as well as proteins of high molecular weight (around 200kDa). Interestingly,  
12 using either anti-N-ter or anti-C-ter PrP<sup>C</sup> antibodies, an 80 kDa biotinylated protein was co-  
13 precipitated with PrP<sup>C</sup> in lipid rafts of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. The presence of this 80  
14 kDa protein within PrP<sup>C</sup> complexes appears to depend on neuronal differentiation, since we  
15 failed to detect this protein co-precipitating with PrP<sup>C</sup> in lipid rafts of the 1C11  
16 neuroepithelial precursor (Fig. 3 and data not shown).

17 Mass spectrometry analysis was then carried out to define the identity of this 80 kDa  
18 PrP<sup>C</sup> partner. Lipid rafts were prepared from 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells as well as from 1C11  
19 precursor. PrP<sup>C</sup> complexes were immunoprecipitated as above and separated on an 8% SDS-  
20 PAGE allowing a better resolution in the 50-100 kDa range of proteins as exemplified in  
21 Figure 3B. Proteins of 80 kDa apparent molecular mass were trypsin-digested and analyzed  
22 with a LC/MS/MS instrument. The experimental peptide fragments were confronted to the  
23 NCBI non-redundant mouse database. Five peptides (aa<sub>53-71</sub>, aa<sub>204-213</sub>, aa<sub>248-260</sub>, aa<sub>274-282</sub>, aa<sub>370-</sub>  
24 <sub>392</sub>) that matched different regions of the TNAP sequence (Fig. 4) were identified with a high

1 score (60.17) in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. In contrast, TNAP peptides were not detected in  
2 immunoprecipitates from 1C11 precursor cells.

3 We took advantage of an anti-TNAP antibody [31] to further study the TNAP-PrP<sup>C</sup>  
4 interaction. Performing the reverse immunoprecipitation with the anti-TNAP antibody did not  
5 allow a clear detection of associated proteins (data not shown). The anti-TNAP polyclonal  
6 antibody may promote a destabilisation of TNAP-PrP<sup>C</sup> complexes. It is also worth noting that  
7 under conditions where biotinylated TNAP was easily revealed in PrP<sup>C</sup> immune-complexes  
8 by streptavidin, the anti-TNAP antibody failed to yield a signal at 80kDa. This suggests that  
9 sensitive technics (biotinylation, mass spectrometry analysis) are required to reveal TNAP co-  
10 precipitating with PrP<sup>C</sup>.

11 We next evaluated the distribution of TNAP at the cell surface of 1C11<sup>5-HT</sup>  
12 serotonergic cells by immunofluorescence. TNAP antibodies yielded a punctate staining of  
13 the membrane, both on cell bodies and on neurites (Fig. 5, B and E). Such a labeling was  
14 reminiscent of the PrP<sup>C</sup> staining (Fig. 5, A and D). The superimposition of the two stainings  
15 showed a partial co-localization of TNAP and PrP<sup>C</sup> at the surface of 1C11<sup>5-HT</sup> neuronal cells  
16 (Fig. 5 C) that is confirmed by scanning confocal analysis (fig. 5 F).

17 As a whole, these data introduce TNAP as a neurospecific PrP<sup>C</sup> partner, in lipid rafts  
18 of either 1C11 serotonergic or noradrenergic progenies. They also indicate that membrane-  
19 bound TNAP and PrP<sup>C</sup> are located in close vicinity within raft domains.

20

### 21 **TNAP expression is restricted to 1C11<sup>5-HT</sup> serotonergic and 1C11<sup>NE</sup> neuronal cells**

22 The restriction of TNAP interaction with PrP<sup>C</sup> to neuronal 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells  
23 prompted us to investigate whether TNAP was present in microdomains of 1C11 precursor  
24 cells. Direct LC/MS/MS analysis of trypsin-digested 80 kDa raft components did not allow  
25 the identification of any peptides corresponding to mouse TNAP sequence in 1C11 precursor

1 cells, while it firmly confirmed the presence of TNAP in microdomains of serotonergic and  
2 noradrenergic differentiated cells. Indeed, three (aa<sub>53-71</sub>, aa<sub>370-391</sub>, aa<sub>392-407</sub>) and five (aa<sub>53-71</sub>,  
3 aa<sub>247-257</sub>, aa<sub>273-282</sub>, aa<sub>370-391</sub>, aa<sub>392-407</sub>) TNAP derived-peptides were respectively identified in  
4 microdomains of serotonergic (score of 40.2) and noradrenergic (score of 70.3) neuronal cells  
5 (see Fig.4 for residues numbering). In addition, Western blot analysis using a TNAP specific  
6 antibody [31] also revealed a unique band of 80 kDa apparent molecular mass in the rafts of  
7 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells, which was absent in 1C11 precursor cells (Fig. 6A).

8         The absence of the TNAP protein in 1C11 cells correlated with a lack of TNAP gene  
9 expression as assessed by RT-PCR analysis. As shown in Figure 6B, TNAP transcripts were  
10 below detectable levels in the 1C11 precursor and were abundant in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup>  
11 neuronal cells.

12         As a whole, these results show that 1C11 precursor cells lack TNAP and that the  
13 expression of this ectoenzyme is restricted to 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> neuronal cells.

#### 15 **A functional TNAP is induced during the differentiation of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells.**

16         We next investigated whether the TNAP interacting with PrP<sup>C</sup> at the cell surface of  
17 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells was functional. To preserve at best the TNAP ectoenzyme natural  
18 microenvironment, we developed a chemiluminescence assay using the CSPD probe. It  
19 allowed us to measure under physiological conditions, TNAP and other phosphatase activities  
20 present at the cell surface of adherent live cells. As shown in Figure 6C (white bars),  
21 phosphatase activities monitored in 1C11 precursor cells were much lower (3 to 4 fold) than  
22 in fully differentiated 1C11<sup>5-HT</sup> neuronal cells. We sought to specify whether the increase in  
23 phosphatase activity associated to neuronal differentiation could be attributed to TNAP. To  
24 this purpose, the chemiluminescent assay was performed in the presence of orthovanadate (1  
25 mM), a phosphatase inhibitor with broad specificity, or tetramisol (5 mM), a specific inhibitor

1 of the TNAP enzyme. Interestingly, TNAP has the particularity of being inhibited by  
2 tetramisol but not by orthovanadate [32] . While not affected by tetramisol (grey bar),  
3 exposure of 1C11 undifferentiated cells to orthovanadate (black bar) fully switched off the  
4 phosphatase activity indicating that a set of phosphatases, distinct from TNAP, is present at  
5 the neuroectodermal precursor stage. By contrast, in 1C11<sup>5-HT</sup> serotonergic cells, tetramisol  
6 inhibited around 65% of phosphatase activities. The remaining activity corresponded roughly  
7 to the level already present in undifferentiated 1C11 cells (Fig. 6C). Noticeably, in 1C11<sup>5-HT</sup>  
8 cells, around 65% of the phosphatase activity which is resistant to orthovanadate fully relates  
9 to TNAP. Similar phosphatase profiles were obtained with 1C11<sup>NE</sup> cells (see Fig. 7B).

10 The time of onset of a functional TNAP among total phosphatase enzymatic activity  
11 was then monitored during the kinetics of serotonergic and noradrenergic differentiation of  
12 1C11 cells (Fig. 7). This is rendered possible by the synchronicity and the homogeneity of  
13 differentiation of 1C11 cells. In 1C11<sup>5-HT</sup> serotonergic and 1C11<sup>NE</sup> noradrenergic  
14 differentiating cells, phosphatase activity levels kept increasing during the time course of both  
15 neuronal differentiation programs, till completion (day 4 for 1C11<sup>5-HT</sup> and day 12 for 1C11<sup>NE</sup>  
16 cells). Such an increase in cell surface phosphatase activities was majorly attributable to an  
17 induction of TNAP as demonstrated by sensitivity to tetramisol (Fig. 7A and B). This TNAP  
18 activity accounted for 60-70% of total phosphatase activities in differentiated cells. Of note,  
19 the induction of a TNAP enzymatic activity during 1C11 bioaminergic differentiation fully  
20 matches the kinetics of expression of TNAP specific mRNA (Fig. 7C).

21 These results demonstrate that a functional TNAP is induced as early as day 3 of both  
22 the serotonergic and noradrenergic neuronal pathways and reaches a maximal activity upon  
23 implementation of a complete bioaminergic phenotype. The onset of TNAP activity at the  
24 surface of bioaminergic cells, which precedes the implementation of a complete phenotype,

1 may confer to this phosphatase a role in the modulation of neuron- or neurotransmitter-  
2 associated specialized functions.

3

#### 4 **TNAP is involved in the control of serotonin and catecholamines synthesis**

5 The specific role of TNAP in the CNS is still elusive. TNAP is known to function as  
6 an ectoenzyme to convert pyridoxal phosphate (PLP) into pyridoxal (PL), ensuring the  
7 passive uptake of this non-phosphorylated form of vitamin B6 into the cells where PL is  
8 converted back to PLP by intracellular kinases. In neuronal cells, PLP is an essential cofactor  
9 of the decarboxylases required for neurotransmitter synthesis i.e. glutamate decarboxylase  
10 (GAD) for GABA and amino acid decarboxylase (AADC) for bioamines (see diagram fig.10).  
11 To date, an involvement of TNAP has been inferred in GABAergic neurotransmission only  
12 [33]. A potential link between a TNAP-dependent control of vitamin B6 metabolism and  
13 serotonin (5-HT) or catecholamine (CA) levels has not been established. We evaluated the  
14 impact of TNAP inhibition on 5-HT and CA synthesis in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. Cells  
15 having implemented a complete phenotype (day 4 for 1C11<sup>5-HT</sup> and day 12 for 1C11<sup>NE</sup>) were  
16 exposed to tetramisol (2.5 mM) for up to 6 hours and cell extracts were collected at various  
17 time-points to measure the levels of bioamines and their precursors.

18 As shown in Figure 8, tetramisol promoted a significant decrease in 5-HT (2 fold) or  
19 dopamine (DA) (1.8 fold), i.e. the AADC products, concomitant with an increase of their  
20 precursors 5-hydroxytryptophan (5-HTP) and dihydroxyphenylalanine (DOPA), respectively.  
21 This effect was observed as soon as 1 h, peaked after 2 h, remained stable over 6 h (Fig. 8A  
22 and B) and vanished after an overnight treatment (data not shown).

23 These data provide direct evidence that TNAP activity may act on 5-HT and CA  
24 synthesis in 1C11<sup>5-HT</sup> and in 1C11<sup>NE</sup> cells and define TNAP as a player in neurotransmitter  
25 metabolism.

1

2 **TNAP modulates the phosphorylation state of laminin and its binding to PrP<sup>C</sup>, in both**  
3 **1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells.**

4 While TNAP activity on phospho-monoesters is well established, there are only few  
5 reports suggesting that TNAP could act on phospho-proteins. TNAP might in fact exert  
6 opposite action to ecto-kinases on extracellular matrix (ECM) substrates. Based on this  
7 assumption, we probed the impact of TNAP inactivation on the phosphorylation of laminin,  
8 selected as a read out as both a target of ecto-kinases [34] and a PrP<sup>C</sup>-partner [16]. As shown  
9 in figure 9A, laminin was barely phosphorylated in 1C11, 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> control cells.  
10 As anticipated from the lack of TNAP expression in 1C11 precursor cells, the level of laminin  
11 phosphorylation was insensitive to tetramisol. In contrast, exposure of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup>  
12 bioaminergic neuronal cells to 2.5 mM tetramisol promoted a raise in laminin  
13 phosphorylation. A five fold increase in the amount of phospho-laminin was quantified at  
14 24h, that persisted over 48h in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> treated cells vs untreated cells (fig. 9B).

15 Immunoprecipitation experiments were further carried out to evaluate the possible  
16 impact of laminin phosphorylation on its interaction with PrP<sup>C</sup>. In agreement with the work of  
17 Graner [16], PrP<sup>C</sup> was found to associate with laminin in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells (fig. 9C,  
18 left panel). Upon exposure of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells to 2.5 mM tetramisol for 24h, the  
19 interaction between laminin and PrP was nearly lost (fig. 9C, right panel). As a whole, these  
20 results identify laminin as a target of the PrP<sup>C</sup>-interacting partner TNAP in neuronal cells. We  
21 may also conclude that, by modulating the phosphorylation level of laminin, TNAP impacts  
22 on the interaction between PrP<sup>C</sup> and laminin.

23

## 1 **Discussion**

2           In the present work, we identify the tissue non-specific alkaline phosphatase, TNAP,  
3 as a partner of PrP<sup>C</sup> in lipid microdomains of 1C11-derived bioaminergic neuronal cells. This  
4 was established through co-immunoprecipitation and mass spectrometry analyses. Three  
5 major observations relate to this partnership: (i) the PrP<sup>C</sup>-TNAP interaction is restricted to the  
6 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> neuronal progenies, (ii) it occurs in lipid rafts where both protagonists,  
7 which are GPI-anchored, preferentially reside, and, (iii) inhibition of TNAP activity alters the  
8 phosphorylation state of the PrP<sup>C</sup>-binding protein laminin, suggesting that PrP and TNAP  
9 could functionally interact.

10           The 1C11 neuronal differentiation model used in the present study has already allowed  
11 to gain information on PrP<sup>C</sup> function. Besides a ubiquitous intracellular signaling coupled to  
12 PrP<sup>C</sup> involved in red-ox equilibrium and cell homeostasis [22], our previous findings have  
13 uncovered some neuronal specific function of PrP<sup>C</sup>. This first relates to the selective  
14 implementation of a PrP<sup>C</sup>-caveolin-Fyn platform governing several signaling pathways  
15 converging on ERK1/2 in the differentiated 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> neuronal cells [3,22]. A  
16 second neurospecific role of PrP<sup>C</sup> is to modulate serotonin receptor intracellular coupling and  
17 crosstalks [25]. Remarkably, both the proper instruction of signal transduction events by PrP<sup>C</sup>  
18 and its interference with serotonin receptor responses involve caveolin. These observations  
19 illustrate the functional implication of PrP<sup>C</sup> location in a subtype of lipid rafts, the caveolae,  
20 involved in cell signaling and capable of internalizing membrane receptors. However, the  
21 cellular and molecular basis accounting for PrP<sup>C</sup> neurospecific function still has to be  
22 characterized. It could rely on the recruitment of a selective subset of PrP<sup>C</sup> isoforms in lipid  
23 rafts. An alternative explanation would be the involvement of additional molecules whose  
24 expression and/or interaction with PrP<sup>C</sup> is restricted to mature neuronal cells. In this context,

1 the present identification of TNAP as a neurospecific PrP<sup>C</sup> partner posits TNAP as one such a  
2 candidate.

3 Besides, our results support the notion that the onset of a functional TNAP  
4 accompanies the serotonergic and noradrenergic differentiation of 1C11 cells. This is  
5 substantiated by (i) the expression of TNAP mRNAs in the differentiated progenies of the  
6 1C11 cell line and the lack of transcripts in 1C11 precursor cells, (ii) the selective  
7 implementation of a tetramisol-sensitive TNAP activity during the kinetics of differentiation  
8 coinciding with TNAP protein expression and, (iii) the participation of this ectophosphatase  
9 to neurotransmitter metabolism. This latter observation is in line with the well-established  
10 TNAP-mediated regulation of pyridoxal phosphate (PLP), a cofactor of decarboxylases  
11 contributing to the last step of some neurotransmitter synthesis (serotonin, norepinephrine,  
12 GABA...). This TNAP associated phosphomonoesterase activity may confer an important role  
13 to this protein in the nervous system, as discussed below.

14 Noteworthy, our experimental design based on lipid raft isolation shows that PrP<sup>C</sup> and  
15 TNAP interact within these specialized microdomains in which they segregate. The location  
16 of PrP<sup>C</sup> in lipid rafts or its interaction with molecules in such microdomains has been  
17 described using other approaches. For instance, Schmitt-Ulms et al. have investigated into  
18 PrP<sup>C</sup> partners in total brain samples. Their analysis confirms that PrP<sup>C</sup> resides in a membrane  
19 environment containing proteins specific of lipid rafts and, in particular, a subset of molecules  
20 that, like PrP<sup>C</sup>, use a GPI-anchor [35]. PrP<sup>C</sup> interacts with GM3 gangliosides present in high  
21 amount in lymphocyte and neuronal lipid rafts [36,37] and with other glycoproteins or  
22 glycolipids [38-40], which co-localize or are enriched with PrP<sup>C</sup> in rafts of neuronal cells.  
23 Whether these partners participate in PrP<sup>C</sup> function is however unknown. Noticeably,  
24 different intracellular signaling molecules such as kinases and adaptors, recruited through  
25 lipid rafts, have been implicated in PrP<sup>C</sup> functional interactions [3,13,20,37,41,42]. Although



1 the functional relevance of PrP<sup>C</sup> compartmentation within rafts has been poorly addressed, it  
2 has recently been established that PrP<sup>C</sup> does recruit NCAM into lipid rafts where it instructs  
3 Fyn activation and subsequent neurite outgrowth and neuronal polarization [20]. Our present  
4 identification of TNAP as a novel raft-specific PrP<sup>C</sup> interacting molecule adds further weight  
5 to the idea that PrP<sup>C</sup> location in rafts deals with its neuronal function. It is now well  
6 established that lipid rafts constitute dynamic sub-membrane structures allowing the  
7 concentration of specific lipids, glycolipids and glycoproteins serving particular functions  
8 [43]. In view of the increasing set of molecules described as interacting with PrP<sup>C</sup> in  
9 membrane microdomains, it is tempting to speculate that PrP<sup>C</sup> takes part to multi-molecular  
10 complexes whose onset is favored by the specific lipid local composition and which may  
11 sustain signal transduction events. Further investigation will be required to determine whether  
12 TNAP functional interaction with PrP<sup>C</sup> occurs directly or indirectly via the intermediate of  
13 other proteins related to neuronal differentiation programs.

14 An interaction of PrP<sup>C</sup> with TNAP may have different implications in neuronal cells in  
15 relation to the various roles envisioned for this ectoenzyme (see fig.10). TNAP is a  
16 homodimeric metalloenzyme that hydrolyses phospho-monoester specific substrates,  
17 phosphoethanolamine (PEA), inorganic phosphate (PPi), an important player in bone  
18 mineralization, and pyridoxal phosphate (PLP), a cofactor of decarboxylases contributing to  
19 neurotransmitter synthesis. However, little is known about the role of TNAP under  
20 physiological conditions and it is only recently that this ecto-phosphatase has been recognized  
21 to be important in the nervous system [29,44]. A role of TNAP in neurotransmission is well  
22 illustrated by the observation that TNAP knock-out mice develop epilepsy due to GABA  
23 deficiency [33]. These defects recall the occurrence of seizures in patients with mutations in  
24 the *ALPL* gene, suffering from severe hypophosphatasia. Moreover, recent data show that  
25 TNAP activity is regulated by sensory experience [29]. Since serotonin containing fibers are

1 present at high density in sensory regions of the brain, the authors suggest that TNAP could  
2 also regulate serotonin or dopamine synthesis and participate in cortical function and neuronal  
3 plasticity by regulating neurotransmitter synthesis. Our data indeed establish a link between  
4 TNAP activity and bioamine synthesis in 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. Hence the interaction of  
5 PrP<sup>C</sup> with TNAP may confer to the prion protein a role in neurotransmitter homeostasis and  
6 neuronal transmission. In this regard, it is worthy to note that TSE-associated  
7 neurodegeneration is accompanied by alterations in neuronal transmission notably involving  
8 the serotonergic system [45].

9        Besides, TNAP could contribute to ectonucleotidase activity in the brain [30,46,47].  
10 Indeed, TNAP has the capacity to dephosphorylate ATP to adenosine in a stepwise manner  
11 [48]. Nucleotide signaling exerts important neuronal function in the development of the  
12 nervous system and in synaptic transmission in adult brain [44]. Interestingly, a change in  
13 nucleotidase activity has been detected in PrP<sup>C/-</sup> mice which exhibit a slower rate of ADP  
14 hydrolysis possibly leading to a lower level of adenosine [49]. Adenosine has an  
15 anticonvulsant effect and this has to be put together with the recent observation that such PrP<sup>C</sup>  
16 deficient mice are more prone to develop seizures in response to convulsant compounds [50].  
17 The susceptibility to seizures and epilepsy recalls the phenotype of TNAP knockout mice.  
18 Possibly, defects in TNAP activity could account for some of the changes in brain  
19 ectonucleotidase activities reported in hippocampal and cortical synaptosomes of mice  
20 lacking PrP<sup>C</sup> [49]. Further investigation into TNAP activity in a PrP<sup>C</sup> null context should help  
21 clarify this issue.

22        Beyond its phospho monoesterase and ectonucleotidase activity, TNAP may also exert  
23 a phosphatase activity on proteins [51]. This is notably supported by the demonstration by  
24 Becq et al that TNAP inhibition enhances the phosphorylation and concomitant activation of  
25 the Cystic Fibrosis Trans-membrane receptor (CFTR) [52]. Interestingly, this ectoenzyme

1 could also have a role on extracellular matrix proteins, as supported by its collagen-binding  
2 domain [53,54]. In line with this, our data define phospho-laminin as a TNAP substrate in  
3 both 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> neuronal cells. To our knowledge, this is the prime evidence that  
4 TNAP may contribute to regulate the phosphorylation state of an ECM protein in neuronal  
5 cells. In contrast, the partnership between PrP<sup>C</sup> and laminin has raised much attention over the  
6 past few years. The interaction of PrP<sup>C</sup> with laminin has been shown to sustain both neurite  
7 outgrowth [16], neuronal differentiation of PC12 cells [55] and memory consolidation [56].  
8 Whether these processes are modulated according to the phosphorylation state of laminin  
9 remain to be investigated. Our data support the notion that the phosphorylation level of  
10 laminin influences its ability to interact with PrP<sup>C</sup> and define TNAP as a novel protagonist in  
11 the PrP<sup>C</sup>-laminin interplay. They add to the current notion that PrP<sup>C</sup> may be part of large  
12 multi-molecular complexes, depending on the cellular context and environment, and thereby  
13 contribute to diverse cellular functions [57]. Resolving the complexity of PrP<sup>C</sup> partners and  
14 functional interactions in neuronal cells should lead to a better understanding of the  
15 neurospecificity of PrP<sup>C</sup> function.

16

## 1 **Materials and methods**

### 2 **Cell culture and reagents**

3 1C11 cells were grown in DMEM medium (Gibco) supplemented with 10% foetal calf  
4 serum (Seromed) and, differentiation into serotonergic (1C11<sup>5-HT</sup>) or noradrenergic (1C11<sup>NE</sup>)  
5 neuronal cells was induced respectively by addition of dibutyryl cyclic AMP (dbcAMP) or  
6 addition of dbcAMP in presence of 2% DMSO as previously described [21]. Unless stated  
7 otherwise, 1C11<sup>5-HT</sup> cells correspond to day 4 of serotonergic differentiation and 1C11<sup>NE</sup> cells  
8 correspond to day 12 of noradrenergic differentiation. The BW5147 mouse myeloma cell line  
9 was grown in RPMI containing 7.5% foetal calf serum (Gibco). For inhibition of TNAP,  
10 tetramisol was added as indicated in the culture medium. Unless indicated, the reagents were  
11 purchased from Sigma.

12

### 13 **Antibodies**

14 Mouse monoclonal antibodies specific of prion protein were from SPI-BIO. SAF32  
15 and SAF34 antibodies recognize an N-ter epitope (a.a. 79-92) while Bar221 is specific of the  
16 C-ter region of PrP<sup>C</sup> (a.a. 140-160). N-CAM was revealed using an anti-pan N-CAM mouse  
17 monoclonal antibody (BD Bioscience). We also used mouse monoclonal anti-caveolin and  
18 anti-flotillin antibodies (Transduction Laboratory) and a rabbit polyclonal antibody to Lck  
19 (Upstate). Preparation of the anti-TNAP antibody as been previously described [31].  
20 Antibody MAB2549 against Laminin-1 was from R&D systems.

21 The secondary reagents used for immunoblot detection were, either goat anti-mouse or  
22 goat anti-rabbit antibodies coupled to horseradish peroxidase (HRP) accordingly to the  
23 primary antibody, or streptavidin HRP to detect biotinylated proteins in immune complexes  
24 and were all purchased from Southern Biotechnology. The secondary antibodies (Molecular

1 Probe) used in immunofluorescence were a goat anti-mouse and a goat anti-rabbit antibodies  
2 coupled to alexa fluo 488 (green) and alexa fluo 594 (red), respectively.

3

#### 4 **Preparation of lipid rafts (GSL) on sucrose gradient and cell surface biotinylation**

5 Purification of the glycosphingolipid (GSL) rich complexes was performed as  
6 described for lymphoblastoid cells [58]. 1C11 adherent cells were washed twice in PBS then  
7 scraped on ice in a small volume of PBS containing a cocktail of protease inhibitors  
8 (Complete<sup>TM</sup> from Roche) and 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) phosphatase inhibitor.  
9 Around  $10^8$  cells were disrupted and homogenized at 4°C in 3 ml of MBS (Mes buffered  
10 saline): 25 mM Mes pH 6.5; 150 mM NaCl, containing 1% triton X-100 (Tx100),  
11 phosphatase and protease inhibitors. The homogenate (HT) was clarified by 1 min  
12 centrifugation at 1000 rpm and brought to a volume of 4 ml at 40% sucrose in MBS-Tx100.  
13 The homogenate in 40% sucrose was transferred to a Sw41 tube (Beckman), overlaid with 4.5  
14 ml of a 30% sucrose solution in MBS (without triton) then, with 2.7 ml of a third layer  
15 containing MBS without sucrose. The step-gradient was centrifuged for 20 h at 180000 g and  
16 at 4°C in a Sw41 rotor (Beckman). The lipid rafts containing GSL complexes appear as an  
17 opaque band 5 mm beneath the 0% - 30% layers interface. They were harvested and diluted to  
18 a volume of 3 ml in MBS. GSL complexes were then pelleted by centrifugation for 1 h at  
19 300000 g in a TL100.3 rotor (Beckman). Such raft preparations were dissolved in 6% SDS-  
20 RIPA buffer (150 mM NaCl, 25 mM Tris HCl pH 7.4, 5 mM EDTA, 0.5% Na-DOC and  
21 0.5% NP40). The different soluble fractions at 40% (F40) and 30% (F30) sucrose as well as  
22 the triton insoluble high-speed pellet (HSP) were collected from the gradient and their protein  
23 concentration was determined using BCA kit (Pierce).

24 For analysis of PrP<sup>C</sup> partners in lipid rafts, membrane proteins were biotinylated prior  
25 to GSL preparation. Cells in monolayer were washed twice with PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  then

1 incubated with EZ-link<sup>TM</sup>-sulfo NHS-LC-biotin (Pierce) at a concentration of 0.5 mg/ml in  
2 PBS for 30 min at 4°C to limit endocytosis of membrane receptors. Adherent biotinylated  
3 cells were washed and GSL were isolated as above, except they were diluted in NET buffer  
4 (150 mM NaCl, 50 mM Tris HCl pH 7.4, 5 mM EDTA) containing 1% Tx-100 (Calbiochem)  
5 and heated 1 h at 37°C in order to improve solubilisation of proteins embedded into  
6 cholesterol and to allow further immunoprecipitation of PrP<sup>C</sup> complexes.

7

### 8 **Immunoprecipitation and western blot analysis**

9 Specific immunoprecipitations were performed using protein A or protein G sepharose  
10 beads covalently linked to anti-PrP<sup>C</sup> IgG2a (SAF34) or IgG1 (Bar221) respectively. This  
11 procedure avoids recovering of IgG in the complexes which is of importance for MS analysis.  
12 We used the Seize<sup>TM</sup>-X protein A (or G) immunoprecipitating kit (Pierce) to prepare  
13 immunoabsorbant according to the manufacturer's recommendations. Anti-PrP coupled-beads  
14 were then incubated overnight at 4°C with biotinylated rafts in lysis buffer containing Tx-100.  
15 Beads were washed 4 times in high salt buffer (NET, 1% Tx100 in 0.5 M NaCl), then twice in  
16 Hepes 40 mM before elution of the immune-complexes in a reducing sample buffer  
17 containing SDS. For analyses in western blot, 2.5 µg of raft proteins were  
18 immunoprecipitated while for further purification of PrP<sup>C</sup> partners for mass spectrometric  
19 analysis, a high amount of raft was used (equivalent to 20-30 µg). Denatured complexes were  
20 run on SDS-PAGE (Bio-Rad). After transfer of proteins from the gel onto nitrocellulose  
21 membrane (Amersham), the membrane was blocked with 1% gelatin in PBS 0.1% Tween 20  
22 (PBST). Detection of PrP<sup>C</sup> and associated proteins was performed using streptavidin-HRP  
23 (Southern Biotechnology) 1/100 000 and the ECL chemiluminescent procedure (Amersham).

24 The same SDS-PAGE and western blot procedures were used to directly detect  
25 proteins in 15 µg of total extract prepared in NET-Tx100 lysis buffer or in 1 µg of raft

1 proteins prepared in 6% RIPA buffer. After blocking, membranes were reacted with the  
2 specific primary antibodies *i.e.* SAF32 (10 µg/ml), anti-N-CAM (2 µg/ml), anti-caveolin  
3 (0.05 µg/ml), anti-flotillin (1 µg/ml), anti-Lck (0.1 µg/ml), anti-TNAP (1/400). Immunoblots  
4 were revealed by specific secondary antibodies coupled to HRP (1/10000) before ECL  
5 staining.

6 To probe an interaction of PrP<sup>C</sup> with laminin, 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells were  
7 incubated with antibodies against laminin-1 (10µg/ml) in PBS containing 0.5% BSA for 1h at  
8 4°C. Cells were washed twice with PBS Ca<sup>2+</sup>/Mg<sup>2+</sup>, scrapped and collected by centrifugation  
9 (10,000g, 3min, 4°C). Pellets were resuspended in NET lysis buffer containing 1% Tx-100.  
10 Lysates were transferred onto protein-A sepharose beads and the last steps of  
11 immunoprecipitation were carried out as described above. SAF32 antibodies were used to  
12 detect PrP.

13

#### 14 **Mass spectrometry**

15 Peptides were generated for mass spectrometry analysis by in-gel trypsin digestion of  
16 proteins. Since the gel was not stained, we used pre-stained standard molecular weight as  
17 reference to evaluate the 80 kDa position. One mm large gel slices excised from 8% SDS-  
18 PAGE and including proteins of interest with an apparent molecular mass of 80 kDa, were  
19 reduced with DTT and alkylated by iodoacetamide treatment. The enzyme digestion was  
20 carried out overnight at 37°C with modified sequencing grade trypsin (Promega, Madison,  
21 WI). Peptides were then extracted from the gel by treatment with a solvent solution  
22 containing 5% formic acid and 50% acetonitrile. The extracts were dried under vacuum and  
23 re-suspended in a minimum volume (10 µl) of a solution at 0.1% formic acid and 5%  
24 acetonitrile and 4 µl of peptide extract were analysed.

1 Mass spectrometric analyses were performed by LC-ESI-MS/MS where a nanoflow  
2 liquid chromatography (LC-Packings nanoflow LC system, Dionex Inc) is coupled to a nano  
3 electrospray ionisation system (ESI) and a tandem mass spectrometer (MS/MS) analyser  
4 (Deca XP LCQ-Ion trap mass spectrometer instrument, Thermo Electron, Waltham, MA).  
5 The system allows peptide extracts to be desalted and concentrated on a capillary peptide trap  
6 (1 mm x 300 µm ID) prior to injection on a C18-resin (LC-Packings, Netherlands) column  
7 (15 cm x 75 µm ID pepMap column). Peptides were eluted at a constant flow rate of 170  
8 nl/min by applying a discontinuous acetonitril gradient (5% - 95%). The column exit is  
9 directly connected to the nanoelectrospray ion sources and the instrument is operated in data-  
10 dependent acquisition mode to automatically switch from MS to MS/MS analysis. MS/MS  
11 spectra were obtained by fragmenting ion peptides by collision-induced dissociation (CID)  
12 using normalized collision energy of 30% in the ion trap.

13 The data files generated by LC-MS/MS were converted to Sequest generic format files  
14 and were confronted to the *mus musculus* NCBI non-redundant database using Bioworks 3.1  
15 Search Engine (ThermoFinnigan). Search parameters for determination of peptide sequences  
16 included carbamidomethyl as fixed modification and oxidized methionine as variable  
17 modification.

18

### 19 **PCR analysis**

20 cDNA were reverse-transcribed using the superscript<sup>TM</sup>II RT kit (Invitrogen) from 5  
21 µg of mRNA prepared from 1C11 cells and its neuronal derivatives by Mini-Prep column  
22 purification (Quiagen). TNAP PCR specific fragment was obtained by 23 cycles of  
23 amplification at 62°C in a thermocycler PTL-100 (MJ research) using the following specific  
24 primers: sense = 5'-GCAGGATTGACCACGGACACTATG-3'; anti-sense = 5'-  
25 TTCTGCTCATGGACGCCGTGAAGC-3'. As an internal control, GAPDH was amplified by



1 18 cycles at 58°C with the 2 primers: sense = 5'-TGAAGGTCGGTGTGAACGGATTTGGC-  
2 3'; anti-sense = 5'-CATGTAGGCCATGAGGTCCACCAC-3'. Specific GAPDH and TNAP  
3 fragments were run on 1% and 1.5% agarose gel respectively and revealed by BET staining.

4

#### 5 **Enzymatic activity of alkaline phosphatase**

6 Phosphatase activity was determined at the surface of intact cells performing  
7 enzymatic test on cells that were cultured in 96 wells-microplates. Cell layers were washed  
8 twice with PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  then incubated with CSPD chemiluminescent substrate (Roche) at  
9 a concentration of 0.25 mM in 200  $\mu\text{l}$  of a physiologic buffer (135 mM NaCl, 4 mM KCl, 1  
10 mM  $\text{CaCl}_2$ , 20 mM HEPES pH 7.5, 5 mM glucose and 1 mM  $\text{MgCl}_2$ ) as described [59]. In  
11 order to discriminate between different phosphatase activities, the substrate was reacted with  
12 cells with or without 5 mM tetramisol, which inhibits TNAP and with or without 1 mM  
13  $\text{Na}_3\text{VO}_4$  which exhibits a larger spectra of phosphatase inhibition, but is not active on TNAP.  
14 Each condition was tested in 6 replicates. Chemiluminescence amplification resulting from  
15 phosphohydrolysis of the CSPD substrate was monitored in a Perkin Elmer reader plate. The  
16 data are given as relative luminescent units (RLU/ $\mu\text{g prot/h}$ ).

17

#### 18 **Membrane immunofluorescence**

19 1C11 cells were cultured on glass cover slips at the bottom of 24 wells-micro plates  
20 and induced to differentiate into 1C11<sup>5-HT</sup> cells. Membrane immunofluorescence was carried  
21 out on intact cells reacted for 1 h at room temperature with SAF32 anti-PrP (10  $\mu\text{g/ml}$ ) and  
22 anti TNAP (1/100) antibodies diluted in PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , 2% FCS and 0.1% sodium azide to  
23 avoid internalization of membrane receptors. After 3 washes in PBS/azide, secondary  
24 fluorescent antibodies were added for 1 h. After washing, cells were fixed with 3.7%  
25 formaldehyde then mounted in fluoromount (Southern Biotechnology). Examination was

1 carried out on an Axiophot microscope (Zeiss) equipped with UV lamp and appropriate  
2 emission filters for epifluorescence and with a camera (Nikon) and video system (Packard  
3 Bell). In addition, sequential acquisition was performed on a scanning confocal microscope  
4 (Leica confocal SP5) at 405, 488 and 561nm.

5

### 6 **Determination of cellular content of bioamines and bioaminergic precursors**

7 1C11<sup>5-HT</sup> or 1C11<sup>NE</sup> cells grown in DMEM supplemented with 10% 5-HT-depleted  
8 FCS were exposed to 2.5 mM tetramisol for up to 24 hours. This tetramisol concentration  
9 allows to fully abrogate TNAP activity (Fig. 7) and lacks any cell toxicity (data not shown).  
10 Cells were washed twice with PBS, scrapped and collected by centrifugation (10,000 g, 3  
11 min, 4°C). The levels of serotonin (5-HT), dopamine (DA) and their precursors 5-  
12 hydroxytryptophan (5-HTP) and dihydroxyphenylalanine (DOPA), respectively, were  
13 measured by HPLC with a coulometric electrode array (ESA Coultronics, ESA Laboratories,  
14 Chelsford, MA), as in [60]. Quantifications were made by reference to calibration curves  
15 obtained with internal standards.

16

### 17 **Phosphorylation of laminin**

18 The phosphorylation state of endogenous laminin was assessed by measuring [ $\gamma$ -<sup>32</sup>P]-  
19 ATP incorporation (specific activity 18.5 Gbq/mmol, Amersham Pharmacia Biotech). Briefly,  
20 1C11, 1C11<sup>5-HT</sup> or 1C11<sup>NE</sup> cells were grown in roller bottles in serum free conditions. [ $\gamma$ -<sup>32</sup>P]-  
21 ATP (1.2 GBq per 10<sup>6</sup> cells) was added to the culture medium 1 hour prior tetramisol (2.5  
22 mM) addition. Spent medium was collected at various time points following tetramisol  
23 treatment, concentrated by ammonium sulfate at 80% saturation and dialyzed against 20mM  
24 Tris-HCl pH 7.5, 0.5 M NaCl, 0.005 % Brij-35 (TNB buffer). Laminin 1 was purified from  
25 the concentrated conditioned medium through affinity chromatography using a protein A-

1 Sepharose column (Biorad) chemically conjugated with anti-laminin 1 antibody. Following  
2 elution, samples were run on a 7% SDS-PAGE and incorporation of radiolabeled phosphate  
3 was quantified using a PhosphorImager (Molecular Dynamics).

4

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10

11

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- 15  
16  
17



1 **Figure Legend**

2 **Figure 1. PrP<sup>C</sup> partitions in lipid rafts of 1C11 cells.** Proteins (10 µg) from different  
3 fractions of 1C11 cells isolated on a discontinuous sucrose gradient, i.e. total homogenate  
4 (HT), the 30% (F30) and 40% (F40) soluble layers, insoluble pellet (HSP) and the raft (GSL)  
5 fraction, were separated on a 12% SDS-PAGE and analyzed by western blotting. The  
6 presence of PrP<sup>C</sup> (A) and caveolin 1 (B) was assessed using SAF32 and C060 monoclonal  
7 antibodies, respectively. Arrows indicate the different forms of PrP<sup>C</sup> (non-, mono and  
8 biglycosylated) and the α and β chains of caveolin 1.

9

10 **Figure 2. PrP<sup>C</sup> is enriched in lipid rafts irrespective of the differentiated state of 1C11**  
11 **cells.** Proteins of total extracts (15 µg) and lipid rafts (1 µg) from 1C11 cells, their neuronal  
12 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> derivatives, and Bw5147 lymphoid cells (used as control) were resolved  
13 by 12% SDS-PAGE and analyzed by Western blot. Detection of PrP<sup>C</sup> (A) and other raft  
14 markers, i.e. flotillin (B), the NCAM isoforms (C) and Lck kinase (D). A 100- to 200-fold  
15 increase in the amount of PrP<sup>C</sup>, flotillin and the GPI-anchored 120 kDa isoform of NCAM  
16 was observed in GSL fractions in 1C11 cells whatever their differentiation state. As expected,  
17 Lck kinase, was only present in lipid rafts of lymphoid cells. Of note, the B, C and D panels  
18 were obtained by stripping and reprobing the same blotted nitrocellulose membrane.

19

20 **Figure 3. PrP<sup>C</sup> interacts with a 80 kDa protein in microdomains of 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup>**  
21 **neuronal cells.** Lipid rafts from 1C11<sup>5-HT</sup>, 1C11<sup>NE</sup> and 1C11 precursor cells were prepared  
22 after biotinylation of membrane proteins. PrP<sup>C</sup> was immunoprecipitated either with an anti-N-  
23 ter monoclonal antibody (SAF34) or an anti-C-ter antibody (Bar221) covalently linked to  
24 sepharose beads. The biotinylated PrP<sup>C</sup> complexes were separated on a 12% SDS-PAGE to  
25 visualize all the PrP<sup>C</sup> species (A) and on an 8% gel to better separate PrP<sup>C</sup> co-

1 immunoprecipitated protein of higher apparent molecular mass (B). Biotinylated proteins  
2 were revealed with streptavidin peroxidase conjugate. The 80 kDa protein that co-  
3 immunoprecipitates with PrP<sup>C</sup> in 1C11<sup>5-HT</sup> is indicated by an arrow.

4

5 **Figure 4. The 80 kDa PrP<sup>C</sup> partner is identified as TNAP by mass spectrometry.** The  
6 position of the 5 peptides identified by mass spectrometry (LC/MS/MS) with the best scores,  
7 is highlighted along the mouse TNAP protein sequence.

8

9 **Figure 5. Co-localization of PrP<sup>C</sup> and TNAP at the surface of 1C11<sup>5-HT</sup> cells.** Membrane  
10 immunofluorescence was performed on 1C11<sup>5-HT</sup> live cells cultured on glass coverslips. PrP<sup>C</sup>  
11 (A and D) was stained with the monoclonal SAF32 antibody and TNAP (B and E) with  
12 polyclonal anti-TNAP antibodies and revealed with alexa green or red labeled secondary  
13 antibodies, respectively. A superimposition of the two fluorescence labelings is shown in  
14 panels (C) and (F). Panels (A), (B) and (C) were obtained by epifluorescence and (D), (E) and  
15 (F) were obtained by sequential acquisition on a scanning confocal microscope.

16

17 **Figure 6. The expression of a functional TNAP is restricted to differentiated**  
18 **serotonergic and noradrenergic 1C11 derived-cells.** In (A), the presence of TNAP in 1 µg  
19 of lipid rafts prepared from 1C11 induced or not to differentiate was revealed by western blot  
20 analysis using an anti-TNAP specific antibody. In (B), the expression of TNAP mRNAs was  
21 evaluated by PCR analysis. TNAP (upper panel) or GAPDH (lower panel) specific fragments  
22 were obtained after amplification by PCR of cDNA synthesized from mRNA isolated from  
23 the 1C11 precursor and the differentiated 1C11<sup>5-HT</sup> and 1C11<sup>NE</sup> cells. In (C), phosphatase  
24 activity at the surface of 1C11 and 1C11<sup>5-HT</sup> cells was measured by luminescence using the  
25 CSPD substrate and expressed as relative luminescent unit (RLU). White bars correspond to

1 total phosphatase activities, black bars to the activity measured in the presence of 1mM  
2 orthovanadate and grey bars in the presence of 5mM tetramisol.

3

4 **Figure 7. TNAP activity is implemented during serotonergic and noradrenergic**  
5 **differentiation of 1C11 cells.** TNAP activity was evaluated in 1C11<sup>5-HT</sup> (A) and 1C11<sup>NE</sup> (B)  
6 cells during the kinetics of differentiation. The histograms represent the level of phosphatase  
7 activity (RLU/ $\mu$ g prot/h) monitored without (white bars) or with orthovanadate (black bars) or  
8 tetramisol (grey bars ) phosphatase inhibitors. The amount of mRNA transcripts specific of  
9 TNAP are shown in panel (C).

10

11 **Figure 8. Involvement of TNAP in serotonin and catecholamine synthesis.** (A) The  
12 intracellular content of 5-HT and its precursor 5-hydroxytryptophan (5-HTP) were measured  
13 in 1C11<sup>5-HT</sup> cells treated with 2.5 mM tetramisol for up to 6 h. (B) 1C11<sup>NE</sup> cells were exposed  
14 to 2.5 mM tetramisol for up to 6 h and their intracellular content of dopamine (DA) and its  
15 precursor dihydroxyphenylalanine (DOPA) were measured and expressed as pmoles /mg of  
16 total protein. Data represent the means  $\pm$  S.E. of three independent experiments performed in  
17 triplicate.

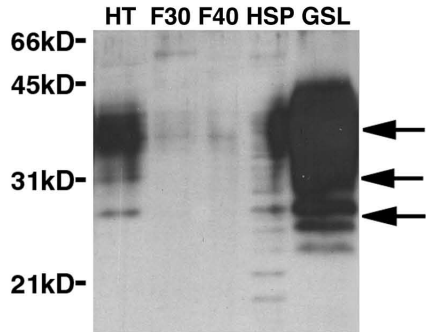
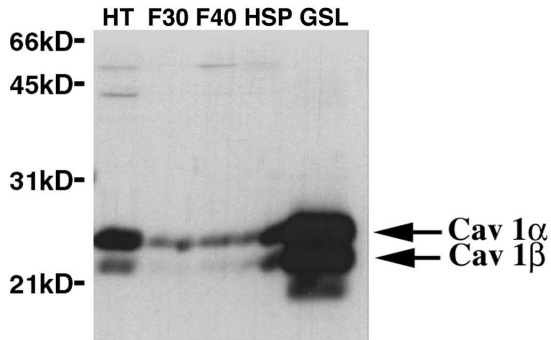
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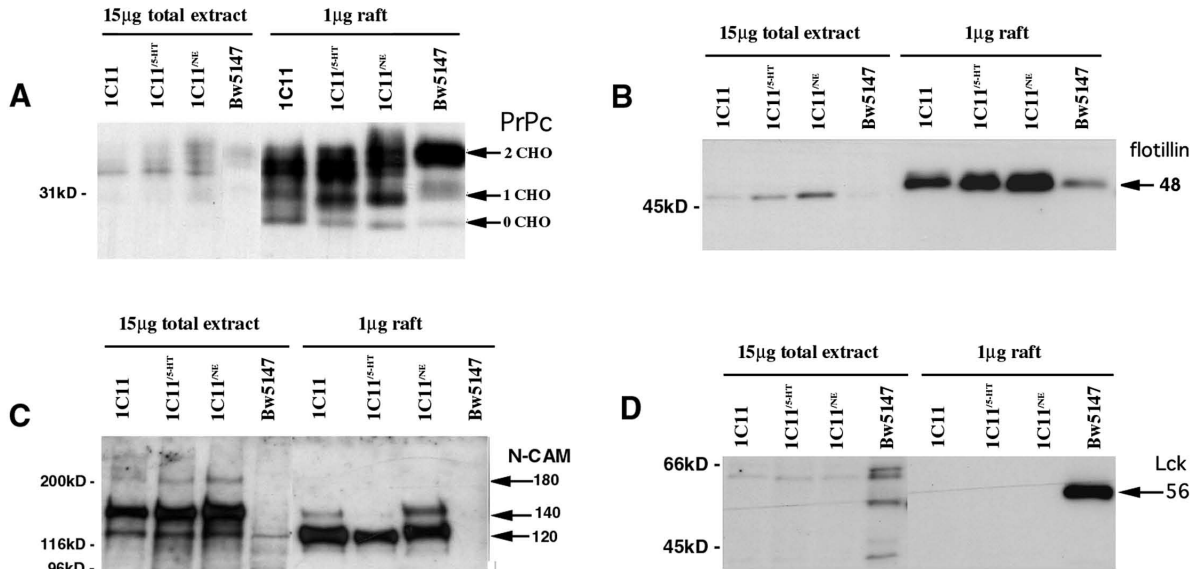
19 **Figure 9. TNAP modulates laminin phosphorylation and binding to PrP<sup>C</sup> in 1C11<sup>5-HT</sup>**  
20 **and 1C11<sup>NE</sup> differentiated cells.** The phosphorylation level of Laminin 1, isolated from spent  
21 medium of undifferentiated 1C11 cells or its neuronal 1C11<sup>5-HT</sup> serotonergic and 1C11<sup>NE</sup>  
22 noradrenergic derivatives pre-incubated with [ $\gamma$ -<sup>32</sup>P]-ATP, was detected by autoradiography  
23 as shown in (A) for untreated cells (control) or at 48h upon TNAP inhibition with tetramisol  
24 (+ tetramisol (2.5 mM)). In (B) the fold increase of phopho-laminin in tetramisol treated vs  
25 untreated cells was quantified at different times with a PhosphorImager and the values

1 correspond to the mean of three independent experiments. (C) Laminin-1 was  
2 immunoprecipitated from 1C11<sup>5-HT</sup> serotonergic and 1C11<sup>NE</sup> noradrenergic cells left untreated  
3 (left) or treated for 24-hr with 2.5 mM tetramisol (right) and immunodetection was carried out  
4 with anti-PrP antibodies (SAF32).

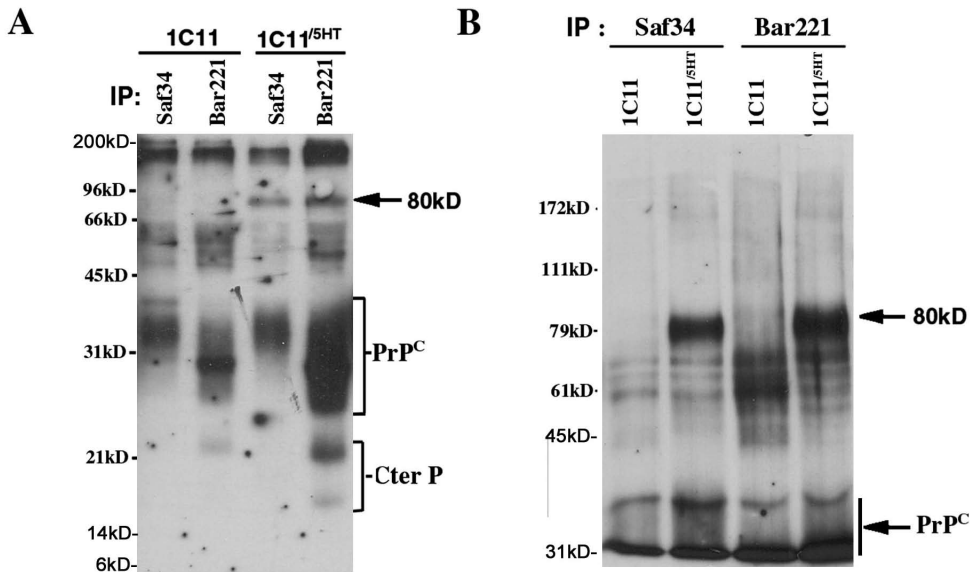
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6 **Figure 10. Diagram depicting possible implications of a PrP<sup>C</sup>-TNAP association in**  
7 **membrane microdomains of neuronal cells.** PrP<sup>C</sup> and TNAP are GPI-anchored membrane  
8 proteins, which majorly reside in rafts. Both have been described to interact with ECM  
9 proteins [16,53,54] and to participate to cell signaling events. PrP<sup>C</sup> can instruct downstream  
10 signaling events, including ERK and CREB activation, by mobilizing a Cav/Fyn complex on  
11 neurites [3,22-24]. In addition, it modulates the coupling of 5-HT receptors, with specific  
12 impact according to G protein-dependent pathway [25]. The TNAP ectophosphatase may  
13 have different substrates. (i) By promoting PLP hydrolysis it contributes to the regulation of  
14 neurotransmitter synthesis [33]. (ii) Its nucleotidase activity may have implications for  
15 purinergic signaling [30,46-48]. (iii) TNAP may be active on phosphoproteins notably of the  
16 cell surface [51,52]. The identification of phospho-laminin as a TNAP substrate uncovers a  
17 novel role of this ectoenzyme in the regulation of ECM molecules. Laminin and the laminin  
18 receptor are important components of the perineural net (PN) and are known partners of PrP<sup>C</sup>.  
19 The interplay between PrP<sup>C</sup>, laminin and TNAP within multiprotein complexes may have  
20 implications for neuronal functions (survival, homeostasis, plasticity).

**A****B****Figure 1**



**Figure 2**



**Figure 3**

1 mispflvlai gtcltnsfvp ekerdpsywr qqagetlkna lklqklntnv aknvimflgd  
61 gmgvstvtaa rilkqqlhhn tgeetrlemd kfpfvalskt yntnaqvps agtataylcg  
121 vkanegtvqv saatertrcn ttqgnevtsi lrwakdagks vgivtttrvn hatpsaayah  
181 sadrdwysdn emppealsqg ckdiayqlmh nikdidving ggrkymypkn rtdveyelde  
241 kargtrldgl dlisiwksfk prkhshyvw nrtellaldp srvdyllglf epgdmqyel  
301 rnnltdpsls emvevalril tknlkgffll veggridhgh hegakqalh eavemdqaig  
361 kagamtsqkd tltvvtadhs hvftfggytp rgnsifglap mvsdtdkkpf tailygngpg  
421 ykvvdgeren vsmvdyahnn yqaqsavplr hethggedva vfakgpmahl lhgvheqnyi  
481 phvmayasci ganldhcawa gsgsapsga lllplavls1 ptlf

**Figure 4**



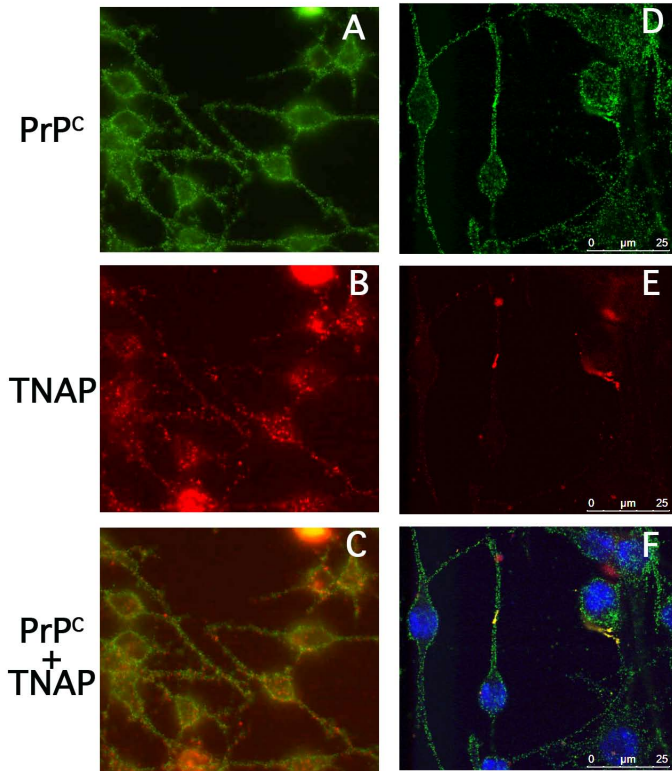
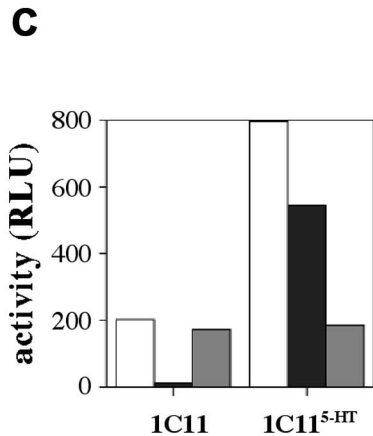
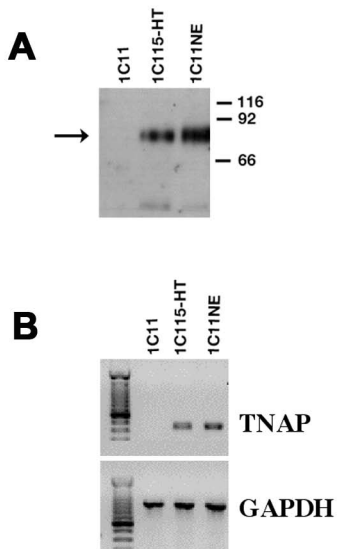


Figure 5



**Figure 6**

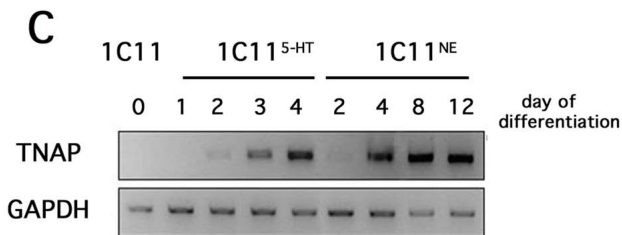
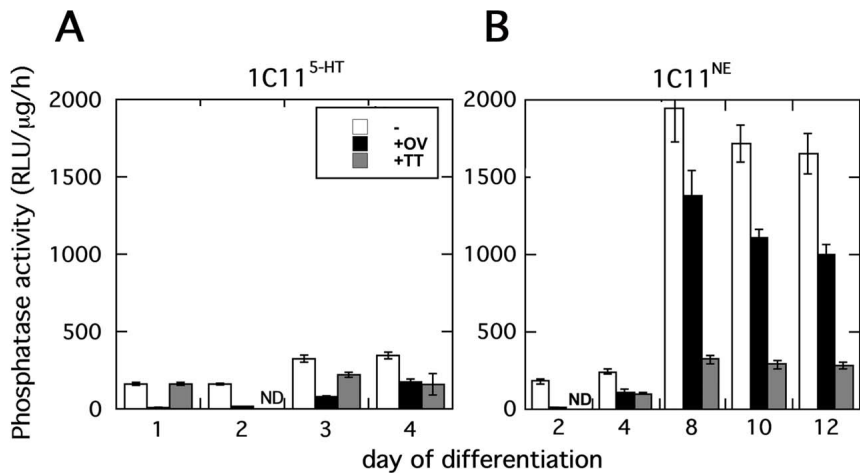


Figure 7

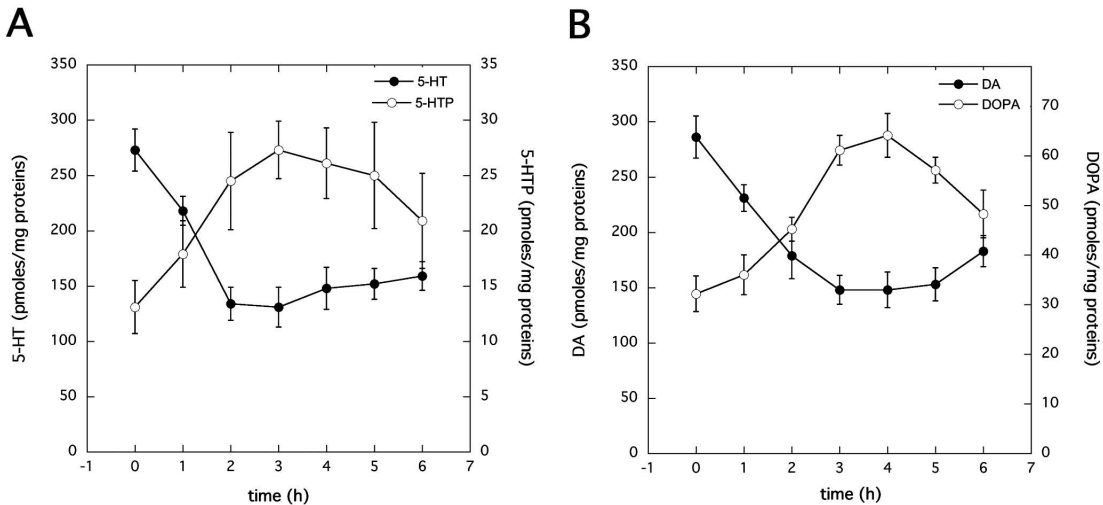


Figure 8

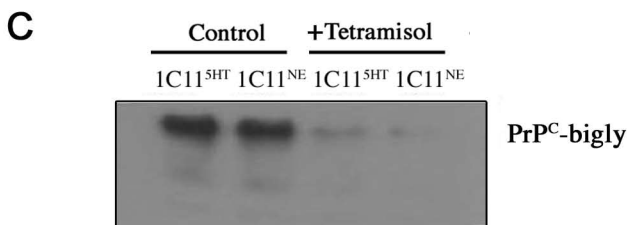
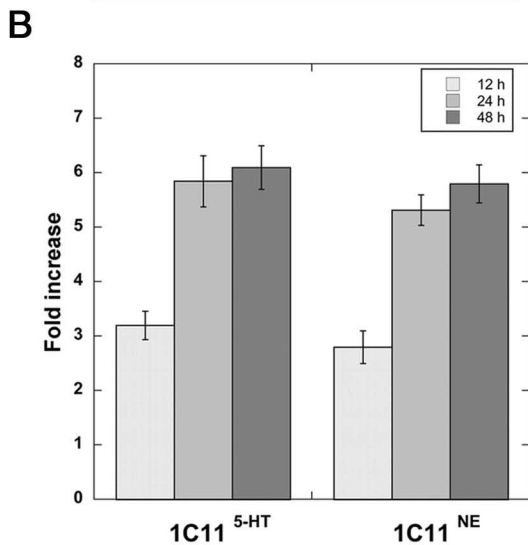
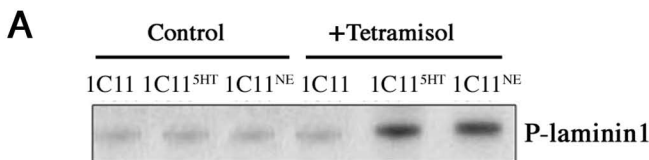


Figure 9

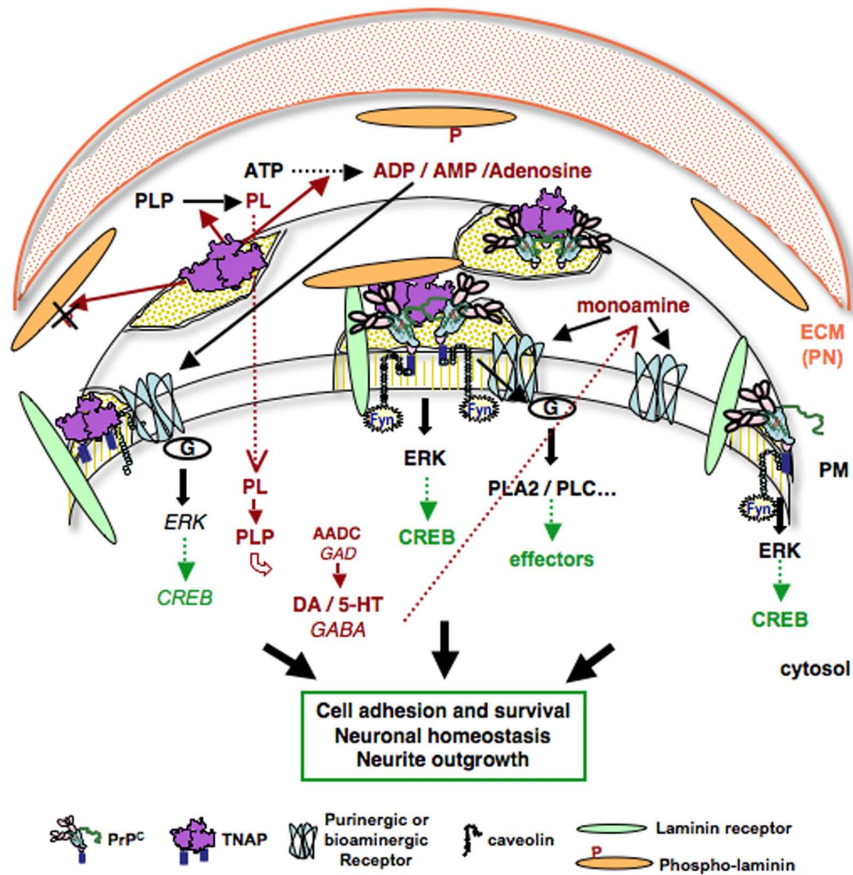


Figure 10