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EFFECT OF PRE-NATAL INTERMITTENT MONOTONOUS AND RHYTHMIC AUDITORY EXPOSURES ON BRAINSTEM NUCLEI PLASTICITY OF ONE DAY-OLD CHICK

Pengaruh Paparan Pendengaran Monotonous Dan Ritmis Intermiten saat Prenatal terhadap Plastisitas Nukleus Batang Otak Ayam Usia Satu Hari

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ABSTRACT

Environmental auditory exposure in a form of both enrichment and stressor environmental can differently modulate the development of auditory system Prenatal chronic noise exposure caused neurogenesis and neuroplasticity disorders in the brain's auditory pathway resulting in neurocognitive impairment. Meanwhile exposure to prenatal music positively modifies morphological and biochemical developments in the brain's auditory pathway that supports neurogenesis and neuroplasticity. This study aimed to determine the plasticity of brainstem nuclei after exposure to music, noise, and a combination of both. This research used an experimental method using 24 female chicks as subjects. Subjects were obtained from eggs after incubation, which were divided into 4 groups: control, music, noise, and combined noise and music. Brain stem nuclear plasticity was measured by total number of nuclei, neuronal nuclear area, and synaptophysin expression as parameters. Prenatal music exposure significantly increased the total number of neurons, neuronal nuclear area, and synaptophysin expression in brain stem nuclei ($p < 0.001$), whereas combined and noise exposure significantly decreased these three plasticity parameters ($p < 0.001$). In conclusion, prenatal music exposure potential to increase neuroplasticity of brainstem nuclei for better neurocognitive function

KEYWORDS:

Pre-Natal, Chronic, Music Auditory Enrichment, Noise Auditory Stress, Synaptophysin, Brainstem Nuclei

ABSTRAK

Paparan pendengaran lingkungan dalam bentuk enrichment dan stresor lingkungan dapat memodulasi perkembangan sistem pendengaran secara berbeda. Paparan kebisingan kronis prenatal menyebabkan gangguan neurogenesis dan neuroplastisitas pada jalur pendengaran otak sehingga mengakibatkan gangguan neurokognitif. Sementara itu, paparan musik prenatal secara positif mengubah perkembangan morfologi dan biokimia pada jalur pendengaran otak yang mendukung neurogenesis dan neuroplastisitas. Penelitian ini bertujuan mengetahui plastisitas nucleus batang otak setelah paparan musik, kebisingan, dan kombinasi keduanya. Penelitian ini menggunakan metode eksperimen dengan menggunakan 24 ekor anak ayam betina sebagai subjeknya. Subjek diperoleh dari telur setelah diinkubasi, yang terbagi dalam 4 kelompok: kontrol, musik, kebisingan, dan gabungan kebisingan dan musik. Plastisitas nukleus batang otak diukur dengan parameter jumah total nukleus, luas neuronal nuclear area, dan ekpresi synaptophysin. Paparan musik prenatal meningkatkan jumlah total neuron, luas neuronal nuclear area, dan ekspresi synaptophysin di inti batang otak secara signifikan ($p< 0,001$), sedangkan paparan bising dan kombinasi secara signifikan menurunkan ketiga parameter plastisitas tersebut ($p < 0.001$). Kesimpulannya, paparan musik prenatal berpotensi meningkatkan neuroplastisitas inti batang otak untuk fungsi neurokognitif yang lebih baik

KATA KUNCI:

Pre-Natal, Kronik, Enrichment Pendengaran dengan Musik, Stres Pendengaran dengan Bising, Synaptophysin, Nukleus Batang Otak

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BACKGROUND

Pre-natal period is a sensitive period since environmental stimuli interact with genes in influencing the formation and refinement of neural connectivity of the developing brain (Linden *et al.,* 2003). Auditory stimulation in a form of both

environmental enrichment, such as music, and stressor, such as noise, can differently modulate the development of auditory system and hippocampus. Both auditory system and hippocampus as its higher order integration of sensory input have direct and indirect connections (Kirste *et al,* 2015). In previous study noise disturb balance because increased excitation and decrease inhibition. Noise exposure also decreased expression of synaptic stability protein. This condition will delay development and maturation of ACx that alter auditory-associated behavior. Noise exposure induce reduction in synaptophysin expression can alter synaptogenesis, decreased expression of PSD 95 that alter excitatory postsynaptic complex, morphology of spine dendritic, synaptogenesis, AMPAR function. Decreased expression gephyrin that alter inhibitory postsynaptic complex, receptors clustering of synaptic (Kumar *et al*., 2014). Synapses are thus of primary importance in understanding the basis of such improved behavioral and morphological correlates. Increased expression of synaptic proteins also reflects synaptic strength, which depends upon target contact and synaptic activity (Garner *et al.,* 2002). one of important synaptic protein is synaptophysin. It is a major integral glycoprotein membrane protein which has weight 38-kDa. Expression of synaptophysin occurs prior as well as parallel to the formation of synapses, and is considered as a marker of synaptogenesis (Plunkett *et al.,* 1998). In the present study, the effect of prenatal combined chronic auditory exposure expression of synaptic proteins in the chick brainstem auditory nuclei nucleus magnocellularis (NM) and nucleus laminaris (NL) during development and at hatching was assessed.

In mammals, fetus may directly elicit stress response to the external stressor. Furthermore, mother's stress hormones may pass through the placenta, followed by placental stress response (Canlon *et al*., 2003). In order to focus on examining the impact of pre-natal chronic auditory stress to fetal brain development directly especially, the confounding factors from mother and placental stress response should be eliminated. One way to do this is by doing research to animals other than mammals, such as in fertilized chicken eggs. It is noteworthy that both chicks and humans are precocious i.e., they can hear extraneous sounds during embryonic/fetal stage and the ability to memorize the auditory cues is well developed in avian as it is in humans (Kumar *et al*., 2014). In natural environment, chicks are attracted to those auditory stimuli that are segmented, repetitive and have short component note ability to perceive musical contents is quite similiar to humans. Their ability to distinguish different styles and rhythms is not elementary rather well refined and sophisticated, which suggests that there is great

similarity in the way the birds and humans hear music (Kauser *et al.,* 2011; Alladi a *et al.,* 2005). As an embryo that independent, fertilized egg of chicken provide an excellent system to study the effects of prenatal acoustic exposure.

METHODS

Subject and Incubation

Isa Brown domestic chicks (*Gallus gallus domesticus*) were used as the experimental model. Fertilized eggs day 0 of healthy chicks, weighing 50–60 g, were obtained from PT Sierad Tbk., and incubated in a double insulated egg incubator. Temperature controlled incubator of 37 ± 1° C and humidity 70% ± 2% was used for incubating the eggs. Tilting of eggs was automatically controlled for 4 times a day and a photoperiodicity of 12:12 h day and night cycle was maintained in 21 days incubation period. The incubator has a forced draft of air for aeration. On day 9.5 of incubation to make access to sound, a part of the eggshell removed keeping the membranes intact at the animal pole over the air sac approximately 2-5 mm size. The fertilized eggs were given prenatal music stimuli through speakers built in the incubator. The sound exposures were for 15 minutes per hour, over a period of 24 hours (total 6 hours per day) from the embryonic day 10 (E10) until hatching.

Experimental Group

The protocol and number of animals used in the experiment was approved by Health Research Ethics Committee of Medical Faculty Universitas Indonesia permit number 142/UN2.Fl/ETIK/2016. The eggs were divided into following four groups. control, music, noise, and combination (noise and music). Control group would not get sound stimulation addition. Second group called music embryos were given auditory stimulation by classical music "Eine Kleine Nachtmusik Serenade in G major No. 13 K. 525 - I. Allegro" within a frequency range of 100– 6300 Hz at 110 dB SPL. The third group got auditory stimulation by noise from recorded sound of meat milling machine in the market. within a frequency range of 100– 6300 Hz at 110 dB SPL. And the last group got combined auditory stimulation between noise and music. The sound exposures were for 15 minutes per hour, over a period of 24 hours (total 6 hours per day) from the embryonic day 10 (E10) until hatching. Except combined group will get 15 minutes noise and 15 minutes music with 15 minutes respectively.

Tissue collecting and Processing

The chicks post hacth 1 from all of group were anesthetized with anesthetic ketamine 40 mg/kg weight and sacrificed by decapitation. Brains were dissected out, the brainstem cut and fixed in 10% normal buffer formalin for 3 days. The next step was dehydration into stratified alcohol to allow the water in the tissues to be removed and using xelene to clearing. Then the tissue is inserted into the liquid paraplast and in the final embeded in to

paraffin blocks. Paraffin blocks cut coronal sections of 5 µm thickness. The section stained by cresyl violet and immunohistochemistry to localize the synaptic proteins synaptophysin.

RESULT

The Total Number of Neurons

The total number of neurons (mean±SD) of the individual brainstem auditory nuclei, *nucleus magnocellularis* (NM) and *nucleus laminaris* (NL) in control, music, noise, and combined group are shown in Table 1. The difference in neuron number among the four groups in the auditory nuclei was significant, when statistically compared with one way ANOVA. Bonferroni test showed that the total number of neurons was increased significantly in both auditory nuclei (NM, $p \leq 0.001$ and NL, $p \leq 0.001$) in the music stimulated group, as compared to the control, noise, and combined group, while it was decreased significantly ($p \leq 0.001$). There was no difference between noise and combined group in number of neurons both of nuclei brainstem NM ($p = 0.565$), and NL ($p = 0.175$) Figure of cresyl violet staining observed in figure 1 for NM and figure 2 for NL. Table 1 showed difference in neuron number both of brainstem nuclei among the group

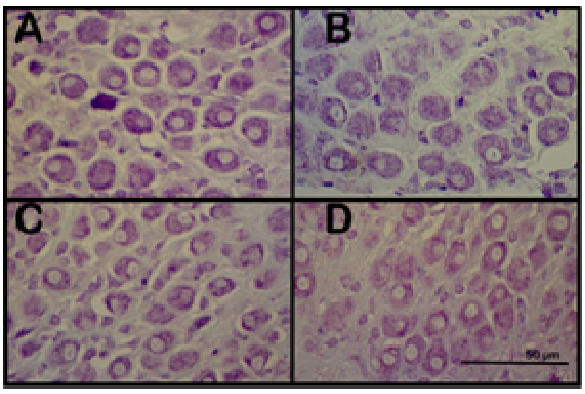

Figure 1. Cresyl violet staining 40x of *nucleus magnocellularis* (NM) A-D control, music, noise, combined

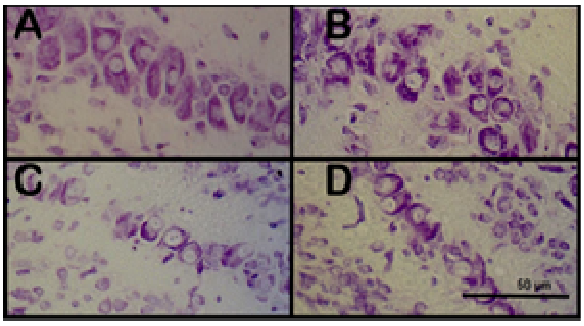

Figure 2. Cresyl violet staining 40x of *nucleus laminaris* (NL) A-D control, music, noise, combined

The Neuronal Nuclear Area (in µm^2)

The mean neuronal nuclear area of NM and NL in four group are shown in table 1. Mean neuronal nuclear area of NM statistical analysis with one way ANOVA showed significant difference. a significant increase in mean neuronal nuclear area of NM was observed in the music group, ($p<$ 0,001) meanwhile in noise and combined group decrease ($p<$ 0,001). In NL, the mean neuronal nuclear area statistical analysis with one way ANOVA showed significant difference ($p \leq 0.001$). Post hoc analysis with Bonferroni test showed a significant increase of nuclear area in the music stimulated group as compared to the control, noise, and combined group. ($p \leq 0.001$). There was no difference between control and combined group in

number of neurons both of nuclei brainstem NM (p= 0.484), and NL (p= 1.000). Meanwhile noise group became the smallest one (p< 0,001). Table 1 showed difference in neuronal nuclear area both of brainstem nuclei among the group.

Table 1. Data of All Group Showing Neuron Number and Neuronal Nuclear Area (μm^2)

Group	Total Neuron Number		Neuronal Nuclear Area	
	Nucleus Magnocellularis	Nucleus Laminaris	Nucleus Magnocellularis	Nucleus Laminaris
Control	374.5 ± 11.66	140.5 ± 8.31	51.66 ± 1.34	47.08 ± 1.05
Music	445.17 ± 18.09	161.5 ± 4.46	59.42 ± 1.44	55.49 ± 0.93
noise	303.5 ± 7.87	112.5 ± 6.47	42.64 ± 1.52	39.74 ± 1.19
Combined	316.67 ± 12.17	122.17 ± 8.49	48.35 ± 1.85	47.09 ± 1.44

Notes: One way ANOVA of both neuron number among and mean neuronal nuclear area of four groups were same, p< 0,001.

Quantitation of Synaptophysin Immunoreactivity

The difference in the staining intensity between the sections among the group was assessed using Image J with IHC profiler plug in. Image analysis of immunostaining sections of control embryos, in both NM as well as NL, showed an increase in synaptophysin immunoreactivity in music group. statistical analysis with one way ANOVA showed significant difference (p< 0.001). and significant decrease in noise group (p< 0.001). There was no significant difference between control group and combined group (p= 137). Figure 3 and 4 are the NM and NL synaptophysin immunohistochemistry staining respectively. Figure 5 is histogram optical density synaptophysin immunohistochemistry on both brainstem nuclei.

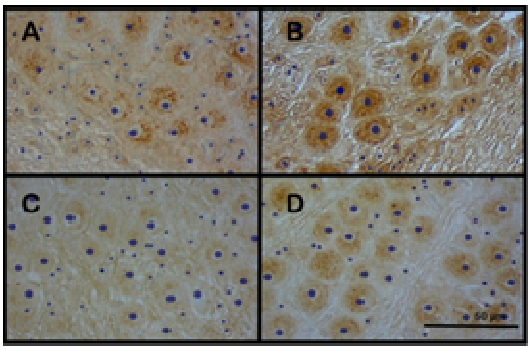

Figure 3. Immunohistochemistry staining showed immunoreactivity to synaptophysin 40x of NM. A-D control, music, noise, combined. The music group has the highest synaptophysin immunoreactivity.

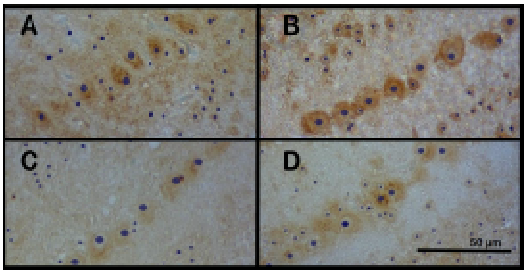

Figure 4. Immunohistochemistry staining showed immunoreactivity to synaptophysin 40x of NL. A-D control, music, noise, combined. The music group has the highest synaptophysin immunoreactivity.

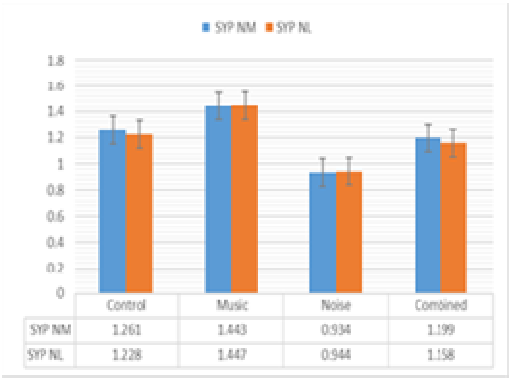

Figure 5. Histogram optical density immunohistochemistry of synaptophysin on both brainstem nuclei.

DISSCUSION

Previous studies imply positive modulation of prenatal music exposure in developmental brain morphology, biochemistry, and behavior. Chronic high decibel noise and music exposures prenatal have different effect on synaptic component of auditory cortex. The study proposed opposite effect of music and noise wherever in music group expression of synaptophysin, PSD-95, and gephyrin increase as synaptic stability that influenced development and maturation of ACx (Kumar *et al.,* 2014).

Expresion of c-Fos and c-Jun in chick brainstem that induced by prenatal music exposure has been reported by Alladi b *et al.* (2005). Under normal conditions c-Fos and c-Jun expression in nucleus magnocellularis and nucleus laminaris was developmentally up-regulated and expression of both the proteins remained higher particularly during the period of cell death. Meanwhile the auditory stimulated groups, c-Fos expression were elevated while c-Jun showed an earlier reduction compared to controls. This opposing pattern of c-Fos and c-Jun expression in response to sound stimulation is indication of cell survival (Alladi b *et al.,* 2005). The other study told effect prenatal auditory enrichment to alter programed cells dead in developing nucleus of chick brainstem. This study noted a peak percentage in the TUNEL-positive cells at embryonic day 12 than reduced at day 16. It noted expression Bcl 2 which higher than control in contrast Bax expression reduced. This study appears to support cell survival (Alladi c *et al.,* 2004).

The chronic effects of sound exposure over the entire developmental period could be studied. This factor of chronicity is important because mild to moderate stress may trigger adaptive mechanism. However, chronic stress gradually cause fatigue and destructive changes, such as over activity of Hypothalamus-Pituitary-Adrenal (HPA) axis or impairment of regulatory function of HPA axis, overproduction of excitatory neurotransmitter and attenuation of control by GABA-ergic inhibitory neurotransmitter (Rodríguez-Manzanares *et al.,* 2005). As we observed in number of neuron combined group there was no significant difference if compare with noise group. This result caused by this may occur due to the glucocorticoid effect. Glucocorticoids are essential for the maturation of the brain in an appropriate amount of need because too little or too much glucocorticoid levels can be bad in brain development. Previous research reports suggest that the administration of synthetic glucocorticoids in pregnant mice can lead several problems: maturation delays in neurons, myelinization processes, glial cell formation and the formation of new blood vessels (Meyer, 1983).

Noisy exposure may increase excessive glucorticoid levels and will not be neutralized by

exposure to music afterwards. Increased levels of glucocorticoids inhibit neurogenesis which results in a decrease in the number of neurons. The size neuronal nuclear area shows transcriptional activity in protein synthesis that may support survival and differentiation of new neurons. Such transcriptional activity supports the development of brainstem auditory nuclei in the early phase of development (Meyer, 1983*)*. Prenatal auditory exposure using music has been reported increased synthesis synaptophysin and syntaxin 1 in chick brainstem auditory nuclei. This condition beneficially enhances positive effect to develop auditory system (Alladi a *et al.,* 2002). In this study we found that there was a significant difference between the mean optical density among the group that immune-positive to synaptophysin. Music group has the highest optical density while the noise group has the smallest optical density. There was a significant difference in the combined group with the music and noise groups but there was no significant difference with the control group. This is in accordance with previous studies of auditory exposure to music increases the value of optical density against synaptophysin and vice versa noise exposure could decrease optical density in different parts of the brain. While optical density in the combined exposure group showed improvement. That may be due to exposure to music after exposure to noise because there is a significant difference with the noise group. This suggests that exposure to music increases the level of synaptophysin associated with an increase in the number of synaptic vesicles as well as the formation of a new axon terminal (Chaudhury and Wadhwa, 2009; Chaudhury *et al.,* 2009).

CONCLUSION

In conclusion, prenatal exposure to music improve the neuroplasticity of brainstem nuclei of the chicks. Neuroplasticity in brainstem is very important because it is the auditory pathway that delivers afferent stimuli to another region in the brain. Although could not prevent neuron cell apoptosis in early development.

ACKNOWLEDGEMENT

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