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Peer-Social Network Development Revealed by the Brain Multivariate Correlation Map with 10 Monoamines and 11 Behaviors

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Abstract

Psychiatric disorders induced by social stress have suggested that monoamines play key roles in a whole brain network. To research an environmentally dependent neural network development of social psychology, we report our model animal study of peer-social behavior learning by screening the relevant monoamines in various brain regions in the light of social brain network. We have originally developed psycho-biological quantification batteries based on multivariate analysis over species, including the current subjects, domestic chicks (*Gallus gallus*). The chicks' peer-social environment was regulated as grouped and isolated from just after hatching. After two weeks, their acquired behavioral features were examined in a social meeting with unfamiliar peers and then, quantified 10 kinds of monoamines in their 13 brain regions at their steady state under off lighted conditions in day-time. The whole brain concentration map visualized regional difference of 10 monoamines. Furthermore, we attempted to map behavioral feature correlation with each monoamine in the whole 13 brain regions. These results showed that isolated chicks expressed (1) significantly higher Dopamine (DA) of left caudal forebrain, (2) moderately higher Norepinephrine (NE) of thalamus-midbrain correlating with immobile and alert behavior. While 3-methoxy-4-hydroxyphenylglycol (MHPG) as well as Normetanephrine (NM) correlated with alert-immobile behavioral feature, Homovanillic Acid (HVA) seemed to weekly correlate with active-affinity behavior. In addition, there existed lateral asymmetry of the correlation generally as left-alert and right-affinity in the lateral part of the brain. These correlations between social behavior and monoamines would possibly contribute to comprehend the developmental mechanism of social brain networks. These findings open a new way in constituting a functional map by multivariate analysis of social behavior and monoamines and their metabolites.

Keywords: Monoamine; Catecholamine; Serotonin; Social disability; Emotion; Memory; Developmental psychiatry; Molecular psychology; Peer interaction

Introduction

Social disabilities are the central symptoms in any psychiatric disorder. Clinical neuropharmacology [1-3] and various molecular researches have comprised a convincing evidence about critical role of monoaminergic neurons in socio-psychological function [4-9]. The neural circuits of limbic system and cortico-limbic system are neural substrates for physiological responses, behavior output, and socio-emotional subjective feeling [10-12], which are supposed to develop as a consequence of continuous adjustment of sensory-motor, emotional and cognitive systems in nervous systems during learning processes [9,13-16]. A deficiency of social experiences in childhood tends to induce severe underdevelopment in monoaminergic neural circuits because of the least experience of the learning [5,17,18]. Although we have much progress in the understanding of kinship interaction and pair bonding mechanisms [7,19], neurobiological mechanism of peer social interaction development is still uncertain. Domestic chicks have been used as an excellent model of social affiliation development since it is a precocial bird which is able to feed by itself from just after hatching and their very early neonatal experience or learning can be manipulated and evaluated [10-12,20-24]. We here report our domestic chick study of peer social behavior learning and the role of monoamines in this process. The goal of this study is an understanding of a social brain map in terms of monoamines levels and their correlation with social behaviors. The correlation mapping is widely used to reveal the inherent complexity of brain as a whole rather than just its individual parts, for example, a functional correlation of the volumetric change in longitudinal MRI study [25,26], task-related cingulated cortex sub-regions by an fMRI study [27], tractography and a resting state functional connectivity combined with graph-theory [28,29]. The social brain map described in this study is based on a multivariate analysis using correlation matrix [30,31] with eleven behavior parameters expressed at social meeting test with unfamiliar subjects and a particular monoamine with a total ten monoamines and their metabolites measured with each sub-region with a total thirteen brain regions from two experimental groups, a socially isolated group and an affiliated group. The statistical significance of the correlation was mapped in sub-regions, which revealed the region-specific modulation of monoamines.

Materials and Methods

Animals

This experimental protocol was approved by the Ethics Review Committee for Animal Experiments of Tokyo University of Agriculture and Technology, TUAT (19-19) that regulates animal care and experimental guidelines of the Japan Neuroscience Society and NIH in USA. Fertilized eggs from domestic chicks (*Gallus gallus domestics*), White Leghorn, Maria strain, were purchased from a local breeder, GHEN Corporation located in Gifu, Japan. They were incubated, and

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hatched as described [32]. Two rearing conditions were set, grouped (Grp, n=6) and isolated (Iso, n=6). As Iso raised condition, each chick was isolated in a sound attenuated incubator surrounded by opaque walls with air circulation (230-270×250-270×220-300 in mm). Iso birds were regarded as peer-social deficit models without any visual or auditory interaction. To minimize compared conditions, we observed only females. Other conditions were essentially the same as described in [32]. The overview of experimental design is shown in Figure 1a.

Behavioral test

A behavioral test per subject was once conducted within 11:00 a.m. and 3:00 p.m. hours on P13 in the different sound proof room from the raised room. The details of the test conditions were essentially the same as described in [32]. The subject chicks underwent the following four serial peer-social contexts: context 1; initial isolation period with no reference chicks, and a masking board in place, context 2; presented with acoustic only cues, ensured by a separation board, context 3; the reference chicks were presented with both visual and acoustic cues, after removing the separation board, context 4 (final); second period of isolation. Each context lasted for 2 minutes. All behavior was recorded using a top video camera (DVD VIDEO CAMERA NTSC-DC40 (Canon)) with an external microphone in the test box.

Behavioral analysis

The recorded WMV files were transferred into WAVE and JPEG files per second using TMPGEnc-4.0XPress software Version4.6.3.268 (Pegasys Inc., Tokyo). The subject's behavior in context 3 was analyzed for this study. In the 10 parameters of Figure 1b, the x, y coordinate of head centre and forehead (in most cases, beak head) position (Figure 1c) were sampled and used to calculate horizontal Velocity (V), head horizontal angle, its rotation velocity (delta Theta) and local preference to define social distance (four equally divided areas, Figure 1d) by Excel (Microsoft, USA). The parameter "facing to peers" was defined as the angle between the beak direction and the perpendicular line to the separating cage wall within 45 degrees (beak-to-separating wall), in reference to where the chicks were placed. We further defined the pecking cage wall (pk-wall) expressed as the frequency of pecking per specified time. Chick calls were classified into four types (Figure 1e) by spectrogram as described in [32].

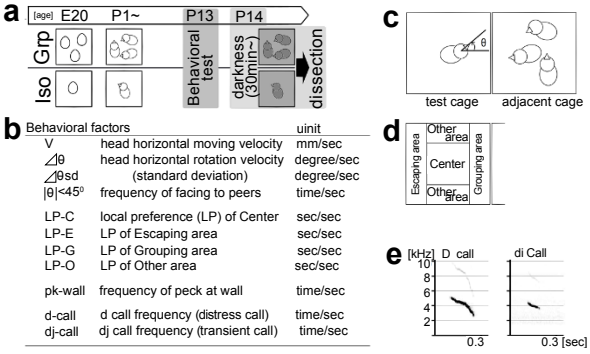

Figure 1: Experimental designs and quantified parameters from behavioral test.

a. The experimental procedure for comparison between group-reared chicks (Grp) and socially isolated chicks (Iso) (see details in Materials and Methods). The age, embryonic day 21 (E21) is usually the day of postnatal day 1 (P1)

b. The list of behavioral factors extracted from the video data during behavior test. The definition of each behavior factor and the test condition were described in detail under Materials and Methods

c. Social meeting test of a subject (left) and three reference peers which were unfamiliar to the test subject (right). The test comprised of four contexts (2min each) and only third context (visually and acoustically interactive) was illustrated here and the behavior data during the context was used for further analysis

d. The definition scheme of local preference (LP-C, E, G and O) was illustrated, which related with personal distance [33,34] and emotional/cognitive response to other environmental condition

e. Each spectrogram example of two kinds of vocal calls: a distress call and an intermediate call. The detailed definition was described in Materials and Methods

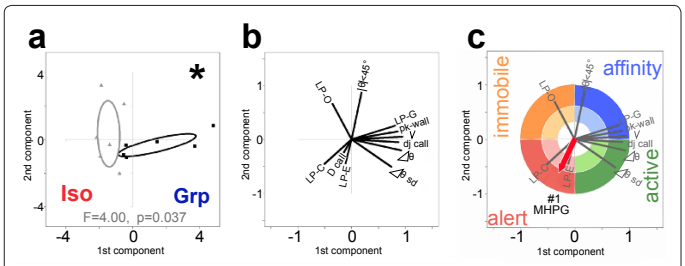

Figure 2: The multivariate analysis of eleven behavioral factors and the correlation analysis with behaviors and a monoamine concentration in a brain sub-region.

a. The data distribution of Grp (right) and Iso (left) behavior during the behavior test in the context 3. Two experimental chick groups were prepared and tested with social interaction behavior at postnatal day 13. The behavior factors were extracted from the video recording, quantified, and the feature vectors of the behavior were computed by principal components analysis as described in Materials and Methods. The principal scores of each subject was plotted in a plane, x axis is the first component score and y axis is the second component score. The plot was assigned to each group and the distribution of the plots (n=6) in either group was demarcated by variance ellipse (asterisk: significantly different by Wilks' lambda with the F and p values)

b. The eleven factor loading vectors in the projected plane a. The factor loading vector was calculated as the correlation of behavior eigen vector with the first or second component of principal components analysis giving the value between -1 and +1 (0 means no correlation) after normalization by eigen value of each component. Only positive value was illustrated in the panel

c. Illustration of four emotional quadrants in the feature space of the behavior. The clustering of the factor loading vectors and emotional expression with each behavior gave four clusters in the feature space and we labeled each cluster by emotional behavior, like active (green), immobile (orange), affinity (blue), and alert (red). The quadrant border was arbitrarily assigned. The correlation of a monoamine (in this case MHPG, a metabolite of norepinephrine) in a brain sub-region (in this case #1, left lateral frontal, Figure 3f) with behaviors was based on Pearson product-moment correlation coefficient (R) computed using the monoamine concentration and the first component score or second component score of each subject, which was derived by PCA of behavior factors (Fig.2a). Thus obtained monoamine vector was color coded according to the location of the vector in four emotional quadrants. The color density indicates as '0.3<R<0.6 (shorter length vector, light)' or 'R>=0.6 (longer length vector, dark)'

Statistics

Statistical analysis was performed using free software R for PCA, multivariate hypothesis testing, Wilks' lambda distribution and one-way ANOVA. In order to integrate 11 behavioral parameters, we used PCA analysis based on a correlation matrix. The details of PCA analysis and the assignment of behavior to emotional state (BOUQUET method) were described in [30-32]. The statistical difference tests between groups of the score plots were performed by Wilks' lambda. To know the approximate contribution of each parameter for the 1st PCA on the 1st and 2nd components projection plane, the factor loading vector was visualized only plus direction with minus vector omitted (Figures 2c and Figure 3g).

Brain monoamine measurement by HPLC

After leaving a subject in the home cage in darkness for 30 minutes

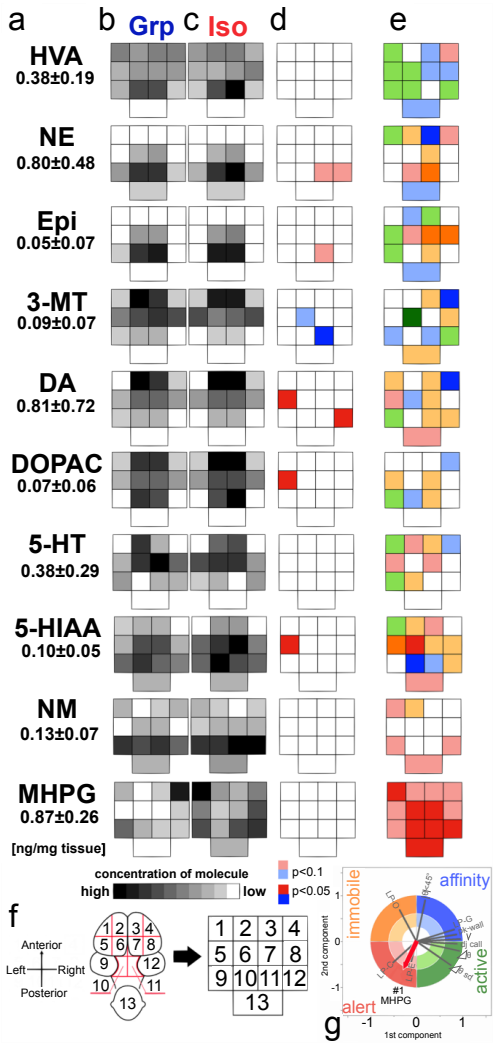

Figure 3: The whole brain maps of 10 monoamines and the correlated behavioral features.

a. Monoamine concentration (average with standard deviation). Monoamine concentration in each brain sub-region from both experimental groups was summated over 13 sub-regions and the average was figured out with the standard deviation. The order of monoamines from top to bottom was according to the emotional color code from active/affinity (green/blue) to alert (red) and not necessarily according to chemically close relation (metabolites)

b, c. Monoamine expression maps in the whole brain, 13 sub-regions. The concentration of each monoamine from each sub-region was sorted according to the values among Grp and Iso subjects together. The order was expressed as 10 steps gray scales (see the bottom common gray-scale) and was mapped. The distribution pattern of each monoamine was different each other and suggested a similar distribution among a monoamine and its metabolites

d. The brain regions that expressed significantly higher monoamine in the comparison between Grp (blue) and Iso (red). (p-value was calculated by Two-tailed paired student t-test with both sides, unequal variance; dark: $p<0.05$, light: $p<0.1$). The group difference was restricted to the limited sub-region

e. The correlation of each monoamine in a brain sub-region with behaviors was illustrated using a color code for each emotion. As illustrated in Figure 2c, Pearson product-moment correlation coefficient (R) was computed using a monoamine concentration in a sub-region and behavior principal components score (the first or second components) of a subject from either Grp and Iso and the R value was used as x or y coordinate value in the PCA plane. The monoamine vector was color-coded according to the emotional quadrant illustration (Figure 3g) and mapped on a sub-region. This method was applied to ten monoamines and thirteen sub-regions. The color density indicates as '$0.3<R<0.6$ (shorter length vector, light)' or '$R>=0.6$ (longer length vector, dark)'

f. The subdivision scheme of a whole brain. First, the cerebellum (region #13) was removed. Then, the first coronal cut was done between the cerebral cortex and the midbrain (tectum)/thalamus and the second coronal cut was done at Interaural 4.72. The sagital cut along rostral-cordal axis was at Lateral 0.00, +/-3.88 [mm] after 'The Chick Brain in Stereotaxic Coordinates' [35]. The conversion from a whole brain outline to the schematic drawing was illustrated.

g. For convenience, the emotional color definitions of Figure 2c was again presented as Figure 3e

within 11:00 a.m. to 3:00 p.m., animals were decapitated and the brain was taken out from the scull. The brain was dissected into 13 regions (Figure 3f) by a surgical blade on a 7% agar block in 0.1M of PBS on ice and the dissected tissues were put in tubes, then immediately frozen in liquid nitrogen, and transferred in -80 degree Celsius within 2 days. Each tissue was homogenized in 500 microL of 0.2M perchloric acid per 100 mg of tissue. After 30 minutes on ice, the samples were spun in a microcentrifuge at 20,000 G for 10 min at 0 degree Celsius. Samples of the supernatant were adjusted at pH 3 with 1M of CH3COONa by pH test paper (ADVANTEC, Universal pH1-11), filtered (13mmMillex-HN, Millipore), then stored in – 80 degree Celsius. The samples were analyzed for 10 kinds of monoamines (Figure 3a) by HPLC (Eicom

EP-700 with electrochemical detection (ECD-300, Eicom), +750mM versus Ag/AgCl). To elute catecholamines from the reverse phase column (3 × 150 mm octadecyl silane column, SC-5ODS,Eicom), a mobile phase (the ratio of 0.1 M citric acetate buffer pH 3.5: methanol: 100 mg/mL of sodium 1-octane sulfonate: 5 mg/ml EDTA-2Na= 850:15:1.9:1) was used at 500 micro L/min, which separated 10 monoamines as follows, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), dihydroxyphenylaceticacid (DOPAC), 3-methoxytyramine (3-MT), homovanillicacid (HVA), norepinephrine (NE), normethanephrine (NM), 3-methoxy-4-hydroxyphenylglycol acid (MHPG), and epinephrine (Epi). The amount of monoamines in the elute was calculated and expressed as [ng/mg tissue] using Isopreterenol as a recovery marker.

Correlation analysis between social behavior and monoamines

The correlation coefficient (R) of Pearson product-moment between monoamine data and either the 1st or 2nd PCA scores was calculated. Then, each monoamine vector was defined by each R value as x or y coordinate value and illustrated together with factor loadings vectors in Figure 2c.

Results

Social behavior features by BOUQUET analysis

Chicks had been reared under two rearing conditions, isolated (Iso) and grouped (Grp) (Figure 1a) and were tested for the acquired social behavior in a meeting context with three unfamiliar peers in the adjacent cage (Figure 1c) on postnatal day 13th (P13). The behavior parameters (Figure 1b) were extracted from video data and the representative parameters were compared between Iso and Grp chicks by BOUQUET [30, 31] multivariate correlation analysis based on PCA and visualizing each PCA score-distribution (Figure 2). Grp and Iso behavior data plots were segregated as positive and negative regions along the component 1st (Figure 2a). Each parametric feature was described in the factor loading positive vectors (Figure 2b). From the non-Parametric Analysis (PCA), some clusters of the factor loading vectors emerged. According to the clusters and the implication of each clusters based on meeting test context (left alone in unfamiliar cage, for example), we set four kinds of psychological translation, active-immobile or affinity-alert and labeled by color codes (Figures 2c and Figure 3g). We used these definitions at the next section.

Brain correlation map between 10 monoamines and 11 behavioral parameters

We measured 10 kinds of monoamines in 13 brain sub-regions of Iso and Grp subjects that were in darkness at home cage in the day-time on postnatal day 14th (Figure 1a). First, a monoamine expression map was constructed (Figures 3b and 3c). Group difference was not widely distributed, but rather limited in a particular sub-region (Figure 3d). In considering well established brain region for monoamine synthesis, the distribution pattern of each monoamine in a whole brain suggested that three major neuromodulators, serotonin (5-HT), Norepinephrine (NE), and Dopamine (DA) differently innervate brain sub-regions and each region showed different turnover rate of them. For example, the DA terminal was high in the medial prefrontal region (#2, 3), but its metabolite DOPAC was high in the midbrain (#10, 11, presumably nigrostratum and ventral tegmentum regions).

Next, we attempted to construct the whole brain correlation map with monoamine and behavioral features in each sub-region (Figure 3e). MHPG (a metabolite of norepinephrine) prominently correlated with 'alert (red)' over whole regions, especially in medial limbic system (#6, 7, 10, and 11) and interestingly left latera/orbitofronal region and right tectum. NM (another norepinephrine metabolite) was partially similar. Meanwhile, HVA (a metabolite of dopamine) moderately correlated with affinity (blue)-active (green) behavioral features almost over whole brain. The left caudal-lateral forebrain (#5) containing sub-nuclei of amygdale and striatum, where DA, DOPAC and 5-HIAA (a metabolite of serotonin) (Figure 3d) was significantly higher in Iso chicks, moderately correlated to alert behavior. The right thalamus-midbrain (#11) showed correlation between affinity behavior and 3-MT, whereas NE and its metabolite EPi, whose original nuclei, locus coeruleus was included in this region, moderately correlated with alert.

Furthermore, there existed lateral brain asymmetry of behavioral features, as left-green and right-blue in HVA (1, 5, 6 versus 3, 7, 8) and 5-HT (1 versus 4), as left-green and right-red in HVA (1 versus 4) and NE (1 versus 4), as left-green and right-orange in Epi (5 versus 8) and DA (9 versus 12).

Discussion

In this study, we explored the possibility of linking micro (monoamines) and macro (behavior) physiology using a statistical brain mapping method and to comprehend the complexity and integrated nature of the social brain network as a whole instead of individual brain parts [10-12]. As the first step to see the applicability of a statistical brain mapping method, we prepared two experimental chick groups, socially isolated (Iso) and affiliated ones (Grp), analyzed their social behavior, and brain ten monoamines in thirteen brain sub-regions. First, we determined the behavior feature space where two groups were well segregated. The feature space was a PCA plane consisting of the first and second components as x and y axes. Secondly, a monoamine concentration in a sub-region was correlated with the first or second component scores of the same subject used for behavior analysis. Next, we calculated the Pearson correlation coefficient (R) with each component and its R value was plotted on the behavior feature plane as x or y coordinates value. Thereby, a monoamine in a sub-region can be related with behavior features in this study. These four emotional features are active, immobile, affinity, and alert. We successfully mapped the correlation of monoamines and emotional features in a whole brain. This map revealed reasonable connectivity of monoaminergic systems, DA to medial frontal region, NE to various brain regions as an alert system, and 5-HT to mesolimbic system. The distribution of monoamines between two experimental groups seemed not much different, but with close examination a couple of difference was observed, for example, higher expressions of DA in left caudal-lateral forebrain and NE in right thalamus-midbrain regions in Iso subjects were statistically significant (Figure 3d). Another prominent result of the brain mapping was the finding of MHPG, a NE metabolite correlated with alert emotion in many brain regions and HVA, a DA metabolite correlated with active/affinity emotion in prefrontal and mesolymbic systems. Although the sample population in this experiment was limited, these results encouraged further study using a statistical brain mapping method to understand a functional brain network as a whole even without brain imaging technology and would be potentially useful for toxicological analysis especially when using animal models.

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