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Down-regulation effects of IFN-α on p11, 5-htr1b and 5-HTR4 protein levels were affected by NH_4CL or MG132 treatment in SH-sy5y cells

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In previous studies, we found interferon-α (IFN-α) could reduce protein levels of p11, 5-hydroxytryptamine receptor 1b (5-HT1b) and 5-hydroxytryptamine receptor 4 (5-HT4), but does not influence their messenger RNA levels in SH-sy5y cells. Thus, we investigated the post-transcriptional modulation of these molecules by IFN-α. SH-sy5y cells were treated with IFN-α, NH_4Cl or MG132 alone or in combination, and then the protein levels of p11, 5-HT1b and 5-HT4 were analyzed by western blots. The regulatory effects of p11 on 5-HT1b and 5-HT4 were also determined in p11 knock-down cells. NH_4Cl but not MG132 could reverse the protein level of p11 in IFN-α-treated SH-sy5y cells. MG132 could recover the protein levels of 5-HT1b and 5-HT4 in p11 knock-down cells. The down-regulation effects of IFN-α on p11, 5-HT1b and 5-HT4 were associated with the lysosome and ubiquitin–proteasome-mediated pathways. p11 was identified as a potent regulator to modulate the ubiquitination of 5-HT1b and 5-HT4. Therefore, it could be potential target therapies in IFN-α-induced depression.

Keywords. 5-Hydroxytryptamine receptor 4; 5-hydroxytryptamine receptor 1b; interferon-α; MG132; NH_4Cl; p11

1. Introduction

Administration of interferon-α (IFN-α), a cytokine demonstrating major antiviral (Boldanova *et al.* 2017) and antitumor (Ives *et al.* 2017) effects, has been used effectively as treatment for a variety of chronic viral infections and malignant tumors, including chronic hepatitis C (Ding *et al.* 2017), chronic hepatitis B (Zhang *et al.* 2017), melanoma (Yu *et al.* 2018) and renal cell carcinoma (Bush *et al.* 2017). Despite its potential therapeutic benefits, administration of IFN-α frequently induces psychiatric side effects such as fatigue (Dowell *et al.* 2016), insomnia (Maddock *et al.* 2004), anxiety (Malaguarnera *et al.* 2016) and cognitive disturbances (Spennati and Pariante 2013), especially depression (Fialho *et al.* 2018). These side effects limit the use of IFN-α greatly (Zahiu and Rimbas 2014).

Studies have found p11 and 5-hydroxytryptamine receptor 1b (5-HT1b) (Svenningsson *et al.* 2006; du Jardin *et al.* 2017) and 5-hydroxytryptamine receptor 4 (5-HT4) (Amigo *et al.* 2016) were involved in the onset of depression. p11 is also known as S100A10, belongs to the S100 EF-hand protein family, and it is widely distributed in all kinds of tissues of the organism specially expressed in the nervous system (Milosevic *et al.* 2017). Studies of the multifunctional protein p11 are shedding light on molecular and cellular mechanisms underlying depression (Verma *et al.* 2007; Seo *et al.* 2018). The p11 protein is responsible for the transport of serotonin receptors from cytoplasm to cytomembrane, such as 5-HT1b, 5-HT1d and 5-HT4. The absence of p11 protein can lead to the lower distribution of serotonin receptors on the membrane of nerve cells, and damage serotonin-signal transduction (Svenningsson *et al.* 2006; Warner-Schmidt *et al.* 2009; Svenningsson 2014). These molecules have been implicated in the regulation of mood and correlated with symptoms of mental depression (Eriksson *et al.* 2013). What's more, it has been claimed that p11 is a new potential target for depression therapy (Zhang *et al.* 2011).

In previous studies, we found that p11, 5-HT1b and 5-HT4 play roles in IFN-α-induced depression. First, IFN-α could reduce protein levels of p11, 5-HT1b and 5-HT4 *in vivo* and *in vitro*. And more importantly, overexpression

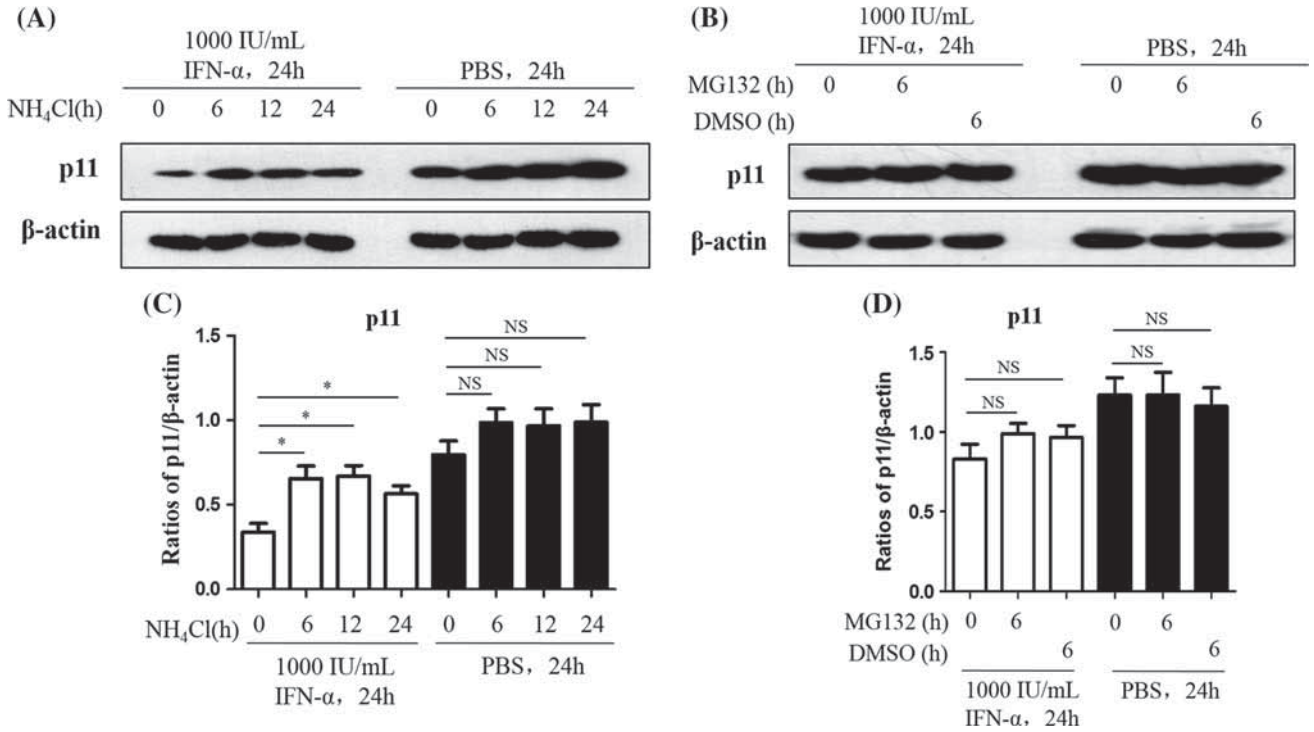

Figure 1. p11 protein levels in SH-sy5y cells after treated with IFN-α plus NH_4Cl or MG132. (**A**) SH-sy5y cells treated with 1000 IU/mL IFN-α-2b for 24 h and NH_4Cl for 0, 6, 12 and 24 h. (**B**) SH-sy5y cells treated with 1000 IU/mL IFN-α for 24 h and MG132 (or DMSO) for 0 and 6 h. p11 and β-actin were detected using western blot. (**C**) p11 protein levels in (**A**) were normalized against β-actin. (**D**) p11 protein levels in (**B**) were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. $^*P<0.05$ and NS, no significant difference.

of p11 was sufficient to reverse the down-regulated effects of IFN-α on protein levels of 5-HT1b and 5-HT4 (Guo *et al.* 2016). We found that IFN-α dramatically reduces the protein levels of p11 and 5-HT1b and 5-HT4 in the SH-sy5y cell line, but there is no difference in the messenger RNA (mRNA) level according to the results of quantitative real-time polymerase chain reaction (PCR) (Guo *et al.* 2016). Thus, mechanisms underlying the down-regulating effects of IFN-α on p11, 5-HT1b and 5-HT4 should be further investigated. As IFN-α had no modulating effects on the mRNA levels of p11, 5-HT1b and 5-HT4, the post-transcriptional modulation of these molecules should be focused on.

It is well known that ubiquitin–proteasome and endosome/lysosome-mediated proteolysis are the two major processes of post-transcriptional modulation of proteins (Matsui *et al.* 2018; Yildirim *et al.* 2018). Naturally, most of the p11 could bind to the N terminal of annexin AII (AnxA2) to form the (AnxA2)2–(p11)2 heterotetramer. Otherwise, the monomer of p11 will be ubiquitinated and degraded quickly (Svenningsson and Greengard 2007). AnxA2 is known to regulate endocytic membrane traffic, and in particular, membrane transport from early-to-late endosomes (Morel *et al.* 2009). 5-HT1b and 5-HT4 all belong to G protein-coupled receptors (GPCRs) (Kozono *et al.* 2017; Yin *et al.* 2018), which are transported to early endosomes and then recycled directly back to the plasma membrane, or transported from early endosomes to an intermediate perinuclear recycling endosome (Sposini and Hanyaloglu 2017). Protein levels of GPCRs also can be degraded through the ubiquitin–proteasome pathway (Skieterska *et al.* 2017). Above all protein levels of p11, 5-HT1b and 5-HT4 may be degraded by the methods of the ubiquitin–proteasome or endosome pathways.

To discover the mechanism underlying the down-regulating effects of IFN-α on protein levels of p11, 5-HT1b and 5-HT4, we treated SH-sy5y cells with NH_4Cl, MG132 and IFN-α. NH_4Cl and MG132 were typical drugs to block lysosome and ubiquitin–proteasome-mediated protein degradation, respectively. We found that treatment with NH_4Cl or MG132 could reverse the down-regulating effects of IFN-α on the protein levels of p11, 5-HT1b and 5-HT4 in SH-sy5y cells. That means, the degradation of p11, 5-HT1b and 5-HT4 was associated with the lysosome and ubiquitin–proteasome-mediated pathways. More importantly, p11 was identified as a potent regulator to modulate the ubiquitination of 5-HT1b and 5-HT4 and therefore it could be potential target therapies in IFN-α-induced depression.

2. Materials and methods

2.1 *Cell culture and drug treatment*

2.1.1. *Cell culture:* Human neuroblastoma cell line (SH-sy5y cell line, Jiahe Biotech Company, Shanghai, China) was

cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 mg/mL streptomycin at 37°C, with 5% CO_2. All experiments were performed when the cells were 80–90% confluent.

2.1.2. *Human recombinant IFN-α-2b treatment:* Cells were refreshed with DMEM and treated with human recombinant IFN-α-2b concentrations (hIFN-α-2b: 0 or 1000 IU/mL) as reported (Motylewska *et al.* 2013). hIFN-α-2b was purchased from Sigma and dissolved and diluted with phosphate-buffered solution (PBS). The cells were harvested at 24 h for protein detection.

2.1.3. *NH_4Cl treatment:* Cells were treated with 20 mM NH_4Cl alone (Zhang *et al.* 2018) or plus with 1000 IU/mL hIFN-α-2b. NH_4Cl was purchased from Sigma and dissolved with PBS. The treated cells were harvested at 0, 6, 12 and 24 h for protein detection.

2.1.4. *MG132 treatment:* Cells were treated with 20 μM MG132 alone (Inoue *et al.* 2018) or plus with 1000 IU/mL hIFN-α-2b. MG132 was dissolved with dimethylsulfoxide (DMSO). MG132 and DMSO were purchased from Sigma. The treated cells were harvested at 0 and 6 h for protein detection.

2.1.5. *Plasmid transfection: Plasmid construction:* To construct p11-cherry plasmid, a 319-bp product of p11 cDNA was amplified using PCR. The forward primer was 5′-CCGAATTCTATGCCATCTCAAATGG-3′ (with an *Eco*RI restriction enzyme site), and the reverse primer was 5′-CGGGATCCCTACTTCTTTCCCTTC-3′ (with a BamHI restriction enzyme site). The PCR products were cut using *Eco*RI and BamHI, followed by cloning into the pRS304:3 m Cherry vector (Addgene, USA), and then p11-cherry was produced. The miRNA-p11 plasmid (sense sequence: 5′-TGACACCTGAGAACTCATGGAAA-3′ and anti-sense: 5′-TTTCCATGAGTACTCTCAGGT-3′) and its corresponding control plasmid (miRNA-control) (sense sequence: 5′-TGACGTCTCCACGCAGTACATTT-3′ and anti-sense: 5′-AAATGTACTGCGCGTGGAGAC-3′) were constructed using the pcDNA6.2-GW/EmGFP-miR vector which was purchased from Invitrogen.

Plasmid transfection: SH-sy5y cells were seeded at a density of 3×10^5 cells per well in six-well plates and incubated in DMEM at 37°C for 24 h. Before transfection, the cells were washed with antibiotic-free DMEM containing 10% FBS. The plasmids were separately transfected into cells with Liposome Transfast^{2000} (Invitrogen, USA), miRNA-p11 and miRNA-control (control). At 5 h after transfection, the cells were washed with PBS and supplemented with complete DMEM. After incubated for another 48 h, hIFNα-2b, NH_4Cl, MG132 or DMSO was added to the medium as indicated. The cells were collected at 48 h for western blots.

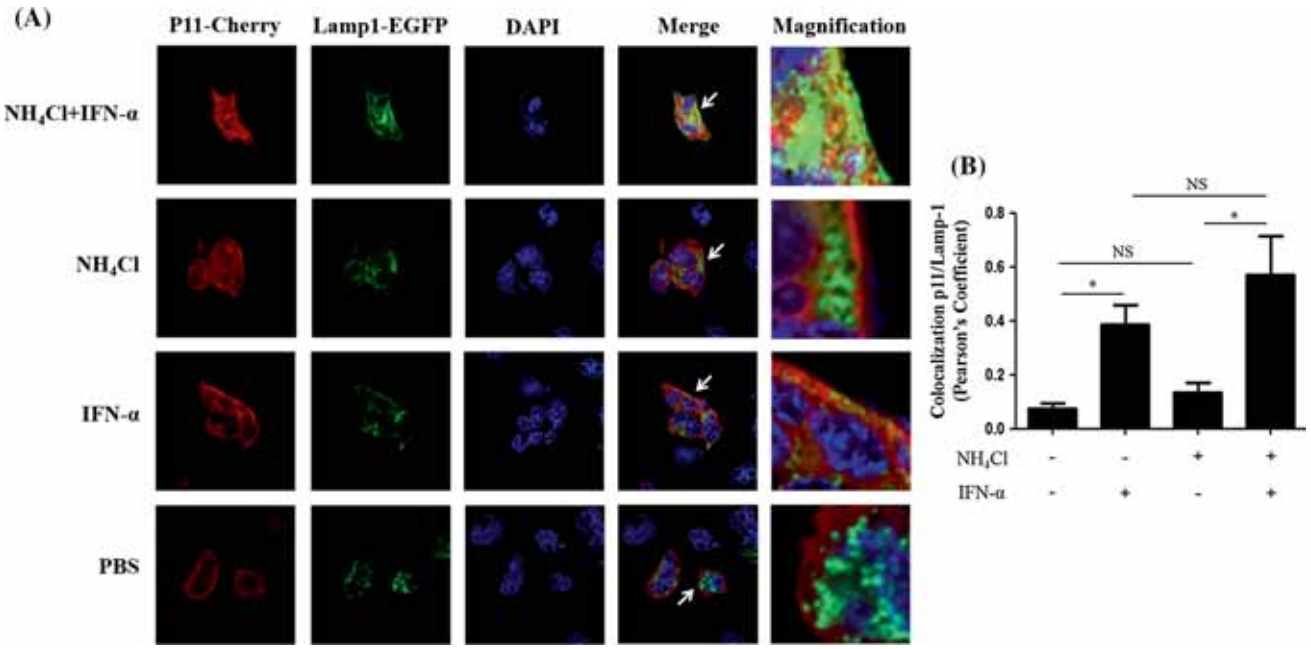

Figure 2. Distributions of p11 and Lamp-1 proteins in SH-sy5y cells treated with hIFN-α-2b (1000 IU/mL) for 24 h and with NH_4Cl for 12 h. The cells were transfected with the p11-cherry vector (in red) and with Lamp-1-EGFP (in green). (**A**) The p11-cherry column in the micrographs shows p11 protein. The Lamp-1-EGFP column in the micrographs shows Lamp-1 protein; DAPI staining shows the cell nuclei. Merged images show the overlay of p11-cherry, Lamp-1-EGFP and DAPI. The magnification shows the enlarged map of the place indicated by the white arrow in the merged images. Confocal microscopy was conducted at 400 × and the scale bar represents 20 μm. (**B**) Histogram of the Pearson's coefficient (represents the co-localization of p11 and Lamp-1). All results were representative of three separate experiments. The data represent mean ± S.E. **P*<0.05 and ***P*<0.01 and NS, no significant difference.

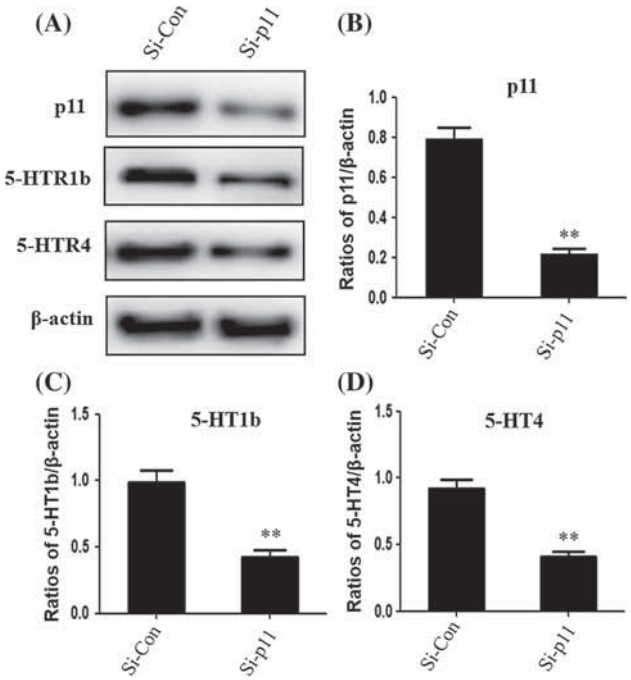

Figure 3. 5-HT1b and 5-HT4 protein levels in SH-sy5y cells after the p11 gene was knock-down. (**A**) Protein levels of p11, 5-HT1b and 5-HT4 were assayed using western blot. (**B**) Protein levels of p11 were normalized against β-actin. (**C**) Protein levels of 5-HT1b were normalized against β-actin. (**D**) Protein levels of 5-HT4 were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. $^{*}P<0.05$ and $^{**}P<0.01$.

2.2 *Western blots*

Cells were collected and lysed in RIPA lysis buffer (Beyotime, Beijing). For electrophoresis, equal amounts of protein samples were added into the wells and electrophoresed for 1.5 h. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were incubated overnight with the primary antibody at 4°C (anti-p11 1:1000, Proteintech; anti-5-HT1b 1:1000, Abcam; anti-5-HT4 1:1000, Abcam and anti-GAPDH 1:2000, Santa Cruz). Subsequently, the membranes were incubated with the secondary antibodies for 1.5 h at room temperature (goat anti-rabbit 1:3000, Santa Cruz and goat anti-mouse 1:3000, Santa Cruz). The western blot was developed using an ECL system (Beyotime, China) and exposed to a radiographic film. The gray scales of the bands were quantified using 'Quantity-One' software.

2.3 *Confocal test*

p11-Cherry plasmid and LAMP1-EGFP (Addgene, USA) were co-transfected into SH-sy5y cells. At 5 h after transfection, the cells were washed with PBS and supplemented with DMEM containing FBS and hIFNα-2b or NH_4Cl was added into the medium. After cultured for 24 h, the cells were washed with PBS and then fixed using -20°C pre-cooled 70% ethanol at room temperature for 10 min. Next, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 20 min and sealed with 20 μL anti-fade mounting medium. Finally, the sections were observed using a laser confocal microscope (Olympus FV1000, Japan).

3. Results

3.1 *Treatment with NH_4Cl but not MG132 could reverse the down-regulating effects of IFN-α on the protein levels of p11 in SH-sy5y cells*

SH-sy5y cells were treated with IFN-α plus NH_4Cl or MG132, and p11 protein levels were measured using western blots. The results showed that the protein levels of p11 in the IFN-α-treated plus 20 mM NH_4Cl group were significantly increased to 93.3% ($P = 0.0231$), 97.1% ($P = 0.0150$) and 66.3% ($P = 0.0328$) for 6, 12 and 24 h separately compared with the controls (figure 1A). No significant differences in the p11 protein levels were found between the IFN-α plus MG132- and IFN-α plus DMSO-treated groups, and between the MG132- and DMSO-treated groups (figure 1B).

3.2 *IFN-α facilitates the distribution of p11 in lysosome*

In previous studies, we had found that IFN-α can down-regulate the protein levels of p11, and treatment with NH_4Cl could reverse the down-regulating effects of IFN-α on the protein levels of p11 in SH-sy5y cells; we suspect these effects were related to lysosome. Lysosomal-associated protein 1 (Lamp1) is known as a glycoprotein from a family of lysosome-associated membrane glycoproteins and it often used as a marker for lysosomes (Pugsley 2017). To identify this speculation, we test the distribution of p11 in lysosome in SH-sy5y cells by a confocal test. Cells were transfected with the p11-cherry vector and Lamp1-GFP vector, and treated with IFN-α and/or NH_4Cl. The co-localizations of p11 and Lamp1 were tested by confocal microscopy. The results indicated that IFN-α treatment could significantly increase the co-localization of p11 (in red) and Lamp1 (in green) compared with the control group. *Pearson's coefficient* increased by 420.3% ($P = 0.0140$) in the IFN-α-treated group compared with the PBS-treated group. And it was increased by 330.0% ($P = 0.0402$) in the NH_4Cl plus IFN-α-treated group compared with the NH_4Cl-treated group (figure 2).

3.3 *Treatment with NH_4Cl or MG132 could reverse the down-regulating effects of IFN-α on the protein levels of 5-HT1b and 5-HT4 in SH-sy5y cells*

To clarify whether the effect of p11 protein expression is directly involved in the regulation of 5-HT1b and 5-HT4

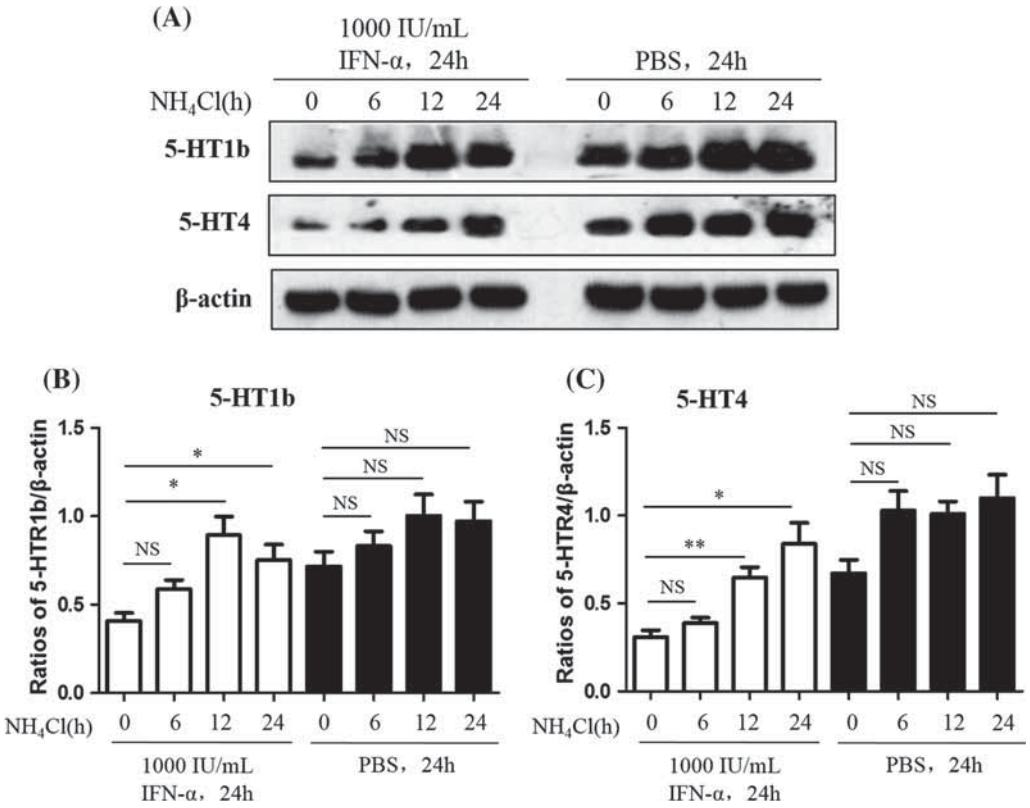

Figure 4. Western blot assayed 5-HT1b and 5-HT4 protein levels in SH-sy5y cells treated with IFN-α plus NH_4Cl. (**A**) SH-sy5y cells treated with 1000 IU/mL IFN-α-2b for 24 h and NH_4Cl for 0, 6, 12 and 24 h. (**B**) 5-HT1b protein levels were normalized against β-actin. (**C**) 5-HT4 protein levels were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. *$P<0.05$ and **$P<0.01$, and NS, no significant difference.

expression. We knock-down the p11 gene in SH-sy5y cells without any treatment, and protein levels of 5-HT1b and 5-HT4 were detected by using western blot. The results exhibited that protein levels of p11 remarkably decreased by 72.7% ($P = 0.0007$) in the p11-Si group compared with the controls (figure 3). Protein levels of 5-HT1b and 5-HT4 were reduced by 57.0% ($P = 0.0067$) and by 55.5% ($P = 0.0025$), respectively, in the p11-Si group compared with the controls (figure 3). This illustrated p11 involved in the regulation of 5-HT1b and 5-HT4 expression directly.

To investigate regulation effects of NH_4Cl or MG132 on protein levels of 5-HT1b and 5-HT4, SH-sy5y cells were treated with IFN-α for 24 h alone or plus NH_4Cl or MG132 for various time durations. The results showed that in the IFN-α-treated group plus with NH_4Cl applied for 12 h, protein levels of 5-HT1b and 5-HT4 significantly up-regulated by 119.2% ($P = 0.0128$) and by 110.3% ($P = 0.0092$), respectively (figure 4). At the same time, there were no significant difference between the NH_4Cl-treated groups and their controls (PBS groups) (figure 4).

The results also exhibit that after MG132 was treated for 6 h, the protein levels of 5-HT1b and 5-HT4 were remarkably increased by 137.4% ($P = 0.0072$) and by 234.4% ($P = 0.0008$), respectively (figure 5). Moreover, there were also no significant difference between the MG132-treated groups and their controls (PBS groups) (figure 5).

The data demonstrated that treatment with NH_4Cl or MG132 could reverse the down-regulating effects of IFN-α on the protein levels of 5-HT1b and 5-HT4.

3.4 *Protein levels of 5-HT1b and 5-HT4 were reduced when p11 gene was silenced, and the effects were reversed in cells treated with MG132 but not NH_4Cl*

In previous studies, we have found that IFN-α can reduce the protein levels of p11, 5-HT1b and 5-THR4, and more importantly overexpression of p11 could reverse the down-regulating effects of IFN-α on 5-HT1b and/or 5-HT4, thus the down-regulating effects were dependent on p11. To identify that these phenomena were related to lysosome or proteasome, p11 was knock-down using Si-RNA technology.

We found as expected protein levels of 5-HT1b and 5-HT4 were all reduced in the Si-p11 groups compared with the controls (figures 6 and 7). Treatment with MG132 can remarkably observably block the down-regulating effects on 5-HT1b and 5-HT4 in the Si-p11 groups (figure 6).

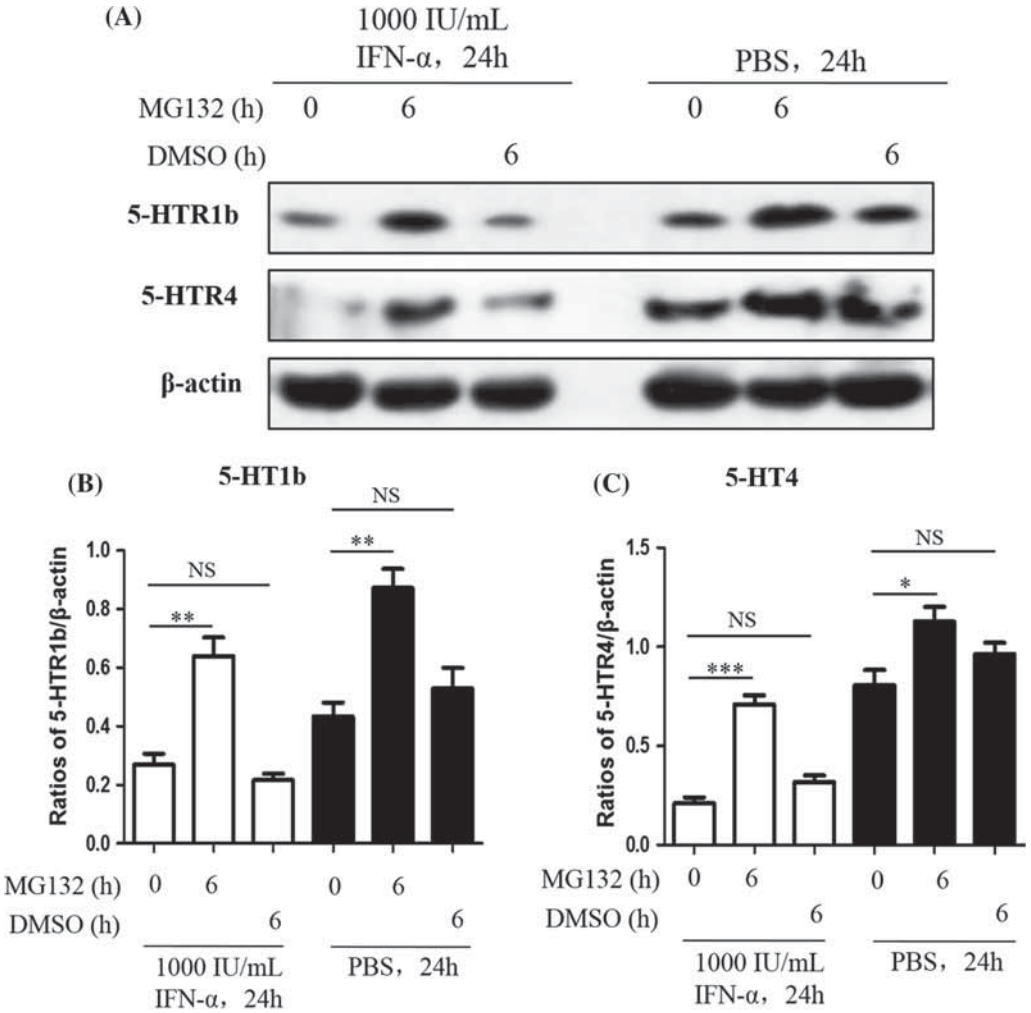

Figure 5. Western blot assayed 5-HT1b and 5-HT4 protein levels in SH-sy5y cells treated with IFN-α plus MG132. (**A**) SH-sy5y cells treated with 1000 IU/mL IFN-α-2b for 24 h and MG132 (or DMSO) for 0 and 6 h. (**B**) 5-HT1b protein levels were normalized against β-actin. (**C**) 5-HT4 protein levels were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. *$P<0.05$ and **$P<0.01$, and NS, no significant difference.

Moreover, treatment with NH_4Cl had no effects on the protein levels of 5-HT1b or 5-HT4 in the Si-p11 groups (figure 7). It indicated that the regulating effects of p11 on 5-HT1b and 5-HT4 were concerned with the proteasome–ubiquitin-related proteolysis pathways but not with the lysosome-mediated pathways.

4. Discussion

Long-term use of IFN-α often leads to mental side effects such as drowsiness, fatigue, anxiety, irritability and anorexia, especially depression (Murakami *et al.* 2016). But, the molecular mechanism underlying IFN-α-induced depression remains unclear. In previous study, we found IFN-α treatment could induce the down-regulation of p11, 5-HT1b and 5-HT4 *in vitro* and *in vivo*. The down-regulation effects of IFN-α on 5-HT1b and 5-HT4 were dependent on p11, and more importantly, the high-expression of p11 could reverse the reductions of 5-HT1b and 5-HT4 in IFN-α-treated cells (Guo *et al.* 2016). We speculated that p11 was acted as a mediator in regulating the protein levels of 5-HT1b and 5-HT4.

In this study, we investigated the post-transcription of p11, 5-HT1b and 5-HT4 after IFN-α treatment, because the drug could not alter the transcriptional efficiency or mRNA stability of these molecules. Protein degradation was mainly through the lysosome pathway or the ubiquitin–proteasome pathway in the cell (Dores and Trejo 2019). Therefore, typical drugs NH_4Cl and MG132 were used to block the two processes, respectively. And effects of the drugs on protein levels of p11, 5-HT1b and 5-HT4 were observed.

We found treatment with NH_4Cl but not MG132 could reverse the down-regulating effects of IFN-α on the protein levels of p11 in SH-sy5y cells (figure 1). Some studies suggested that p11 with monomers can be degraded though the ubiquitin–proteasome pathway. Most of the p11 bounds

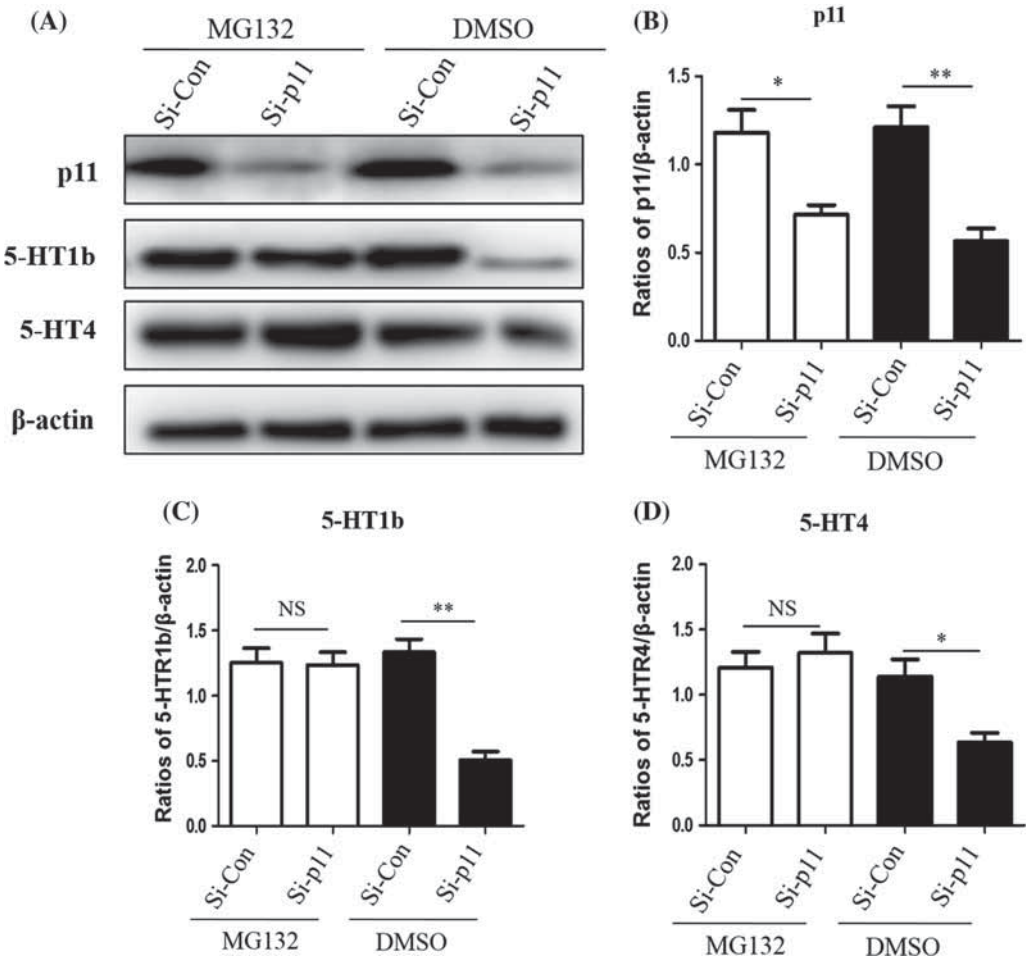

Figure 6. p11, 5-HT1b and 5-HT4 protein levels in SH-sy5y cells transfected with the Si-p11 vector after MG132 treatment. After plasmid transfection for 48 h, total proteins were extracted; MG132 or DMSO was added to the medium 6 h before the cells were collected. p11, 5-HT1b and 5-HT4 protein levels were analyzed using western blotting. (**A**) SH-sy5y cells were transfected with Si-p11, using the Si-control vector as the control. The p11 (**B**), 5-HT1b (**C**) and 5-HT4 (**D**) protein levels in the Si-p11 transfection groups were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. *$P<0.05$, **$P<0.01$ and NS, no significant difference.

with AnxA2 to form a tetramer, and this structure could block the ubiquitination of p11 to avoid being degraded in cells (He *et al.* 2008). Our result indicated that the down-regulation effects of IFN-α on p11 protein were also possibility dependent on lysosome-mediated degradation.

To confirm that IFN-α can facilitate p11 to enter into lysozymes, we conducted a fluorescence plasmid transfection test; p11-Cherry vector (red) and LAMP1-GFP vector (green) were co-transfected into SH-sy5y cells. This result showed that the co-localization of red and green in the NH_4Cl plus IFN-α-treated group was significantly higher than that in the NH_4Cl group (figure 2). This suggests that IFN-α treatment can promote the distribution of p11 in lysozyme.

Interestingly, researchers had reported that the distribution of p11 was followed by AnxA2, but it rarely exists in lysosomes and late endosomes (Mayran *et al.* 2003). AnxA2 mainly exists in cell membrane, endoplasmic reticulum, early endometrium and other organelles, and its distribution depends on cholesterol. AnxA2 can also be present in late endosome or lysosome if these organelles also have cholesterol induced by drugs (Zeuschner *et al.* 2001; Morel and Gruenberg 2007). In this study, we found that p11 can be distributed in the lysosome, which is likely to be transported into the lysosome by AnxA2 under the action of IFN-α. In this process, AnxA2, cholesterol and other substances may also exist in the lysosome. This is a possible mechanism for the degradation of p11.

To observe post-transcriptional modification of 5-HT1b and 5-HT4, cells were treated with NH_4Cl or MG132 and their protein expressions were observed. We found that treatment with NH_4Cl or MG132 could reverse the down-regulating effects of IFN-α on the protein levels of 5-HT1b and 5-HT4 in SH-sy5y cells (figure 4). This indicates that the degradation of 5-HT1b and 5-HT4 was associated with both the lysosome and ubiquitin–proteasome pathways.

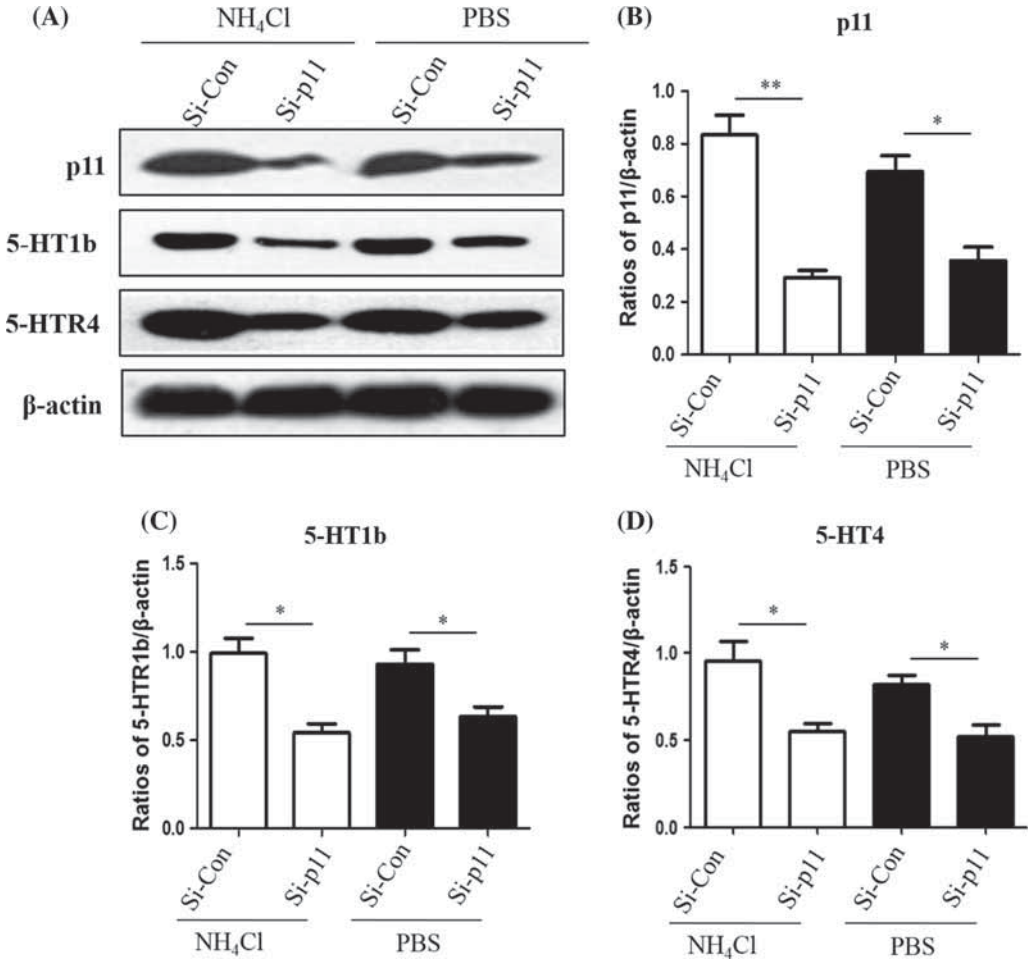

Figure 7. p11, 5-HT1b and 5-HT4 protein levels in SH-sy5y cells transfected with the Si-p11 vector after NH_4Cl treatment. NH_4Cl or PBS was added to the medium 12 h before the cells were collected. Total proteins were extracted at 48 h and the p11, 5-HT1b and 5-HT4 protein levels were analyzed using western blotting. (**A**) SH-sy5y cells were transfected with Si-p11, using the Si-control vector as the control. The p11 (**B**), 5-HT1b (**C**) and 5-HT4 (**D**) protein levels in the Si-p11 transfection groups were normalized against β-actin. Each group, $n = 3$. The data represent mean ± S.E. compared with the controls. $^*P<0.05$, $^{**}P<0.01$ and NS, no significant difference.

To distinguish whether the down-regulation effects were directly or indirectly mediated by the lysosome or ubiquitin–proteasome pathways, the expression of p11 was knock-down and the protein levels of 5-HT1b and 5-HT4 were observed. The results demonstrate that protein levels of 5-HT1b and 5-HT4 were reduced when the p11 gene was silenced (figures 6 and 7). It indicated that the protein levels of 5-HT1b and 5-HT4 are dependent on p11, and the presence of p11 can inhibit the degradation of 5-HT1b and 5-HT4. p11 can be an important regulator to stabilize 5-HT1b and 5-HT4.

The experiment also observed that the down-regulating effects on 5-HT1b and 5-HT4 could be reversed in cells treated with MG132 (figure 6) but not with NH_4Cl (figure 7). This indicated that the lysosome pathway is not directly involved in the protein degradation of 5-HT1b and 5-HT4, while the ubiquitin–proteasome pathway directly takes part in the process. This illustrated that the degradation of 5-HT1b and 5-HT4 was increased in the case of p11 silencing. Studies on the ubiquitination and degradation of 5-HT receptors are rare, and it is reported that the 5-HT7 receptor can be ubiquitinated and degraded (Matthys *et al.* 2012). Similar to 5-HT7, both 5-HT1b and 5-HT4 also serve as GPCRs with analogous structures and may be degraded by ubiquitination.

Studies have found that p11 can directly bind with 5-HT1b (Svenningsson *et al.* 2006) and 5-HT4 (Warner-Schmidt *et al.* 2009). It was not difficult to speculate that this combination may block the ubiquitination of 5-HT1b and 5-HT4 (due to the effect of steric hindrance), and thus avoiding degradation in proteasome. In the absence of p11, the obstruction disappeared and 5-HT1b or 5-HT4 was degraded by the ubiquitin–proteasome pathway. Therefore, MG132 could reverse the protein levels of 5-HT1b and 5-HT4 when the p11 gene was silenced. Thus, p11 was likely to be a regulator of the ubiquitination of 5-HT1b and 5-HT4.

To sum up, we speculated that IFN-α caused the internalization and transport of p11 to late endosome/lysosome,

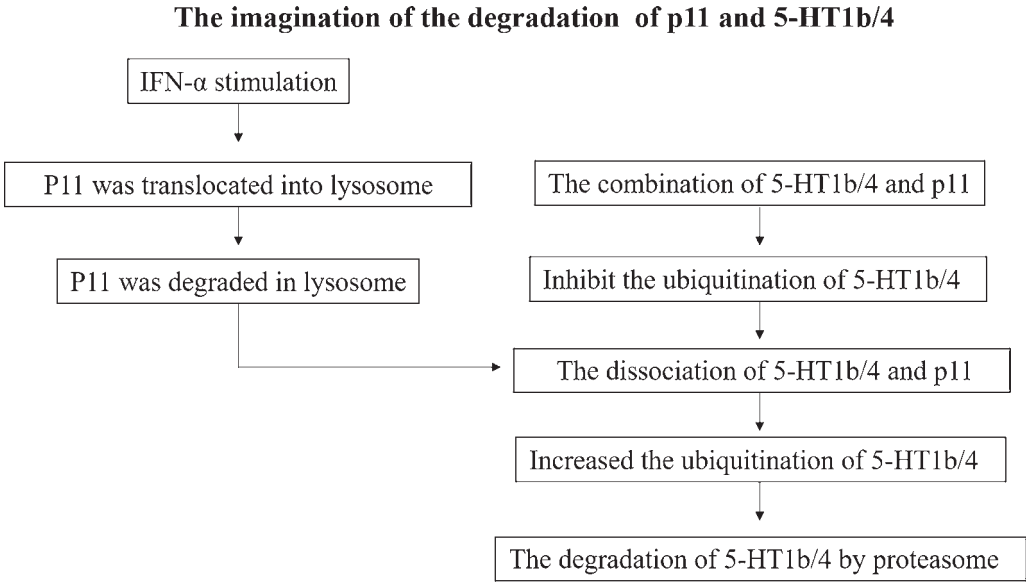

Figure 8. Process of the degradation of p11 and 5-HT1b/4. First, p11 was translocated into the lysosome of the cell after the stimulation of IFN-α. Second, p11 was degraded in the lysosome. Third, it induced the decreased protein levels of p11, and the combinations of p11 and 5-HT1b/4 were dissociated. Fourth, 5-HT1b/4 loses the protection of p11, and then the ubiquitination of 5-HT1b/4 was increased. Finally, 5-HT1b/4 was degraded by proteasome.

and was degraded. And subsequently, 5-HT1b and 5-HT4 could not form complexes with p11, leading to ubiquitination and degradation of 5-HT1b and 5-HT4 (figure 8).

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