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Different Types of Early Life Stress Affected Histone H4 Acetylation in the Left Prefrontal Cortex of Mice in Different Pathways

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Abstract

Histone H4 acetylation is an epigenetic change that regulates gene expression and is linked to a variety of neurological functions. The purpose of this study was to see how different types of early life stress affected histone H4 acetylation in the left prefrontal cortex (PFC) of mice. Histone acetylation levels were determined using Western blot analysis in four groups: control, control isolation, chronic stress, and chronic unpredictable stress. The control group comprised of mice that were not disturbed from birth until weaning, whereas the control isolation group was isolated after weaning. The chronic stress group was separated from their mothers on a daily basis, whereas the chronic unpredictable stress group was separated from their mothers on specified weekdays. Stress, according to our findings, may raise histone modification levels, whereas routine and repetitive stress situations may produce habituation behavior as a compensatory mechanism. Notably, this study provides preliminary evidence that different types of stress alter H4 acetylation in the PFC in mice. Understanding the molecular mechanisms governing histone acetylation in response to early life stress can offer light on the neurobiological processes at work and help to create tailored therapies for stress-related illnesses.

Keywords: Histone, prefrontal cortex, early life stress, epigenetics, Western Blot

Introduction

Early life stress affects the brain function in the children and carried out until adulthood[1]. It associated with the worse progress of chronic disease such depression and

cardiovascular disease [2]. Early life stress has been claimed to increase the risk of stress sensitivity in the adulthood [3-6], reduce developmental flexibility as well as fitness ramification [7], increase the risk of pathological condition [8] increase the risk of pathological condition[8, 9] and affect the reward mechanism in the brain in many species[10-12].

Rodent models are often used to characterize early life stress[2]. Maternal separation (MS), an early life stress model in rodent, has been proposed to be equivalent with children neglect in human. This model disrupts the emotional behavior in rodent as well as neuronal function[13]. Early life stress leads to epigenetic modification of target gene that related to emotional behaviour [14-16]. Epigenetic indicates an interaction between genes and environment[17]. Epigenetic consists of DNA methylation and other modification without change any DNA sequence[18]. Although DNA methylation is common and relatively fast mechanism in higher organism, Histone modification is still the main regulatory mechanism in many eucharyotic organisms. These two mechanisms are two independent processes [19]. Chromatin appears in several forms and is composed of genomic DNA, protein and RNA. The protein content of chromatin is composed primarily of core histones that are packaged into nucleosomes resulting in the condensation of the DNA. Several epigenetic mechanisms regulate the stability of the nucleosomes and the protein-protein interactions that modify the transcriptional activity of the DNA. Interestingly, epigenetic control of gene expression has recently emerged as a relevant mechanism involved in the regulation of many different biological processes including that of muscle development, muscle mass maintenance, function, and phenotype in health and disease [20].

When histones are acetylated, phosphorylated, and methylated (depending on the methylated residue), less condensed chromatin structure giving access to the transcriptional machinery and co-activators. When DNA and histones are methylated (depending on the methylated residue, and in the simultaneous absence of histone acetylation and phosphorylation), chromatin being less accessible to the transcriptional processes. In addition, transcriptional co-repressors bind to the methylated DNA. Together, these changes result in transcriptional silencing[19, 21, 22]. However, epigenetic change or gene expression induced by stress may induce the structural behavioral changes. McCoy and colleagues found that the maternal separation's offspring elicit DNA methylation and histone modification [23]. Histone acetylation barely appears in the stress mice. In experiment with maternal separation from day 14 to 16 (acute stress), the mice show high increase of H4 acetylation[24].

this study aimed to investigate whether acetylation of histone H4 is modified in left part of prefrontal cortex of adult C57Bl/6 mice. Mice are tested on paradigm of chronic stress. In concequence, we modify the methode of maternal separation to eliminate the bias caused by stress during weaning. This modification is important to observe whether the mice have stress in the first separation during the weaning in PND 21, hence, we create one more control group with isolation cage's feature.

Materials and method

the application of the method to see the histone H4 acetylation in early life stress mice. Each bloting process contain 11 samples with various group.

Material

C571Bl/6 left part of mice's prefrontal cortex

DC protein Assay:

- Reagent A, reagent S, reagent B
- BSA solution

Buffer:

Type of buffer	Composition
Extraction buffer	(for 10 µl) 1 ml of 0,1 M Tris/HCl pH 8,0 +0,2 ml of 0,01 M EDTA + 5 ml of 10 % SDS + 100 µl of 1 fold Halt Protease Inhibitor Cocktail
Loading buffer	(for 10 µl) 5,7 µl EP+DTT + 3,8 µl sample buffer + 0,2 µl 1 M DTT + 0,3 µl Bromphenolblue
Running buffer	(for 1 L) Tribase 30 g + glycine 144 g + 10 % SDS 100 ml
Transfer buffer	(10 fold for 1 L): Tribase 30,27 g +glycine 144 g + ddH2O 1L
TBST solution	1x TBS + 0.1 % Tween

Blotting devices with polyvinylidene difluoride (PVDF) membrane.

Blocking solution

Red alert staining

Luminata crescendo

G-maschine (genesys software) for detection/analysis

Method

We categorized the samples to get different effects depend on what condition that we apply to the animals. The mouse strain that we used is C57Bl/6. The control isolation group differed from the control group to observe some effects in alone or together with other offspring in a cage after the separation. The maternal separation with weekend also differed from the maternal separation without weekend. This difference is aiming to see whether routine separation makes the offspring able to cope the stress better than the non-routine separation.

Animal preparation

Four groups are defined, samples are prepared:

a. **Control group**: Animals are kept undisturbed since the first day of birth (PND1) until weaning (PND-21). The mother and the offspring are kept in one cage until weaning day (PND 21). After weaning in day 21, the offspring are grouped into two cages based on sex.
b. **Control Isolation group**: Animals are kept undisturbed since the first day of birth (PND-1) until weaning (PND-21) The mother and the offspring are kept in one cage until weaning day (PND 21). After weaning in day 21, each offspring will place one cage.
c. Chronic stress : The offspring are separated from its mother 3 hours a day, 7 days a week in separate cage from post natal day 1 (PND-1) until Postnatal day 21 (PND-21). Because we also separate them in the weekend, so we call this group by **Maternal separation Week End + (MSWE+)**
d. Chronic unpredictable stress : The offspring are separated from its mother 3 hours a day, 5 days a week in separate cage from post natal day 1 (PND-1) until postnatal day 21 (PND-21). Because we only kept them away in 5 days (without Weekend) we call this group by **Maternal Separation Week End – (MSWE -)**

   The separation of the animal should consider the coziness aspects including water, food, contamination of the worker and also the maternal conditioning (so the mother will recognize their offspring immediately after back from separation). Before we did the brain dissection, the animals have to do the behavior tests which

are water test/forced to swim (PND 62-63) and anhedonia /sucrose test (PND 80). Brain dissection is done in PND 100.

Brain Dissection

For our experiment, the left part of prefrontal cortex of the mice are taken and put in the tubes. The sample stored in the liquid nitrogen in -80°C.

Whole Protein extraction

The proteins in the prefrontal cortex of the mice were extracted in order to get the protein fraction. It is important to homogenize them properly by using sonication machine otherwise the samples can not represent all the proteins in the prefrontal cortex region. The homogenization process should not leave any tissue or other solid material.

Firstly 0,5 ml tubes with 100 µL of extraction Buffer without DTT were prepared, the tubes are put on the container with ice. The tissues were homogenized by using sonication machine until the solution become homogen. The sample solutions were centrifuged with 14000 G in 5 minutes in approximate 20°C. Supernatant was discarded into the new tube.

Concentration determination

Tubes were prepared and filled by 25 µl of Reagent A/S and 200 ml of Reagan B (Reagan A/S contains 1 ml Reagent A and 20 µl Reagent S). New tubes that had been added with reagent were filled by 1 µl of the each sample solution. The tubes were shaked well and stored in dark for 15 minutes. Concentration determination is a pivotal step to measure the correct amount of samples that should inserted into SDS page. The goal is to determine the absolute protein concentration in the sample to load a defined amount to the gel. The next goal is to ensure that the absolute quantity of protein loaded is in a range that is sufficient for visualization but does not exceed the capacity of the gel. We also performed the standard curve to calculate the protein concentration in the samples. This processes used nanodrop photometry as quantification device. Each sample was tested couple times in nanodrop. The mean was taken as final result and projected onto a graph to observe the tendency. For determination of loading control, prepared standard curve which contained blank solution and 2,5, 5, 10, 20 and 30 µg/µl solution. Each solution contained 45 µl water except for 20 µg/µl solution (15 µl of water) and 30 µg/µl that had no water added. the 30 µg/µl solution was mixed with 18,75 µl of BSA 1,6 µg/µl solution.

And 20 µg/µl solution with 75 µl of BSA solution. The 45 µl from mixed 20 µg/µl solution was mixed with 10 µg/µl solution. Other 45 µl from 10 µg/µl solution was mixed with 5 µg/µl solution. The same procedure to 2,5 µg/µl was repeated.

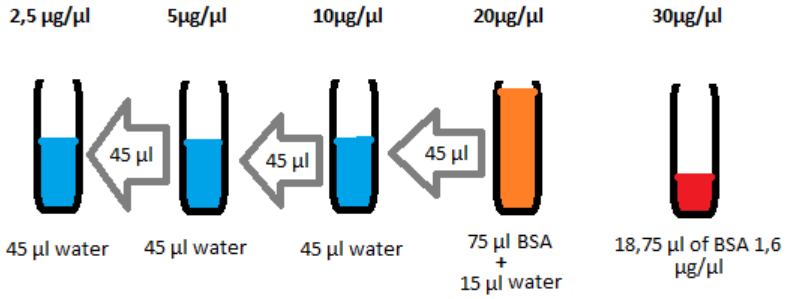

Fig 2. Pippeting scheme of standard curve solution

After incubation in the dark, the sample solution was pippeted into nanodrop ca. 1,3 µl in 75 n wavelength of UV. The test was repeated several times and recorded. The result was projected onto a graph to see the tendency of the standard curve. The standard curve shows the shape as follow. The good standard curve is indicated by R^2 that close to one (in our case 0.9941 which means good enough).

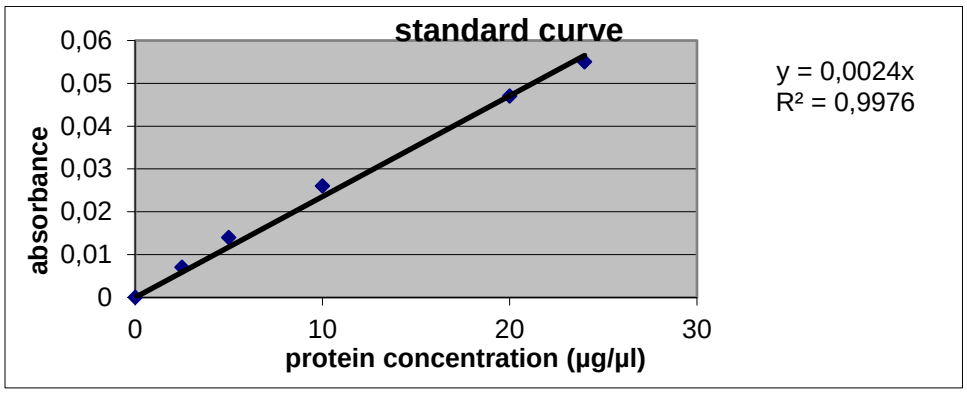

Fig.3 Standard curve of tested solution. The tendency of the curve remains linier (R^2 = 0.9941)

From the graph above we can conclude that the acceptable amount of protein concentration is between 0 – 30 µg/µl. A standard curve was used to comprised of two-

fold serial dilutions of control run in parallel to verify the linearity of the assay and to quantify the results.

We use the equation of the standard curve to calculate the protein concentration of the samples. The equation is y = 0,0024x . E.g for sample number 707, the absorbance is 0.022, therefore the protein concentration is x= 0.022 / 0.0024 = 9.166666 µg/µl. and so on to the other samples absorbances.

SDS Page/electrophoresis

SDS page is a critical step when we transfer the sample proteins into SDS page's device and charge it with 75 mV. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the gel. Smaller proteins migrate faster through this gel and the proteins are thus separated according to size (usually measured in kilodalton, kDa). The important aspect of this step is to carefully load the samples to avoid the mixture between each sample which will make the result less reliable.

The loading buffer was made based on following calculation: Since SDS pocket's volume is 20 µl per lane, we need to make solution of protein together with loading buffer as much as 20 µl. We decided to load a defined amount of protein as much as 25 µg/20 µl or 50 µg/20 µl respectively. From the concentration determination's part we know how many protein that we should load into SDS pocket. This amount of protein is obtained from the nanodrop measurement. Since we want to prepare 25 µg/20 µl solution, the loading buffer is the rest volume after reduced by protein. E.g for sample number 707, the protein concentration is 9,166666 µg/µl, consequently we need 5,454 µl of protein for 50 µg. therefore the volume of loading buffer is = 20 – 5,454 = 14,545 µl. And so on with the other samples. New eppendorf tubes for the sample (with the labels) were filled with loading buffer based on nanodrop measurement. The eppendorf tubes were filled again with samples based on calculation of nanodrop. Sample should be shaked first in the shaker. The eppendorf tubes then were cooked in the heater for 5 minutes in 95°C. Meanwhile the SDS page was prepared by putting two gels into the device. The striker at the bottom of the gel was pulled out. The space between the gels was filled by running buffer to see whether there is a leak or no. When there is no leak, the device was put into container in the right direction (Positive charge with positive part and negative charge with negative part). The combs was taken from the gels carefully and the container was filled by running buffer until "2 gels" border. The sample solutions (sample + loading buffer) were loaded to the pockets in the gels. Each pocket contained 20 µl of sample solution, except for marker (only 10 µl). Make sure that the solution does not spread to neighboring

pocket or outside its own pocket. We did the same for the opposite gel. If needed, the container should be moved carefully so the samples from one side do not spread anywhere else. After finishing, the container was closed by the lid and charged for 1 hour with 75 V. The charge will flow from the negative charge to the positive charge. The charge was stopped when the protein solution reaches very close to the bottom of the gel.

Blotting/ protein transfer

Blotting process is a process to transfer the protein from SDS page into the prepared polyvinylidene difluoride (PVDF) membrane. This step is important because the antibody cannot bind to the gel. Therefore PVDF membrane is needed (We also used nitrocellulose membrane at the end of the experiment). This procedure was done by putting the gel side by side with the membrane in the certain sequence with the filter paper, sponges and membran. The charge is flown from negative charge to positive charge. This charge led the protein in the gel moved to the membrane that sticked with the gel. The sequence of the gel,membrane and position of the board in the device is important because they determine the direction of the printed membrane. The short exposure to methanol was also important to keep the membrane in negative charge. Overexposure will damage the membrane.

The membranes and the filter papers were prepared with the equal size as the gel. More size for the filter paper. The filter papers and sponges were soaked in the transfer buffer. Every surface should contacted with methanol and shortly transfer the membrane into transfer buffer. SDS page's devices were opened to access the gels. The gel was carefully transfered into Blotting device with sequence: Black board-sponge-filter paper-membrane-gel-filter paper-sponge-transparent board. The blotting device was locked and put into blotting chamber with the positive part in the board facing the positive part in the chamber and respectively. The chamber was put into container. The container was filled by the transfer buffer until blotting sign. Lid was closed and chamber was charged for 2 hours in 75 V. The container should be observed. If it too hot, container would be put on the ice. After 2 hours, membrane was transported into small container and stained by red alert in 5 minutes. Red alert is useful to see the band so we can easily cut it for antibody incubation. Red alert was recycled and the membrane was washed with distilled water. If needed red alert staining can be repeated. If necessary, membrane can be put in G machine and the manual photograph can be taken.

Antibody test is the crucial test to see the bond of the protein with the antibody. The basic principle is to attached the correspondent protein with their antibody (primary antibody) and later secondary antibody after incubation for one night. The primary antibodies are generated when a host species or immune cell culture is exposed to protein of interest (or a part of it). In this experiment we used the commercial antibodies. After rinsing the primary antibody with TBST, we applied secondary antibody. Secondary antibody targeted species-specific portion of the primary antibody. Secondary antibody will bind to primary antibody and enhance the signal by using peroxidase. The peroxidase-linked secondary antibody will bind the luminata crescendo and trigger the luminescence in the dark chamber.

Firstly the membrane was cut into three pieces based on the location of HPRT and nucleolin. The membrane was cut near those two protein's bands (approximately 15 kda and 70 kda) and put in different boxes. Membranes were labelled with H4 , HPRT and Nucleolin. The membranes were put slightly in methanol and transfered directly into distilled water Secondly, water was poured out and the membranes were washed 3 times 5 minutes by TBST. After washing step with TBST, the blocking solution was poured and the membranes were incubated for 1 hour. Blocking solution is used to prevent the interaction between the membrane and the antibody. It binds to whole surface of the membrane and make no place for antibody to bind the membrane except for the binding site from the specific target protein. Blocking solution (for 6 ml/10 fold) = 0,6 ml blocking solution (1 fold) + 5,4 ml TBST. After 1 hour, the blocking solution was poured out and replaced by primary antibody, based on its type of membrane. Primary antibodies:

1. Rabbit α-H4ac= 1 : 4000
2. Rabbit α-nucleolin = 1: 1000
3. Rabbit α-HPRT = 1 :500 (Abcam)
4. Mouse α HPRT (Santa Cruz)=1:100 or 1:200
5. Antirabbit antibody (secondary antibody) = 1:4000
6. Antimouse antibody = 1:4000 (Secondary antibody for mouse α HPRT(santa cruz))

The membranes were Incubated overnight on the shaker at 4°C. The day after, antibody's solutions were recycled into their tubes and the membranes were washed by TBST 3 times 10 minutes to remove unbound primary antibody outside the binding site. After the last TBST , secondary antibody (anti rabbit antibody) with concentration 1:4000 was added in the blocking solution (6 ml in total). They were Incubated it for 1 hour. The secondary antibody was poured away and the membranes were washed three times 10 minutes with TBST. The membrane was put in the G-box and poured by Luminata crescendo evenly to every surface of the membrane in order to enhance the signal from

the peroxide in secondary antibody. Using Genesys program and also the G-machine, the band of each protein can be detected. We clicked Auto capture with 1-minute exposure. The membrane's pieceswere taken from the boxes and arranged like the original membrane. the photograph was recorded and saved.

Signal detection and result interpretation

In this last step, the data need to be intepreted to show the overall picture of experiment. We decide to take single experiment as single unit for control group, means that each experiment (with eleven samples) represent one control group's ratio. In this step, we optimize the normalization process. In order to avoid miscalculating because incorrect loading doses in the beginning of experiment, we use the ratio of normalization and observe the difference between the samples from this ratio instead directly from the absorbance values. Since the ratios of acH4 with nucleolin or HPRT are always constant, the calculating step become more reliable than if we only use acH4 data.

Selected pictures of the membrane was opened in the Genesys application. The bands were arranged into given columns so the membrane can be grouped based on the sample that loaded. After the columns fix properly with the band, result was locked and saved. The raw volume of each band will appear in the lower part of monitor. The raw volume appeared into excel array. Raw volume of acH4 were divided by both nucleolin and HPRT to get the ratio of acH4: nucleolin and acH4: HPRT. From this ratios, control group values were selected and mean of them were found. The ratio of nucleolin and HPRT were divided by the mean of control group value to get the normalization ratios for nucleolin and HPRT. Normalized values of nucleolin and HPRT were separated into each group of sample (E.g control with control, MSW+ with MSW+ and so on) and their means were calculated. Finally, the standard deviation from the single value of sample was calculated and put into a diagram. We used the ratio instead of solely acH4 because the normalization tends to be more stable than acH4. Therefore, by making ratio between nucleolin /HPRT with acH4, we can avoid any miscalculating step in loading processes during experiment.

We used the unpaired T-test two tailed as a statistics test because we compared two groups which might contain matching data-points or might not and there is no basis to assume that there may be a significant difference between the groups.

Results

a. *Test of HPRT and nucleolin in prefrontal cortex's whole protein extracts for Western Blot quantification*

We tested two normalization proteins (loading controls) by Western Blot in order to have an internal control for the target proteins (H4) within the samples. Firstly, we tested antibodies against two normalization proteins in this test: nucleolin and HRPT. Nucleolin is a 130 kDa protein that can be found in the region of nucleolus.

Initially we test HPRT's antibody. The samples that we used were whole protein extraction from left and right part of prefrontal cortex of adult animal. We got the result as described in the figure 5.

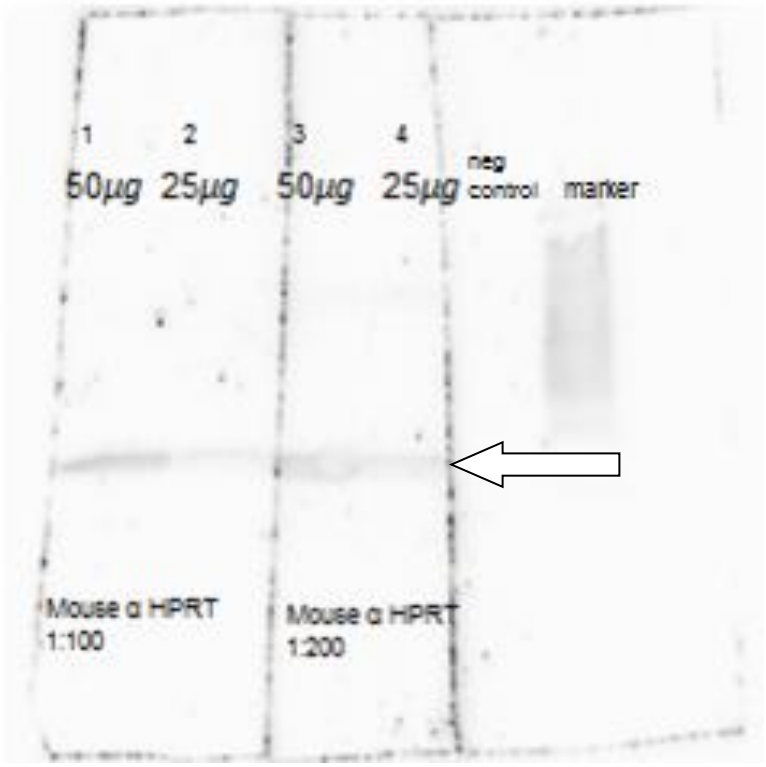

FIG. 5 Blotting test with HPRT's mouse antibody (1:100 and 1;200) from Santa Cruz (HPRT band shown by the arrow) from left part of prefrontal cortex of early life stress C571Bl/6 *mice(PND-100). Each lane represents one sample (from right to the left: 25 µg, 50 µl, 25 µg, 50 µg)(The border between lanes is not so clear in the figure) . The HPRT band is located at approximate 23 kda.*

Secondly, we use the same membrane to test the nucleolin antibody. In histone extraction experiment, nucleolin band did not appear when it tested in adult animal.

But our experiment used whole extraction of the adult animal, prefrontal cortex. We aimed to confirm the presence of nucleolin band in the whole protein extraction's samples and also see whether the antibody work. The result is shown in figure 6

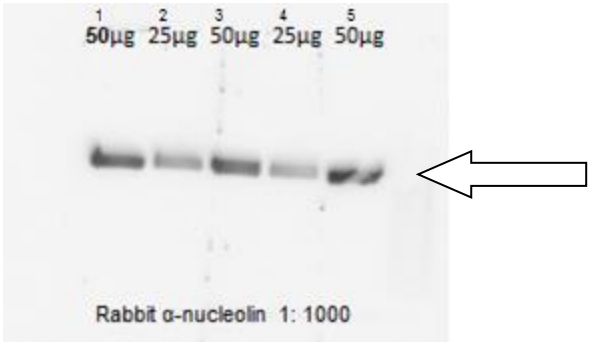

FIG.6 Blott Test with Rabbit alpha nucleolin 1: 1000 (nucleolin band is shown by the arrow) from left part of prefrontal cortex of early life stress C571Bl/6 *mice(PND-100). Each lane represents one sample (from left to right: 50* µg, 25 µg, 50 µg, 25 µg and 50 µg) *The nucleolin band is located at approximate130 kda.*

As shown in figure 6, nucleolin shown a bright band at 130 kDa. It is also shown different intensity in different loading dose. This indicates that signal depending on loaded protein amount. Furthermore, nucleolin has a strong band which makes nucleolin work effectively as normalization protein.

Futhermore, we tested HPRT from Abcam's company and nucleolin together in the same membrane to see the overall view. The result can be seen in figure 7

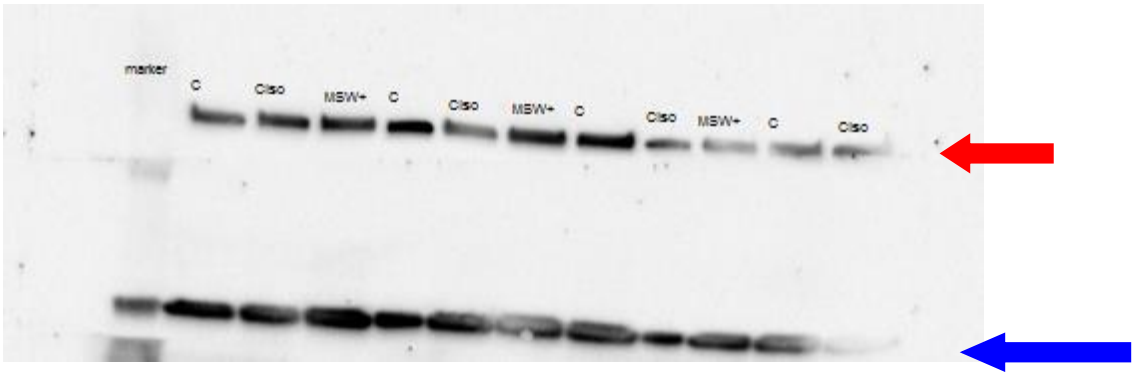

FIG 7. Blot Test with the nucleolin antibody and HPRT antbody from Abcam (which are shown in the correspondence arrows: red arrow = nucleolin, Blue arrow = HPRT) of the left part prefrontal cortex of early life stress C571Bl/6 *mice (PND 100). Each lane represents one sample from total eleven samples.*

b. Application of Western blot in histone H4 acetylation in early life stress mice

Next, we determined acetylated H4 protein of the prefrontal cortex from different groups of mice, twice per sample. Each experiment contained 11 samples. Due to technical problem, we only used two-thirds of total samples.

First blot we tested Control group, Control Isolated group and MSW+ group. In second blot we tested Control group and MSW- group. After applying all steps which have been mentioned in the method, we got the results from the first experiment as follow

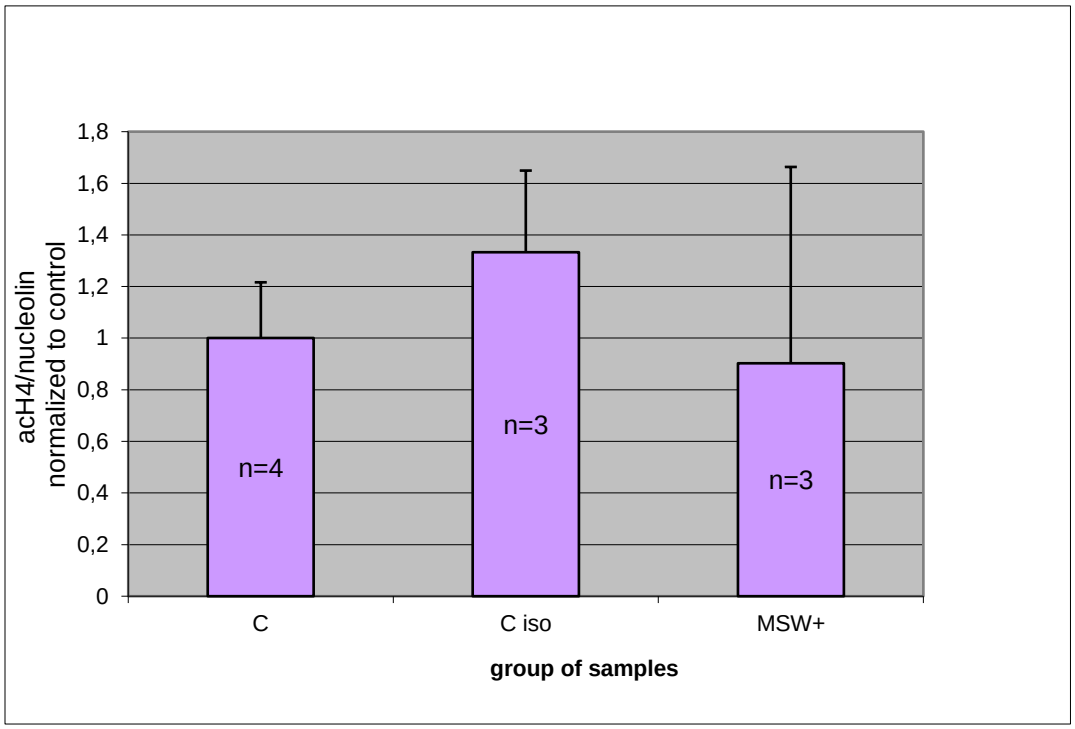

FIG 8. Relative value normalized to nucleolin of control (C), control isolation (C iso)and Maternal Separation with weekend (MSW+) group in Blot one. P value C and C iso =0.15536768. P value C and MSW+ =0.8131649. P value Ciso and MSW+ =0.4167778

	C	C iso	MSW+
acH4/nucleolin normalized to control	1.24637727	1.03035321	0.36672873
	1.08724713	1.30799262	0.56964774
	0.74257397	1.66106363	1.77299686
	0.92380163		

mean	1	1.33313649	0.90312444
sd	0.21632396	0.3161061	0.76013324

TABLE 1. Relative value normalized to nucleolin of control (C), control isolation (C iso) and (MSW+) group with Nucleolin ratio P value C and C iso =0.15536768. P value C and MSW+ =0.8131649. P value Ciso and MSW+ =0.4167778

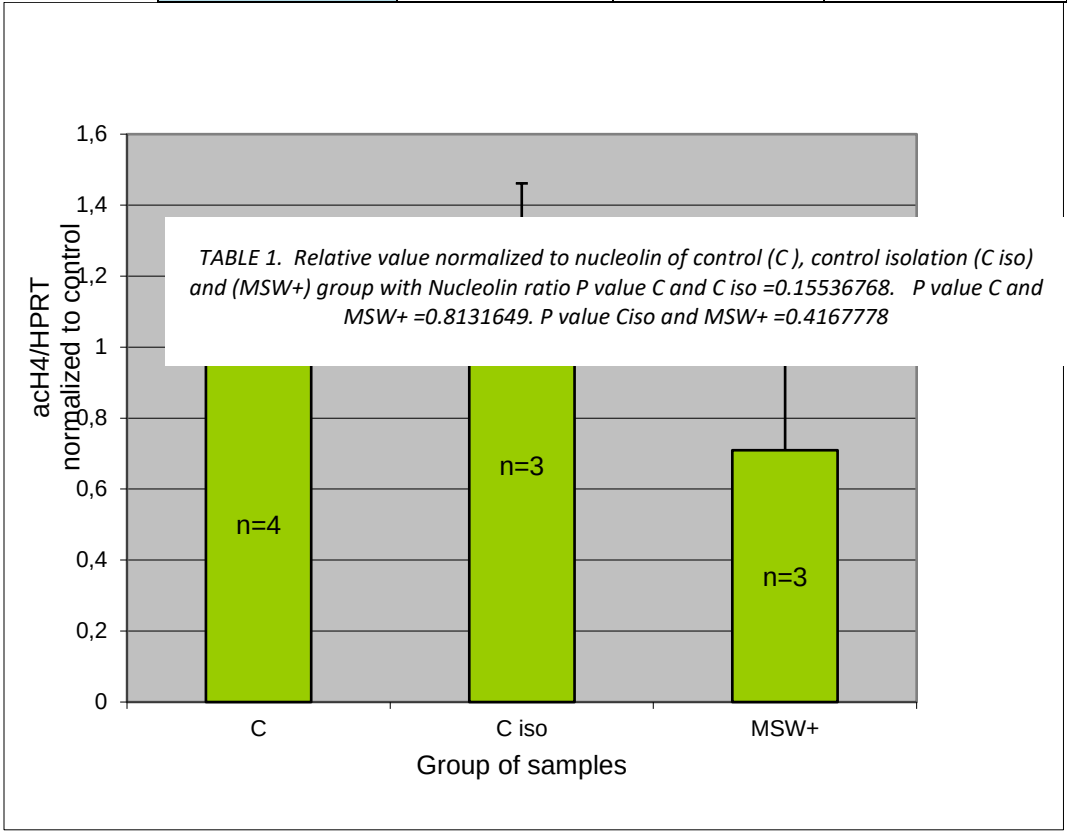

FIG 9. Relative value normalized to HPRT of C , C iso and MSW+ group with HPRT. P value C and Ciso =0.5723426; P value C and MSW+=0,1921924; p value Ciso and MSW+=0,1847495

TABLE 2. Relative value normalized to HPRT of control (C), control isolation (C iso) and Maternal Separation with weekend (MSW+) groupin Blot one. P value C group and Ciso group=0.5723426; Pvalue C and MSW+=0,1921924; p value Ciso and MSW+=0,1847495

The animals for each experiment belong to 1 litter per group. The outcome of two normalization proteins seems similar one to another. This indicates that normalization work perfectly. The Ciso groups tend to increase compare to control group in both experiments (fig 8 and fig 9). In contrast MSW+ groups tend to decrease compare to control group in both experiments (fig 8 and fig 9). The tendency can be observed furthermore in each single value in table 1 and table 2.

We used the unpaired T-test two tailed as a statistics test because we compared two groups which might contain matching data-points or might not and there is no basic to assume that there may be a significant difference between the groups.

	C	C iso	MSW +
acH4/HPRT normalized to control	0.98268451	1.0110412	0.30269285
	1.17260853	0.96814462	0.98477007
	1.01484678	1.20864054	0.84176079
	0.82986018		
mean	1	1.33313649	0.70974124
sd	0.14054835	0.12827308	0.35969321

The big standard deviation of the MSW+ group might be caused by the the samples divided into two main levels which then make the distribution of the data is not concentrated in one level. This can be seen from the figure 8 and table 1 from nucleolin group. The distribution of the samples is spread evenly.

From second blot, we also did the same method but using different samples. In this second blot, we used control (C) and MSW-samples. Both nucleolin and HPRT's results from this second blot are concluded in following graphs and tables.

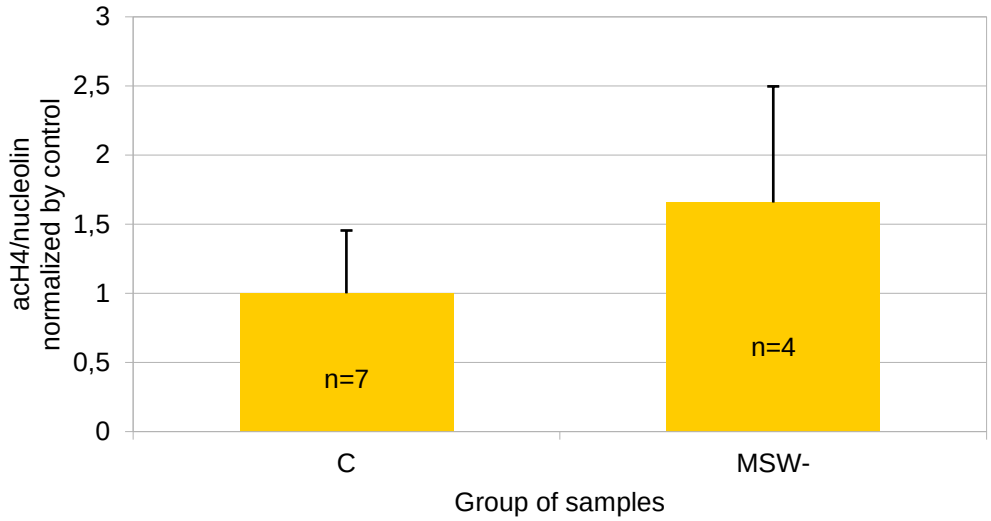

*FIG 10. Relative value normalized to nucleolin of control (C) and MSW- group with Nucleolin. P value C and MSW- =*0.12020115.

*TABLE 3. Relative value normalized to nucleolin of control (C) and maternal separation without weekend (MSW-) group with Nucleolin. P value=*0.12020115. *Normalized ratio of nucleolin was gained from division of the nucleolin ratio and abH4 with the mean of ratio from the control group of the nucleolin ratio*

	C	MSW-
acH4/HPRT normalized to control	1.18600454	2.35133766
	0.45759802	2.6815961
	1.38435614	1.02913858
	1.796129	1.33796263
	1.28760829	
	0.5049065	
	0.38339751	
mean	1	1.85000874
SD	0.55051239	0.7913885

*TABLE 4. Relative value normalized to HPRT of control (C) and (MSW-) group with HPRT. P value=*0.06346306.

	C	MSW-
acH4/nucleolin normalized to control	0.91744902	1.89716229
	0.49937855	2.74252913
	1.16435751	0.96217238
	1.64239913	1.02644864
	1.12509554	
	1.30887168	
	0.34244858	
mean	1	1.65707811
SD	0.45470705	0.83992695

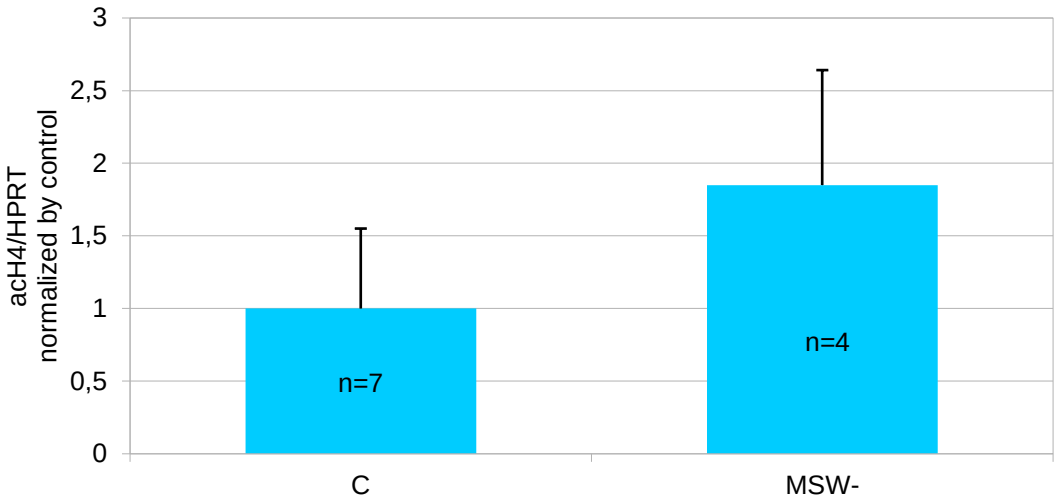

*FIG 11. Relative value normalized to HPRT of control (C) and MSW- group with HPRT. P value C and MSW- =*0.06346306.

In this second blot, number of control group is very large. In contrast, number of control group in first blot is relatively small. We only tested two groups, control group and MSW- group. The tendencies of both experiments (nucleolin and HPRT) in this second blot also remain similar. The MSW- groups tend to increase compared to control group. This is different from MSW+ group in first blot. This might indicate that the MSW- have opposite reaction to the MSW+ although we cannot clearly mention this because both groups are compared to the different control group which come from different litter and have different litter size (C group in first blot n=4; C group in second blot n=7)

- *Test for the litter effect*

However, the variance might also caused by the mother behavior in nursing the pups and also the litter size which are differ from each blot. Therefore, we want to see if there is a difference between litters. We used the the ratio of acH4 and nucleolin of control groups from both experiment as comparison. The data are shown here

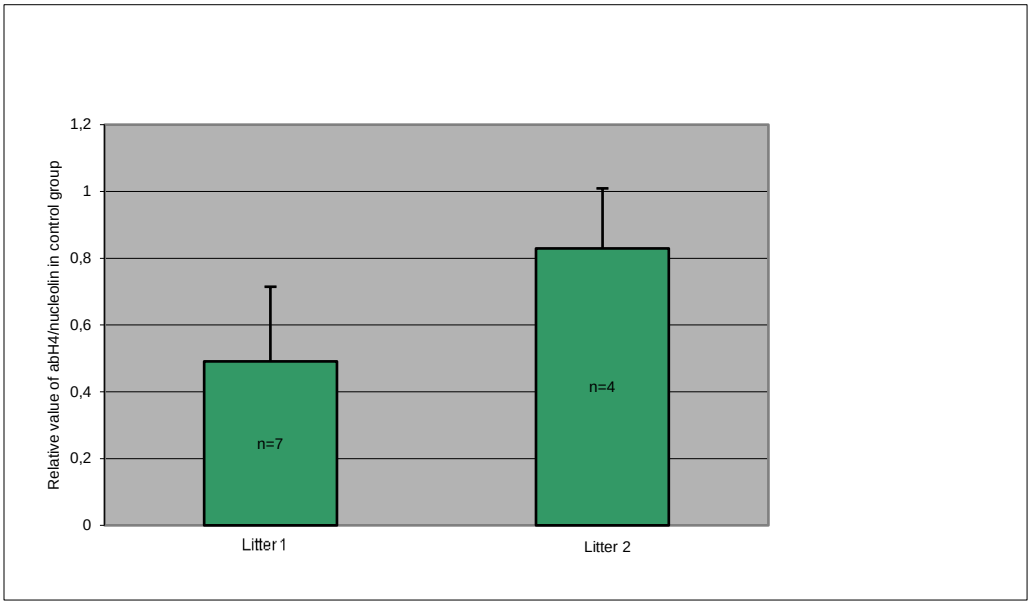

FIG 12. comparison between the ratio of nucleolin in first and second experiment

	First blot	Second blot
acH4/ nucleolin of control group	0.45068755	1.03413235
	0.24531467	0.9021004
	0.57197885	0.61612145
	0.80681197	0.76648795
	0.55269181	
	0.64296998	
	0.1682244	
mean control	0.49123989	0.82971054
sd	0.22337024	0.17948627

TABLE 5. comparison between the ratio of nucleolin of control group in first and second experiment

From the graph we can barely see the evidence of the litter effect. This effect might caused by different aspects including nursing behavior of the mother and also litter size. Therefore we can not compare side by side between nucleolin ratio from blot

one and blot two. The same procedure was also done to HPRT. By taking the ratio value of normalization of HPRT we gain the graph and table as follow

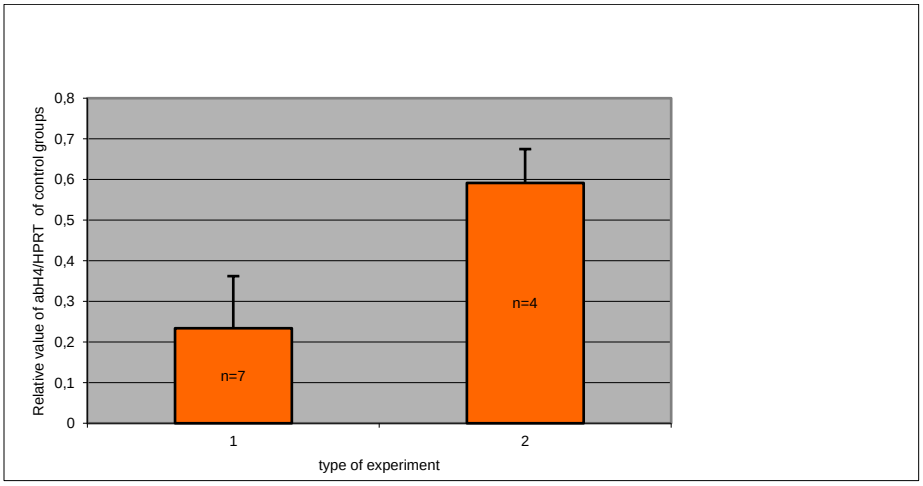

FIG 12. Comparison between the ratio of HPRT of control group in first and second experiment.

TABLE 6. comparison between the ratio of HPRT of control group in first and second experiment

Ratio of acH4/HPRT of control group	0.1068632	0.6937361
	0.32328971	0.60040144
	0.41945133	0.49096007
	0.30069611	
	0.11791119	
	0.0895351	
mean control HPRT	0.23353074	0.59161782
sd	0.12856156	0.08315091

This data, similar to table 5, also shows comparison between acH4/HPRT in control group in two blots. The graphs show the same tendency as nucleolin group in fig.11. In HPRT, the difference between acH4/HPRT ratio of control group is immensely high. This result indicate high variability between two litter. The unbalance distribution of samples between two blots may responsible on this difference, as well in nucleolin comparison in figure 11. First blot had more control groups than second blot. The litter size might play the important role in this difference, but the maternal behavior should not be forgotten as well.

Discussion

Application of Western blot in histone H4 acetylation in early life stress mice

Overall result showed that H4 acetylation is regulated by different type of stresses in Prefrontal cortex (PFC) of the mice. The PFC is a region of the brain involved in higher-order cognitive processes and emotional regulation. It undergoes extensive maturation during childhood and adolescence, making it particularly vulnerable to the effects of early life stress. Animal and human studies have shown that Early life stress can induce structural and functional alterations in the PFC, including changes in neuronal morphology, synaptic connectivity, and neurotransmitter systems [25-27]. These alterations contribute to deficits in PFC-dependent functions, such as working memory, impulse control, and emotion regulation, which are frequently observed in individuals exposed to early life stress[25, 28]. Consequently, investigating the molecular mechanisms that modulate PFC plasticity in response to early life stress is crucial for understanding the etiology of associated behavioral and cognitive impairments.

The control isolated (C iso) group shows higher amount of histone acetylation compared to control group (Fig 8 and Fig 9). These two groups (C group and C iso group) have similarity because both of them are not separated from their mother until PND 21. The difference is only in the isolation chamber in the control isolated group after PND 21. This isolation chamber might be a first and stressful experience for the pups. This method has similarity with acute stress method where the animal for the first time exposed to the stress. This result supports the conclusion from Xie's (2013) who said that the maternal separation can increase the acetylation of H4 in acute stressed animal[24].

In other hand, MSW- group tends to increase compared to control group (Fig 8 and Fig 9). It is contradicting to MSW+ (Fig 8 and Fig 9). It might related with predictable and unpredictable stress. In this case predictable stress was represented by MSW+ group and unpredictable stress was represented by MSW- group. As mentioned, the separation of the pups from its mother in MSW- group happens within 5 days, and the rest 2 days, they will be let together in their mother's cage. Maternal separation during early postnatal development in mice can result in enduring behavioral impairments, encompassing anxiety and depression-like behaviors. The effects are frequently intensified when coupled with additional stressors, such as maternal stress, and can be passed to later generations via non-genomic pathways[29, 30]. Extended parental

separation notably heightens vulnerability to depression-like behaviors when mice encounter stress in adulthood[31, 32]. These two undisturbed days might cause the animals cannot predict when they will be picked up from the cage. Every new week, they are taken from their cage to the isolated cage, which might be a relatively new environment to them since they have two days free of disturbance. Considering those pups always in the save environment with their mother and other pups, the isolation chamber which have no family, might be associated as unsafe condition by the pups which trigger the stress level. This result is contradicting with Xie (2013) who states that h4 acetylation increases after acute stress. Xie also mention that the corticosterone level is increase after unpredictable acute stress. It is obvious that time of stress exposure play the pivotal role in this difference[24].

Furthermore, the MSW+ tends to decrease (Fig 8 and Fig 9) and the experiment with MSW- tends to increase (Fig 10 and Fig 11). The MSW+ group are the group of animals that separated regularly also in the week end. This daily activity might lead the animals realize that they will soon re-unite with their mother couple hours again like they supposed to be every day. This result contradicts with Xie (2013) experiment which shown the significant increase in acetylated H4 in stress animal[24]. However, Xie focused on acute mechanism of stress and our experiment held based on chronic stress paradigm. On the other side, our result supports the experiment from Ferland (2011) that mentioned the chronic stress significantly decrease the acetylation of histone 4 (H4) at Lys12 in CA3 and Dentate gyrus of Chronic variable stress animals compared to control animals[33]. Although Ferland did the experiment on the chronic stress, the method was not based on maternal separation but more physical-induced stress. Moreover, the difference within MSW+ and MSW- group make us speculate that unpredictable stress and predictable stress seems different in term of their effect to H4 acetylation

However, the variance of two control groups is relatively high (Fig 10 and Fig 11). We can easily see the tendency from each control from each experiment. This variability might come from the difference of litter size. The first control groups have 7 samples and the second control groups have 4 samples. The other possibility is the maternal behavior that differs among those two experiments. Mother's behavior like nursing and licking might differs between litters. Nevertheless, the comparison between two control groups make us assume that the difference between those two controls exist.

To sum up, this experiment was made to see whether there is any change in prefrontal cortex of histone acetylation after various type of stress in the early life development. From the result we speculate that stress might increase the amount of histone

modification. On the other hand, we also speculate routine and repetitive stress environment might also lead the habituation behavior, a compensation mechanism to overcome stress. Though out this experiment, we get the first idea that H4 acetylation is regulated by different type of stresses in prefrontal cortex in mice. However, this experiment had several weaknesses. Firstly, the technical problem due to pipetting may make some samples are mixed during experiment. Those make the result less reliable. Secondly, the membranes that we used were not homogenous due to defect on its surface. This may lead the distribution of antibodies and other substrates were not very diffuse which eventually affect the final measurement.

Conclusion

We tested HPRT and nucleolin antibody to validate them. Consequently, we applied the method for acH4 quantification and found the same trend for both of normalization proteins. And we finally, we indicate the first evidence for acH4 regulation by testing the method to the early life stress mice.

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