The Role of Posterior Pallial Amygdala in Mediating Motor Behaviors in Pigeons

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Abstract

The posterior pallial amygdala (PoA) is located on the basolateral caudal telencephalon, including the basal division of PoA (PoAb) and the compact division of PoA (PoAc). PoA plays a vital role in emotion regulation and is considered a part of the amygdala in birds. However, the regulatory functions responsible for motor behaviors and emotions between PoAb and PoAc are poorly understood. Therefore, we studied the structure and function of PoA by tract-tracing methods, constant current electrical stimulation, and different dopamine receptor drug injections in pigeons (*Columba livia*). PoAb connects reciprocally with two nuclear groups in the cerebrum: 1) a continuum comprising the temporo-parieto-occipitalis, corticoidea dorsolateralis, hippocampus, and parahippocampalis areas and 2) rostral areas of the hemisphere, including the nucleus septalis lateralis and nucleus taeniae amygdalae.

Extratelencephalic projections of PoAb terminate in the lateral hypothalamic nucleus and are scattered in many limbic midbrain regions. PoAb and PoAc mainly mediated the turning movement. D1 agonist and D2 antagonist could significantly reduce the latency period for entering into the central area and increase the stay time in the central area, whereas D1 antagonist and D2 agonist had the opposite effect. PoAb and PoAc are important brain areas that mediate turning behavior.

Introduction

The arcopallium/posterior pallial amygdala complex, previously known as the archistriatum (A), is present in the avian posterior ventral telencephalon¹. After the Avian Brain Nomenclature Forum held at Duke University in July 2002, the Forum renamed most of the structures in the archistriatum that shared premotor characteristics to arcopallium, and for the remaining parts of the archistriatum that were assumed to constitute the amygdala, the Forum appended amygdala to their names to distinguish them from the arcopallial nuclei¹. The arcopallium/posterior pallial amygdala complex plays an essential role in controlling fear reactions in avians^{2–6}. However, limited information is available about the contribution of its subdivisions.

As parts of the arcopallium/posterior pallial amygdala complex, the posterior pallial amygdala (PoA) comprises the basal division of PoA (PoAb) and the compact division of PoA (PoAc). At present, PoAc shows some similarities in the receptor expression profile of the mammalian lateral regions of the amygdala⁷. However, there are few studies on PoAb^{7–9}, and the role of PoAb and PoAc in mediating motor behaviors of birds remains unknown. Hence, these issues will be discussed in the present study.

As a common bird in daily lives, homing pigeons (*Columba livia domestica*) have many outstanding abilities, including homing and good orientation discrimination, making them an ideal model for studying neurological mechanisms. In this study, we defined the afferent and efferent projections of PoAb in the pigeon using cholera toxin subunit B (CTB) and biotinylated dextran amine (BDA). Subsequently, we investigated the effects of PoA on the mediation of motor behavior in pigeons via electrical stimulation. Lastly, we also tested the effects of different dopamine D1 and D2 receptor agonists and antagonists on forward behavior.

Results

Fiber connections of PoAb

Retrograde labeling by CTB. The center of the injection site of the right PoAb was placed at A 6.25, L 7.6, and D 6.8 according to the atlas of Pigeon¹³, and the diffusion of the tracer was mostly restricted within PoAb (Figure 1b). Labeled areas outside PoAb included many regions of the forebrain. Around PoAb, numerous labelings were found in the cortex piriformis (CPI) from approximately A 5.00 to A 7.50, fewer labelings were found in the arcopallium dorsale (AD) and arcopallium intermedium (AI), and sparse labelings were found in the arcopallium anterius (AA) (Figure 2c–f). Other labeled areas outside PoAb were divided into three streams in the telencephalon. Two of them were from the nidopallium caudoventrale, in which an extensively labeled area was found to be separated. One of them traveled dorsolaterally, along the way through the parahippocampalis (APH) and hippocampus (Hp) areas to the dorsolateral corticoid (CDL) and temporo–parieto–occipitalis (TPO) areas (Figure 2c–g); in contrast, the other ran ventrally and turned rostrally, passing through the nidopallium caudolaterale (NCL), dorsal mesopallium (MD), and ventral mesopallium (MV). Labeled neurons proceeding rostrally extended to the hyperpallium apicale (HA), hyperpallium densocellulare, hyperpallium ventrale (HV), and hyperpallium intercalatum (HI) of the rostral telencephalon (Figure 2a–c). They converged in the pallium at about A 7.0. The third course ran basal and turned rostrally. En route, it branched to nucleus taeniae amygdalae (TnA), nucleus septalis lateralis (SL) and the anterior commissura (AC). Labeled neurons proceeding further rostrally extended to the basorostral pallial nucleus.

Numerous labeled neurons were observed in the nucleus dorsomedialis anterior thalami (DMA) (Figure 2d–f) of the diencephalon. In contrast, fewer labeled neurons were observed in the radix mesencephalicus nervi trigemini (RxVM) (Figure 1e, Figure 2i), tectum opticum (TeO) (Figure 2e) and nucleus preopticus anterior (POA). Sparsely distributed labeled neurons were observed in the lateral hypothalamic nucleus (LHy) (Figure 2c) and nucleus rotundus.

In the midbrain, labeled neurons were widely scattered to a different site. CTB-labeled neurons were distributed in a continuous region stratum griseum central (SGC), stratum griseum et fibrosum superficiale (SGF) (Figure 1c–e, Figure 2g–i), nucleus isthmi, pars magnocellularis (Imc) (Figure 1c–d), nucleus isthmi, pars parvocellularis (Ipc) (Figure 1c–d), and formatio reticularis medialis mesencephali. Fewer labeled neurons were observed in the substantia grisea centralis (GCt) (Figure 2g–h), nucleus intercollicularis (ICo) (Figure 2g–h), substantia grisea et fibrosa periventricularis (SGP). Sparse labeling was observed in the locus ceruleus (LoC), nucleus pontis lateralis (PL), stratum album central (SAC) (Figure 1c–e, Figure 2h–i), nucleus subpretectalis (SP) (Figure 2f), and ventral tegmental area (VTA).

Anterograde labeling by BDA. The injection of BDA to the right PoAb was located at A 5.75, L 7.60, D 6.10, and diffusion of the tracer was restricted within the nucleus (Figure 1g). Similar to CTB-labeled neurons, around PoAb, many BDA-labeled fibers were found in CPI, AD, and AI. Moderate numbers of BDA-

labeled fibers were observed in AA and arcopallium mediale (Figure 2c–f). In contrast, PoAc contained few labeled fibers. Rostral to the injection, projections from PoAb were divided into four streams: one traveled dorsolaterally of the forebrain, along the way through APH and Hp to CDL and TPO, and among them, CDL extensively contained BDA-labeled fibers (Figure 2c–g); in contrast, the other ran ventrally and turned rostrally, and the BDA-labeled fibers forming the continuum were found to exist extensively throughout NCL, MD, and MV. Labeled fibers extended to the HA, proceeding further rostrally. Less numerous labeled fibers existed in HV, and very sparsely labeled areas were observed in HI of the rostral telencephalon (Figure 2a–c). The first two streams joined in the pallium, similar to the findings observed for CTB labeling. The third stream ascending fiber bundle extended rostrally toward the basal forebrain, and on this pathway, many fibers appeared in TnA (Figure 2e). Moderate numbers of fibers appeared in nucleus septalis lateralis and tuberculum olfactorium, and more rostrally, terminated in AC at approximately A 7.75. Using AC as a turning point, the contralateral bundle repeated its ipsilateral in a mirror or symmetric fashion. The fourth stream ran through the diencephalon and midbrain *via* a descending fiber bundle.

In the diencephalon, the main course of the descending pathway was primarily through the following areas: DMA and DMP in the rostral diencephalon (Figure 2e–f); then TeO and POM in the caudal diencephalon; and finally, a limited number of fibers in nIV, LHy, and RxVM.

In the midbrain, the BDA-labeled areas were much more than the CTB-labeled areas. The descending pathway labeled many areas along its course, and the labeled areas were divided into two streams. Ventrally, it projected massively to the limbic midbrain. There were several labeled varicose fibers or terminals in fasciculus longitudinalis medialis, SGC, SGF, and SP (Figure 1h–j, Figure 2g–i). In addition, BDA-labeled neurons were frequently observed in GCt, SAC, SGP, and Sop (Figure 2g–i). The other labeled fibers were scattered in the midbrain, and these areas received a moderate number of labeled fibers from the descending pathway, including lmc, lpc, OM and LoC (Figure 1j). Few labeled fibers were distributed sparsely in torus semicircularis, VTA, and tractus vestibule–mesencephalicus.

In the CTB and BDA injections, three pigeons each were targeted to PoAb. In each case, two were largely confined to the PoAb and with successful labeling, so they were selected to describe and depict the results. And the summary Figures and Table 1 represent composite results. The general patterns of the afferent and efferent projections of PoAb are summarized in Figure 3 and Table 1.

Table 1

Distribution of labeling of BDA and CTB injection in PoAb

Sites	Number of CTB - labeled neurons		Density of BDA - labeled fibers and terminals	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Telencephalon				
AA (arcopallium anterius)	+	-	++	-
AC (anterior commissura)	+++	+++	+++	+++
AD (arcopallium dorsale)	++	++	+++	+
AI (arcopallium intermedium)	++	++	+++	+
AM (arcopallium mediale)	-	-	++	-
APH (area parahippocampalis)	+++	+++	+++	+++
BaS (basorostral pallial nucleus)	+	+	-	-
CDL (area corticoidea dorsolateralis)	+++	+++	+++	+++
CPi (cortex piriformis)	+++	+++	+++	+++
HA (hyperpallium apicale)	++	+	+++	+++
HD (hyperpallium densocellulare)	++	+	-	-
HI (hyperpallium intercalatum)	+	-	++	-
Hp (hippocampus)	++	++	+++	+
HV (hyperpallium ventrale)	-	-	+	+
MD (mesopallium dorsale)	+++	+++	+++	+++
MV (mesopallium ventrale)	+++	+++	+++	+++
NCL (nidopallium caudolaterale)	+++	+++	+++	+++
NCV (nidopallium caudoventrale)	+++	+++	++	++
SL (nucleus septalis lateralis)	++	++	++	++
TnA (nucleus taeniae amygdalae)	+	+	+++	-
TPO (area temporo-parieto-occipitalis)	+++	-	+++	-
Tuo (tuberculum olfactorium)	-	-	++	++
Diencephalon				
Distribution of Labeling of BDA and CTB Injection in PoAb (Table 1 continued)				
DMA (nucleus dorsomedialis anterior thalami)	++	+++	+++	+++

DMP (nucleus dorsomedialis posterior thalami)	-	-	++	++
nIV (nucleus nervi trochlearis)	-	-	+	+
LHy (lateral hypothalamic nucleus)	+	+	+	+
POA (nucleus preopticus anterior)	++	++	-	-
POM (nucleus preopticus medialis)	-	-	++	++
Rt (nucleus rotundus)	+	+	-	-
RxVM (radix mesencephalicus nervi trigemini)	++	++	+	+
TeO (tectum opticum)	++	++	++	++
Midbrain				
Distribution of Labeling of BDA and CTB Injection in PoAb (Table. 1 continued)				
FLM (fasciculus longitudinalis medialis)	-	-	+++	+++
FRM (formatio reticularis medialis mesencephali)	+++	+++	-	-
GCt (substantia grisea centralis)	++	++	++	++
Ico (nucleus intercollicularis)	++	-	-	-
Imc (nucleus isthmi, pars magnocellularis)	+++	+++	++	++
Ipc (Nucleus isthmi, pars parvocellularis)	+++	+++	++	++
LoC (locus ceruleus)	+	+	++	++
OM (tractus occipito-mesencephalicus)	-	-	++	++
PL (nucleus pontis lateralis)	+	+	-	-
PT (nucleus pretectalis)	-	+	-	-
SAC (stratum album central)	+	+	++	++
SGC (stratum griseum central)	+++	+++	+++	+++
SGF (stratum griseum et fibrosum superficialia)	+++	+++	+++	+++
SGP (substantia grisea et fibrosa periventricularis)	++	-	++	++
Sop (stratum opticum)	-	-	++	++
SP (nucleus subpretectalis)	+	+	+++	+++

SpL (nucleus spiriformis lateralis)	-	+	-	-
ToS (torus semicircularis)	-	-	+	+
TVM (tractus vestibule–mesencephalicus)	-	-	+	+
VTA (ventral tegmental area)	+	+	+	+

Number of labeled neurons and density of labeled fibers and terminals: +++, numerous; ++, moderate; +, few; -, absent.

Abbreviations: BDA, biotinylated dextran amine; CTB, cholera toxin subunit B; PoAb, basal division of posterior pallial amygdala

The effects of electrical stimulation on motor behaviors

Before the electrical stimulus, the pigeons stays in place, generally accompanied by turning its head, or raising its head or pecking feathers in the arena. A series of new different motor behaviors were induced after the electrical stimulation of PoA, among which ipsilateral lateral movement, forward movement, contralateral lateral movement, backward movement, *etc.*, occurred more frequently (Figure 4a, 4d). The different motor behaviors elicited in the arena were defined using a previous study¹⁴ with slight modifications (see Table2 for details). Figure 4c showed that PoAb mainly mediated ipsilateral lateral movement (75.00%) and seldomly mediated contralateral lateral movement (1.85%). PoAc was more complex; the behavioral response of it was as follows: ipsilateral lateral movement (78.89%), contralateral lateral movement (3.89%), forward movement (1.11%), and backward movement (2.78%). The result indicated that PoA mainly mediated the turning movement behavior in pigeons. There was no significant difference in the ipsilateral lateral movement response rate between PoAc and PoAb (Figure 4f). The locations of the stimuli in PoAc and PoAb are presented in Figure 4b and Figure 4e, respectively.

Table 2

Definitions of some motor behaviors in the arena

Motor behaviors	Definition of the motor behaviors
Ipsilateral lateral movement	Alternate stepping of both feet towards right side of the body
Forward movement	Alternate stepping of both feet and moving forward
Contralateral lateral movement	Alternate stepping of both feet towards left side of the body
Backward movement	Alternate stepping of both feet and moving backward
The number of steps generated by different behaviors is generally 2-3 steps.	

The effects of different drugs on forward behavior

Personalized screening. According to the SMART output data, the total scores of 25 pigeons were obtained. A normality test was performed on the total scores of each pigeon, and the distribution was found to be non-normal ($P > 0.05$, $N = 25$, Figure 5a). Eight pigeons did not enter the central area (the total score was 0.00) (Figure 5b), and nine pigeons were very active in the central area (the total score was > 1.50 , Figure 5d). Combining the locomotion trajectories of the pigeons in the experimental box captured by SMART, we finally selected those individuals of which the total scores between 0.90 and 1.50. Consequently, eight pigeons were used for the drug injection experiment (Figure 5c, 5e).

The effects of D1 and D2 receptor agonists and antagonists on the latency of entering central area in pigeons. The effects of D1 and D2 receptor agonists and antagonists on the latency period of pigeons entering the central area were different (Figure 5). D1 agonist decreased the latency period of pigeons for entering the central area ($F_{3,28} = 14.78$, $P < 0.001$, Figure 5f). When the concentration of D1 agonist was $0.5 \mu\text{g}/\mu\text{L}$, the range of the latency period of pigeons for entering the central area decreased by 44.61% ($P < 0.001$), but there was no significant difference in the other groups. In contrast, D1 antagonist increased the latency period of pigeons for entering the central area ($F_{3,28} = 27.58$, $P < 0.001$, Figure 5g). When the concentration of D1 antagonist was $0.1 \mu\text{g}/\mu\text{L}$ and $0.5 \mu\text{g}/\mu\text{L}$, the latency period increased significantly, and their ranges increased by 35.46% ($P < 0.01$) and 71.61% ($P < 0.001$), respectively. For $0.02 \mu\text{g}/\mu\text{L}$ concentration of D1 antagonist, the latency period did not increase significantly. D2 agonist also increased the latency period of pigeons for entering the central area ($F_{3,28} = 25.63$, $P < 0.001$, Figure 5h). The ranges of the latency period increased by 45.24% ($P < 0.001$) and 81.40% ($P < 0.001$) when the concentrations of D2 agonist were $0.1 \mu\text{g}/\mu\text{L}$ and $0.5 \mu\text{g}/\mu\text{L}$, respectively. However, $0.02 \mu\text{g}/\mu\text{L}$ D2 agonist exhibited no significant influence. D2 antagonist decreased the latency period of pigeons for entering the central area ($F_{3,28} = 7.74$, $P < 0.001$, Figure 5i). The latency period of pigeons for entering the central area was decreased by 26.46% ($P < 0.001$) when the concentration of D2 antagonist was $0.02 \mu\text{g}/\mu\text{L}$; but when the concentrations of D2 antagonist were $0.1 \mu\text{g}/\mu\text{L}$ and $0.5 \mu\text{g}/\mu\text{L}$, the latency period was decreased by 16.69% ($P < 0.05$) and 18.89% ($P < 0.05$), respectively. Overall, D1 agonist and D2 antagonist can reduce the latency period of pigeons for entering the central area. In contrast, D1 antagonists and D2 agonists could increase the latency period of pigeons for entering the central area.

The effects of D1 and D2 receptor agonists and antagonists on the stay time of pigeon in the central area. D1 and D2 receptor agonists and antagonists have different effects on the stay time of pigeons in the central area (Figure 5). D1 agonist increased the stay time of pigeons in the central area ($F_{3,28} = 11.59$, $P < 0.001$, Figure 5j). When the concentration of D1 agonist was $0.1 \mu\text{g}/\mu\text{L}$ and $0.5 \mu\text{g}/\mu\text{L}$, the stay time of pigeons in the central area increased by 58.12% ($P < 0.001$) and 62.03% ($P < 0.001$), respectively,

but when the concentration of D1 agonist was 0.02 µg/µL, no significant difference was noted in the stay time. D1 antagonist decreased the stay time of pigeons in the central area ($F_{3,28} = 26.19$, $P < 0.001$, Figure 5k). The stay time of pigeons in the central area decreased by 40.21% ($P < 0.001$) when the concentration of D1 antagonist was 0.1 µg/µL. D2 agonist had a significant impact on the stay time of pigeons in the central area ($F_{3,28} = 34.64$, $P < 0.001$, Figure 5l). When the concentration of D2 agonist was 0.02 µg/µL, the stay time in the central area increased by 53.72% ($P < 0.001$); whereas it decreased by 12.53% when the concentration of D2 agonist was 0.5 µg/µL, and no significant difference was noted. D2 antagonist increased the stay time of pigeons in the central area ($F_{3,28} = 81.74$, $P < 0.001$, Figure 5m). The stay time of pigeons in the central area increased by 25.92% ($P < 0.01$) and 92.19% ($P < 0.001$) when the concentration of D2 antagonist was 0.1 µg/µL and 0.5 µg/µL, respectively; however, 0.02 µg/µL D2 antagonist did not result in significant changes in the stay time. In summary, with increase in drug concentration, D1 agonist and D2 antagonist significantly increased the stay time of pigeons in the central area. In contrast, D1 antagonist decreased the stay time of pigeons in the central area. Further, D2 agonist increased the stay time of pigeons in the central area at a low concentration but decreased the stay time at a high concentration.

Discussion

The present study revealed the fiber connections of PoAb, which innervate most of the telencephalon, especially the pallium. Concerning descending pathways, PoAb projects primarily to the midbrain. The labeling areas are consistent with those reported by previous researchers in forebrain^{6,8-9,15-20}. Compared with PoAb, PoAc has few connections with the pallium. As for the downstream pathways, PoAc connects primarily with the diencephalon and projects less on the lateral hypothalamus. The connections between PoAb and pallium were confirmed in pigeons, which are in accordance with the findings in chickens or mallards, in spite of the projections between PoA and the ventral basal ganglia or medial striatum have not been proven in chickens or mallards²¹⁻²². However, the tectal layers and the associated isthmus complex were not described ascending projections to the pallium in other studies. But the present study shows that PoAb connects reciprocally with SGC, SGF, and SAC and so on. This may be caused by different development process methods or false positive findings. And we did not draw the controversial brain areas in the summary diagram. As the second major vertebrates, birds have no corpus callosum (CC). The connection of PoAb with many areas in the brain of pigeons suggests that it plays an essential role in interhemispheric exchange, which consequently indicates that PoAb compensates for the absence of CC in birds.

The results of electrical stimulation indicated that PoAb can single-handedly mediate ipsilateral lateral movement, whereas PoAc is different. The differences in PoAb and PoAc with regard to connectivity result in differences in their mediation of motor behaviors. PoAb has closer connections with pallium than does PoAc. As for the descending pathways, PoAb connects with many locomotor brain regions in the midbrain, whereas PoAc has little connections with locomotor brain regions in the midbrain, most of which are one-way projections. PoAc has significantly more connections with other brain regions within

the amygdala of birds than does PoAb⁸. Perhaps the close connections within the amygdala lead to the complexity of PoAc behavior. Moreover, the molecular mechanisms of PoAb and PoAc on the mediation of motor behavior in pigeons remain to be studied.

Each pigeon's total score exhibited whether the activity time and activity degree of the pigeon in the central area were random or not. Compared with mice, pigeons have poor ability to explore new environments; thus, the total scores of most pigeons were 0.00, and only few pigeons were very active. The reason for the significant difference may be that the brains of birds and mammals have evolved differently.

The effects of different drugs on forward behavior revealed that D1 agonist and D2 antagonist could promote forward behavior, whereas D1 antagonist and D2 agonist have inhibitory effects. Our results corroborated those of previous studies in Wistar rats, hens, and Japanese quail^{11, 23–25}. However, compared with reptiles, the effects of D1 and D2 receptor agonists and antagonists were partly in accordance with those on pigeons^{12, 26–27}; this could be attributed to the difference in species. Since experiments in this part mainly analyze the movement of pigeons, the forward behavior was directly related to the pigeon movement. Besides, PoAc was characterized by high tyrosine hydroxylase (TH) immunoreactivity, whereas PoAb has negligible numbers of TH positive cells^{7, 9}. Thus, we chose the dominant site of forward behavior in PoAc for drug injection. The PoA, TnA, area subpallialis amygdalae (SpA), bed nucleus of the stria terminalis, and pars lateralis (BSTL) were considered to form the avian amygdala⁷. Dopamine plays an important role in the regulation of emotions²⁸. By comparing the behavioral differences between the experimental group and the control group, the role of PoAc in the regulation of forward behavior is studied. The results of this part further prove that PoAc affects the behavior of pigeons by regulating their emotions.

Overall, PoAb connects with many more areas in the cerebrum than PoAc, suggesting that the role of PoAb was exchanged between the right and left hemispheres. The function of PoAb and PoAc is to mediate the turning behavior, and both of them can be used as important brain areas to regulate turning behavior. The effects of different drugs on forward behavior proved that PoAc affects motor behaviors by regulating emotion. Our study will provide a theoretical basis for controlling motor behavior in birds and enrich the theoretical system of emotion-related brain regions and behaviors in birds.

Methods

Animals.

Overall, 65 adult pigeons (body mass, 350–450 g) were used in this study. All animals (unknown sexes) were acclimatized to a wire cage (95 × 80 × 65 cm³) under a normal day/night light cycle with food and water *ad libitum*. The feedstuff was composed of wheat, corn, sorghum, and soybean at the ratio of 1:1:1:1, and the cages were cleaned twice a week. All experimental procedures and animal housing and manipulations were approved by and carried out under the Life Science Ethical Review Committee of

Zhengzhou University (Henan, China). All efforts were made to minimize the suffering of the animals throughout the experimental procedures.

Declarations: The study was carried out in compliance with the ARRIVE guidelines, and all methods were carried out in accordance with relevant guidelines and regulations.

Tract-tracing of PoAb pathway

Surgical procedure. The pigeons were anesthetized by injecting 3% pelltobarbitalum natricum (0.12 ml/100 g body mass) intraperitoneally. After the pain reflex disappeared, the pigeons' heads were placed in a stereotaxic holder customized for the pigeons. The skin above the surgical field was removed carefully, part of the skull was removed using a dental drill, the dura mater and arachnoid mater were moved, and the brain tissue was exposed. Subsequently, the injection cannula (O.D.0.41 × I.D.0.25; R.W.D. Life Science, Shenzhen, China) was implanted. When the PoAb was reached, the gaps between the cannula and the cranium were sealed with EC glue and fixed with dental cement after the glue solidified. Enrofloxacin solution (5%) was used in the surgical area to help recovery. The surgery area was cleaned regularly. The pigeons were fed separately during 5-7 days of recovery, after which they were used in the following experiments.

In vivo injections. After recovery, each pigeon received unilateral injections of one tracer, BDA (10,000 molecular weight; Thermo Fisher, Eugene, OR, USA) or CTB (low salt; List Biological Laboratories, Campbell, CA, USA) on the right hemisphere (Figure 1a, 1f). BDA (10% in phosphate-buffered saline [PBS], pH 7.4) was used for anterograde labeling, whereas CTB (1% in PBS) was used for retrograde labeling. At each injection site, 150nl of the tracer was applied at each depth using a mechanical pressure device (R.W.D. Life Science, Shenzhen, China). After 2 days (for CTB injections) or 7 days (for BDA injections) of survival, the pigeons were deeply anesthetized using pelltobarbitalum natricum (100 mg·kg⁻¹) and perfused with physiological salt solution, followed by cardiac perfusion with 4% formaldehyde. Their brains were extracted and postfixed overnight in 4% paraformaldehyde at 4°C and subsequently transferred to 30% sucrose in PBS at 4°C for 3 days. Brains were cut at the coronal plane in 35-μm thick slices using a freezing microtome (Leica CM1950, Germany).

Immunohistochemistry. BDA and CTB fluorescence staining was used to demonstrate anterogradely labeled fibers and retrogradely labeled neurons.

For CTB fluorescence staining, slices were washed with PBS before (3 × 10 min) and after (1 × 5 min) incubation in 10% normal goat serum (30 min; 1:10 in PBS). Subsequently, they were incubated with polyclonal rabbit anti-CTB antibody (Abcam, RRID: AB_34992; 1:1,000 in PBS) for 12-14 h at 4°C. Then, the slices were washed in PBS (3 × 10 min) and incubated with the secondary antibody Alexa Fluor®488-conjugated AffiniPure Goat Anti-Rabbit IgG (Wuhan service biotechnology; 1:200 in PBS) for 1 h at 37°C. Finally, the slices were washed in PBS (3 × 10 min), mounted on glass slides, and covered using a coverslip with fluoromount (Wuhan service biotechnology, Wuhan, China).

For BDA fluorescence staining, after the slices were incubated for 30 min in 10% normal goat serum, they were incubated with Alexa 594 streptavidin (BioLegend Way San Diego, CA; 1:200 in PBS) for 50 min at 37°C. Finally, the slices were washed in PBS (3 × 10 min), mounted on glass slides, and covered using a coverslip with fluoromount.

The effects of PoA electrical stimulation on motor behaviors

Microelectrode implants. The electrode (diameter = 120 μm; stainless steel wire, Teflon insulation) was implanted into PoAb and PoAc, respectively ($N = 3$). All processes were performed as described above.

Electrical stimulation protocols. After recovery, the behavioral responses of the pigeons to electrical stimulation were observed. The subjects were given 5 min for adaptation, and then electrical stimulation (stimulation intensity = 0.3–0.8 mA; interstimulus interval = 3–5 min; frequency = 20 Hz) generated by an YC - 2 - S bipolar programmed stimulator (Chengdu Instrument Factory, China) was applied *via* stainless steel electrodes (diameter = 120 μm; impedance range < 0.5 MΩ). The test was done in an arena made of polymathic methacrylate (diameter × height, 80 × 80 cm²). The responsive behaviors evoked by different stimulation intensities were recorded using a SONY HDR-CX220 camcorder (Sony Corporation, Tokyo, Japan).

Histology. After three test trials (inter-experiment interval was 3 days), the pigeons were deeply anesthetized and then perfused. Subsequently, their brains were removed from the skulls, postfixed, and dehydrated. Lastly, histological analyses were performed by Nissl and hematoxylin and eosin staining. Light microscopy was used to identify the stimulation sites of the sections.

The effects of different drugs on forward behavior

Personalized screening. The pigeons should be screened to avoid the impact of personalized differences before the experiment. According to the method used for studying jungle crow's response to mirror-image stimulation¹⁰, the size of the experimental box was 100 × 100 × 40 cm³ (length × width × height), and the video equipment was installed 120 cm above the experimental box. The experimental box was divided into two areas: the central area (within the red box) and the edge area (outside the red box). The central area was square with an area of 33.33 × 33.33 cm², accounting for 1/9 of the whole area.

The pigeons were placed in the edge area of the experimental box when the recording device was working. Each pigeon was recorded for 15 min. The first 5 min recorded the pigeons' adaptation process to the experimental box, and the last 10 min constituted the actual personalized screening videotape. Subsequently, 75% alcohol was used to spray the whole experimental box, and the next experiment was recorded after 30 min. SMART 3.0 Panlab video analysis software (Henan Baiquan Bioscientific Co.,limited, China) was used to collect the data on movement behavior of each pigeon ($N =$

25) automatically; the data mainly included movement time, movement speed, and movement distance of the pigeon in various areas of the experimental box; the movement trajectory of the pigeon was also captured. The personality screening criteria were determined by the movement time, distance, and activity degree in each area of the experimental box. The personality score of each pigeon was calculated as follows: (movement distance in the central area $\times$ 9 / total distance) / (movement time in the central area $\times$ 9 / total time). The pigeon motion trajectory map obtained by SMART software was used on an auxiliary basis.

Stereotaxic implantation of the injection cannula. After personalized screening, the pigeons ($N = 8$) were implanted with an injection cannula (O.D.0.41 $\times$ I.D.0.25) at the site of PoAc (A 5.00 mm; L 7.60 mm). All processes were performed as described above.

Drug treatments. After cannula implantation, the recovered pigeons were administered four different drugs ($N = 8$): SKF-38393 (D1 +: D1 agonist), SCH-23390 (D1 -: D1 antagonist), quinpirole (D2 +: D2 agonist), and raclopride (D2 -: D2 antagonist); all drugs were bought from Sigma-Aldrich (St Louis, USA). According to the methods in Japanese quail¹¹ and male green anoles¹², three concentration gradients were set for each drug: 0.02 $\mu\text{g}/\mu\text{L}$ (low), 0.1 $\mu\text{g}/\mu\text{L}$ (medium), 0.5 $\mu\text{g}/\mu\text{L}$ (high); 0.9% saline was used as the control. Because the four drugs can be metabolized autonomously in pigeons, two injections can be performed for each drug.

First, the pigeons in the control group were injected 1 μL of saline, and their movement behaviors were recorded in the experimental box for 30 min; then the pigeons were injected 1 μL of 0.02 $\mu\text{g}/\mu\text{L}$ D1 agonist (low concentration), and their movement behaviors were recorded for 30 min after 15 min of drug absorption time. After 1 day, 1 μL of saline was injected as control, following which 1 μL of 0.1 $\mu\text{g}/\mu\text{L}$ D1 agonist (medium concentration) was injected; after 15 min, their movement behaviors were recorded for 30 min. Another day later, 1 μL of saline was injected as control, and then 1 μL of 0.5 $\mu\text{g}/\mu\text{L}$ D1 agonist (high concentration) was injected; after 15 min, their movement behaviors were recorded for 30 min. Different concentrations of D1 antagonist, D2 agonist, and D2 antagonist were injected; the experimental procedure was the same as that for D1 agonist, and the interval between the injection of different drugs was 4 days.

Data recording and analysis.

The terminology used in the present study is based on the Avian Brain Nomenclature Forum^{1,13}.

Photomicrographs were captured using a digital camera (Olympus, Japan). The layout of micrographs, lettering, and printing, and schematic illustrations of labeled neurons and fibers were performed using Adobe Photoshop 7.0J (Tokyo, Japan) and Adobe Illustrator 10.0J (Tokyo, Japan).

All data were analyzed on IBM SPSS Statistics 24 (Chicago, USA). Data were recorded on SMART 3.0 software, which included all relevant data and animal behavior trajectory data. The data standardization

method was as follows: for each animal, (data of each treatment/sum of four treatments) × 100%. The Kolmogorov–Smirnov test was used to test normality of the data, and one-way ANOVA was used to analyze the drug administration data. Data of all groups are represented as mean ± standard error of the mean.

Declarations

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Authors' contributions

XM Tian collected and analyzed data and wrote the initial draft of the paper. YH Shi collected the data. YF Zhang analyzed the data. YJ Wang and MK Li were responsible for image processing. H Cheng and ZL Wang planned the experiments, analyzed the data, and helped write the paper.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Peer Review

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Figures

Figure 1

Fiber connections of PoAb. Schematic of in vivo CTB injections (a) and BDA injections (f) into PoAb. (b): Injection site of CTB in the PoAb at A 6.25. (c): Ipsilateral CTB-labeled neurons in Imc at A 3.5. (d): Contralateral CTB-labeled neurons in Imc at A 3.5. (e): Schematic of CTB-labeled at A 1.25. (g): Injection site of BDA in the PoAb at A 5.75. (h): Ipsilateral labeled fibers in SP at A 5.25. (i): Contralateral labeled fibers in SP at A 5.25. (j): Schematic of BDA-labeled at A 2.75. Scale bars = 200 μ m in (c), (d), (g); 100 μ m

Figure 3

Models of main pathways of PoAb in the pigeon brain. Arrowheads at end of arrows show reciprocal connections, whereas those present midway indicate one-way projections. Abbreviations: AD, arcopallium dorsale; AI, arcopallium intermedium; APH, area parahippocampalis; BaS, basorostral pallial nucleus; CDL, area corticoidea dorsolateralis; CPi, cortex piriformis; DMA, nucleus dorsomedialis anterior thalami; DMP, nucleus dorsomedialis posterior thalami; FLM, fasciculus longitudinalis medialis; FRM, formatio reticularis medialis mesencephali; H, hyperpallium; Imc, nucleus isthmi, pars magnocellularis; Ipc, nucleus isthmi, pars parvocellularis; LoC, locus ceruleus; M, mesopallium; N, nidopallium; OM, tractus occipito-mesencephalicus; POA, nucleus preopticus anterior; PoAb, basal division of posterior pallial amygdala; POM, nucleus preopticus medialis; RxVM, radix mesencephalicus nervi trigemini; SL, nucleus septalis lateralis; SP, nucleus subpretectalis; TnA, nucleus taeniae amygdalae; TPO, area temporo-parieto-occipitalis; Tuo, tuberculum olfactorium

Figure 4

Effects of electrical stimulation on motor behaviors. Schematic of electrical stimulation of PoAc (a) and PoAb (d). Coronal slice of PoAc Nissl staining at A 5.0 (b) and PoAb hematoxylin and eosin staining at A 5.5 (e). (c): Response rate of four different behaviors after electrical stimulation of PoAc and PoAb. (f): Response rate of ipsilateral lateral movement of PoAc and PoAb at different electrical stimulation intensities. Scale bars = 500 μ m in (b) and (e). Abbreviations: AD, arcopallium dorsale; AI, arcopallium intermedium; AM, arcopallium mediale; AMm, arcopallium mediale, pars magnocellularis; AMP, arcopallium mediale, pars parvocellularis; CPI, cortex piriformis; NCV, nidopallium caudoventrale; PoAb, basal division of posterior pallial amygdala; PoAc, compact division of posterior pallial amygdala; TnA, nucleus taeniae amygdalae

Figure 5

Effects of different drugs on forward behavior. (a): Schematic of personalized screening. (b-d): Locomotion trajectory of different pigeons. (e): Schematic of in vivo injections of different drugs into PoAc. (f-i): Effects of D1 and D2 receptor agonists and antagonists on the latency period of pigeons for entering the central area. (j-m): Effects of D1 and D2 receptor agonists and antagonists on the stay time of pigeons in the central area. Data of all groups are presented as mean $\pm$ SE. Abbreviations: PoAc, compact division of posterior pallial amygdala; SE, standard error of mean