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Research article

The effect of prenatal sensory stimulation on the parvalbumin neurons in entopallium and visual Wulst

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ABSTRACT

Conserved genetic mechanisms participate in developing brain asymmetries in vertebrates, but the effect of prenatal sensory stimulation on lateralised neural circuits is less understood. In birds, lateralisation emerges from the interaction of genetic factors and prenatal experience. Light exposure during a sensitive stage of embryonic development asymmetrically stimulates the two eyes, causing lateralised visual functions. However, nothing is known about its effects on the cell composition of visual areas. Here, we investigated the effects of sensory stimulation on the neuroanatomy of the two primary visual areas of the bird telencephalon (entopallium and visual Wulst) by analysing the density of parvalbumin neurons in domestic chicks. We found that both areas are sensitive to light incubation, showing denser parvalbumin neurons in the left hemisphere of light-incubated chicks than dark-incubated chicks. Light-incubated chicks also showed denser parvalbumin neurons in the left hemisphere than in the right one. In contrast, no asymmetry was present in dark-incubated chicks. We show that prenatal experience influences neural compositions in the telencephalon.

1. Introduction

The development of lateralisation in vertebrates is influenced by shared genetic mechanisms, conserved from birds to humans. One of the most intriguing aspects of brain hemispheric asymmetries, however, is that they are also shaped by sensory stimulation during the prenatal period [1]. Compared to mammals, research in oviparous species like birds, can more easily reveal how visual lateralisation emerges from the interaction of genetic and environmental factors [1–5]. Under the influence of the genes of the Nodal cascade, chick embryos turn within the egg so that body covers the left eye, while the right eye is directly behind the eggshell [2,3]. By exposing the eggs to light, we can stimulate the right eye system only. Since avian species present an almost complete decussation at the optic chiasm, most of the fibres originating from the stimulated eye reach the left hemisphere. Light exposure during a sensitive stage of embryonic development thus causes neuroanatomical and behavioural lateralisation. Although the effects of this phenomenon on behaviour have been extensively studied [5,6], research on the effects of light stimulation in embryos on neuroanatomy is much

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scarcer.

If domestic chick eggs are exposed to light, the thalamofugal projections, which constitutes one of the two visual pathways to birds' telencephalon (Fig. 1) present an asymmetry, with the left thalamus sending more bilateral projections to the telencephalon than the right one (see below [7,8]). This was once believed to be the only neuroanatomical asymmetry caused in domestic chicks by light exposure, and the development of behavioural lateralisation was also attributed uniquely to this factor [9–11]. In the thalamofugal pathway, visual fibres project from the retina to the opposite thalamus (nucleus opticus principalis thalami, OPT) and from there to the ipsilateral visual Wulst [12–14]. This structure is thalamofugal recipient in the telencephalon and is considered a homolog to the primary visual cortex of mammals [15]. Within the thalamofugal pathway, a minority of recrossing projections go from each OPT to the opposite Wulst, carrying visual information from the ipsilateral eye [1,14,16]. In light-incubated chicks, more fibres recross to the right Wulst from the left OPT than vice versa. This confers to the right Wulst a potential advantage for integrating information from the two eyes [7,8].

However, most visual projections reach the avian telencephalon through the tectofugal visual pathway. Thus, asymmetric light exposure could be expected to have a prominent effect on the structures of this pathway. Despite that, until recently [17], there had been no convincing demonstrations that light incubation could affect chicks' tectofugal pathway (but see Ref. [7] for suggestive trends). In the tectofugal pathway, retinal projections reach to the contralateral optic tectum (avian the superior colliculus). Visual fibres then go from each tectum to its ipsilateral nucleus rotundus in the thalamus (equivalent to the pulvinar complex in mammals). Each tectum also sends fewer fibres that recross towards the other hemisphere, reaching the opposite rotundus [12,18]. The rotundus then projects to the entopallium in the forebrain. The entopallium, the functional equivalent to the extrastriate visual cortex of mammals [14,18], has a crucial role in a variety of visual mechanisms, including intensity, form, pattern and motion discrimination [15,19–28].

This led to the hypothesis that light exposure affects both visual pathways, which has been tested in two recent electrophysiological studies on the two primary visual inputs of the forebrain: the visual Wulst and the entopallium [16,17]. In both structures, light exposure of the incubating eggs increased the responsiveness of single visual neurons and induced some forms of lateralisation. Interestingly, in both structures, asymmetries in the neuronal responses between the two hemispheres chicks were found for chicks that had been not exposed to light during incubation. This is in line with the recent evidence that some forms of lateralisation emerge also without light exposure [27–41].

Since light exposure affects neural responses in the entopallium and visual Wulst, this should be reflected in the anatomy of the neural circuits of both these structures. Tracing studies have shown that exposure to light changes the projections reaching the visual Wulst [1,7,8], which may be responsible for the effects observed in this structure [16,17]. However, no clear light-dependent effect has been found in the tectofugal projections that provide visual input to the entopallium. Moreover, how light incubation affects the cell composition in the visual Wulst or the entopallium is unknown.

In this work, we study the effects of light on the neuroanatomy of the visual Wulst and entopallium by analysing the density of parvalbumin-expressing neurons. Parvalbumin (PV) is a calcium-binding protein. In the mammalian cortex, parvalbumin-expressing neurons make up about 40 % of GABAergic interneurons [42]. They are implicated in inhibitory functions and visual attention control

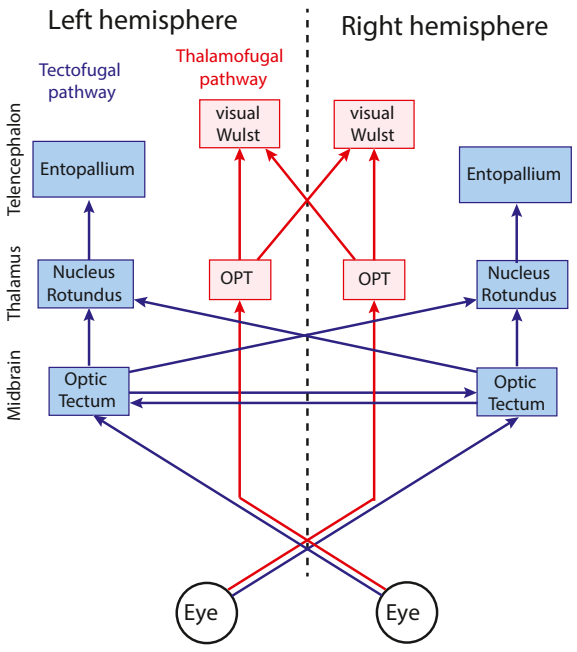

Fig. 1. Major visual pathways to the telencephalon in chicks. The stations of the thalamofugal pathway are in red, and those of the tectofugal pathway are in blue. OPT – nucleus opticus principals thalami.

[43–46]. Moreover, in mammals, the maturation of parvalbumin-expressing neurons has a pivotal role in experience-dependent plasticity during critical periods [47,48]. This is particularly relevant for our purposes since, in chicks, the emergence of light-dependent lateralisation is restricted to a critical period of embryonic development [1]. In mammals, parvalbumin mediation of critical periods has been well investigated in sensory areas, such as the visual cortex [47,48], making entopallium and Wulst interesting candidates for the study of PV expression. Moreover, given their functional role, the lateralisation of PV circuits may determine the known asymmetries between the two eye systems in attention allocation and response inhibition in chicks [49].

For this purpose, we labelled parvalbumin immunoreactive (PV-ir) cells in the entopallium and the visual Wulst of a large sample of 20 dark- and 20 light-incubated chicks. We investigated whether light affects the density of parvalbumin neurons in each of the two brain regions of interest. We also assessed both spontaneous and light-induced brain hemispheric asymmetries, comparing the density of parvalbumin neurons in the two hemispheres.

2. Results

We successfully stained 40 brains. The parvalbumin neural somata were clearly distinguishable from the background (Fig. 2a–f) and visualised anatomical structures in the telencephalon. The entopallium core region contained a high density of parvalbumin neurons, which gradually decreased in the surrounding region. Low densities of parvalbumin neurons were also present in the nidopallium.

All layers of the visual Wulst contained parvalbumin expressing neurons (Fig. 2a–f). The hyperpallium apicale (HA) showed a relatively homogeneous distribution with a moderate density of parvalbumin neurons. The nucleus interstitialis hyperpallii apicale (IHA) showed the highest density, transitioning into the hyperpallium densocellulare (HD) with a gradual decrease. Even lower

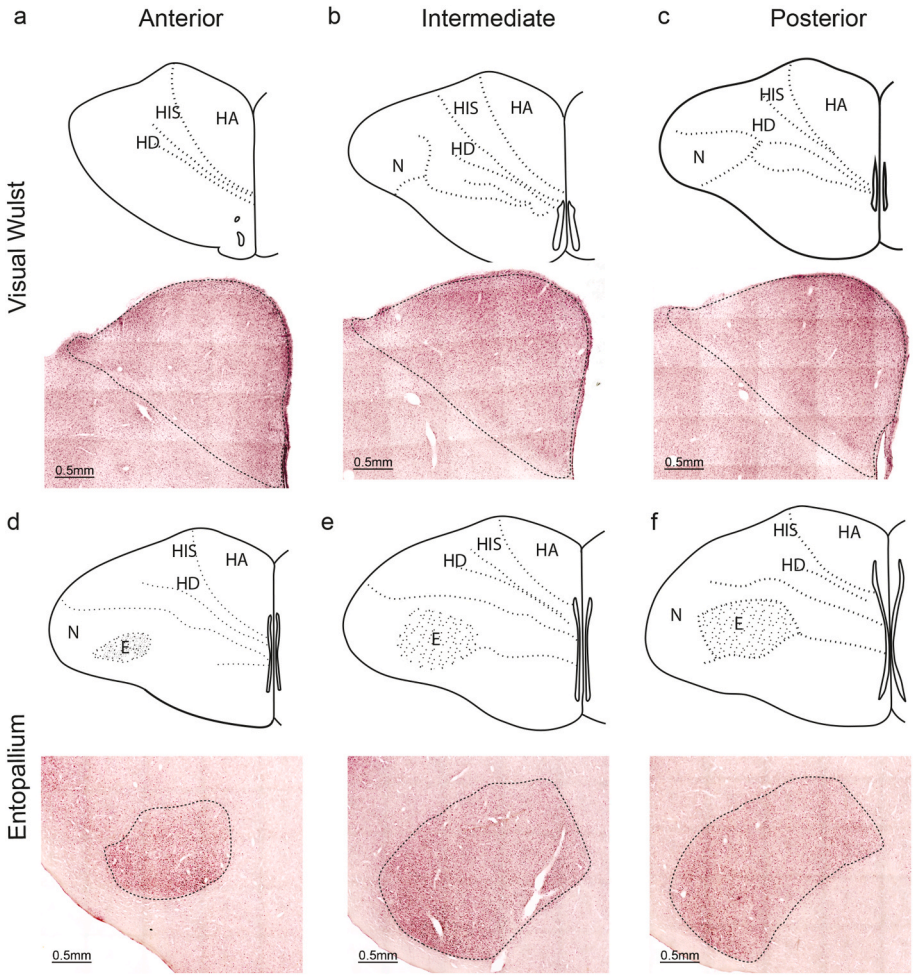

Fig. 2. Regions of interest. Schematic drawing and photomicrographs of coronal sections showing examples of parvalbumin labelling in the anterior, intermediate and posterior regions of the visual Wulst comprising HA, HIS and HD (a–c), and entopallium (d–f). HA – hyperpallium apicale, HIS – nucleus interstitialis hyperpallii apicale, HD – hyperpallium densocellulare, E – entopallium, N – nidopallium. Dashed black lines define the areas chosen for counting.

densities of parvalbumin neurons were present in the mesopallium.

The repeated measures ANOVA run on the entopallium showed a significant interaction of hemisphere*incubation condition ($F_{(1,32)} = 8.251$, $p = 0.007$). The same interaction also emerged for the visual Wulst ($F_{(1,33)} = 5.311$, $p = 0.028$). Thus, in both brain regions, parvalbumin neuron densities in the two brain hemispheres varied as a function of the incubation conditions (dark-vs. light-incubated eggs). All the other main effects and interactions were not significant for either of the two regions (Table 1).

Post hoc analysis of the entopallium revealed a significant brain hemispheric lateralisation, but only in the light-incubated chicks. Here, a higher density of parvalbumin neurons was present in the left entopallium compared to the right ($t_{(18)} = 2.440$, $p = 0.025$; see Fig. 3a and Table 2). In line with that, only the left entopallium showed a significant difference between the light-incubated and the dark-incubated group ($t_{(34)} = 2.811$, $p = 0.008$; Fig. 3a). In contrast, in the right entopallium, no significant difference emerged comparing the two groups ($t_{(34)} = 0.207$, $p = 0.838$) (Fig. 3a). The dark-incubated chicks did not show any significant difference in the densities of parvalbumin neurons between the left and right entopallium ($t_{(16)} = -1.349$, $p = 0.196$) (Fig. 3a).

Similar results were found in the visual Wulst. In the light-incubated group, we found higher parvalbumin neuron density in the left as in the right Wulst ($t_{(18)} = 2.707$, $p = 0.014$; Fig. 3b), while no significant difference between left and right Wulst was found in dark-incubated chicks ($t_{(17)} = -0.671$, $p = 0.511$; Fig. 3b). Moreover, a significant difference between the incubation conditions emerging only in the left hemisphere ($t_{(35)} = 2.128$, $p = 0.040$) but not in the right one ($t_{(35)} = -0.346$, $p = 0.732$) (Fig. 3b–Table 2).

3. Discussion

As hypothesised, we found that both the entopallium and the visual Wulst are sensitive to light exposure during the late embryonical development stage. This is shown by the emergence of a light-dependent asymmetry. In both these areas, we observed a significantly higher density of parvalbumin neurons in the left hemisphere of light-incubated chicks than in that of dark-incubated animals. As a consequence, an asymmetry emerged in chicks that had been exposed to light in egg. In the left hemisphere, the density of parvalbumin-labelled neurons was higher than in the right one. In contrast, the parvalbumin neurons of the right hemisphere were unaffected by light incubation and no clear asymmetry was present in the dark incubation group.

Embryonic exposure to light thus affects in a similar way the neuroanatomy of the telencephalic recipients of both the thalamofugal and the tectofugal pathways. This contrasts with the view that in chicks, light-dependent lateralisation effects would be limited to the thalamofugal pathway [9–11]. Contrary to primates, in birds, the thalamofugal pathway carries only a minority of the visual projections, while the rest reaches the telencephalon through the tectofugal pathway [12,18]. Since both pathways are shaped by light exposure in chicks, the effects of light incubation are more pervasive than previously believed, modulating almost the totality of visual inputs to the telencephalon [5,6]. This is consistent with the widespread lateralisation effects reported by behavioural studies in light-incubated chicks [5,50]. Our results show that neural composition in the entopallium and the Wulst can be modulated by environmental stimulation rather than being purely genetically predetermined. This is also consistent with recent electrophysiological evidence that in-egg light exposure increases visual responsiveness in both the telencephalic visual recipients of chicks [16,17]. Our findings further reveal the mechanisms by which prenatal stimulation can have long-lasting effects on neural and cognitive development, highlighting the crucial contribution of research in egg-laying species to this endeavour.

In both the left visual Wulst and entopallium, light incubation increased the density of the parvalbumin neurons. Stronger light effects on the left hemisphere are consistent with the avian visual pathways' anatomy: the left telencephalon receives mostly the visual projections from the right eye system, which received light stimulation during incubation. That this affects the density of parvalbumin neurons is a novel finding, the functional implications of which are not trivial. At least in mammals, parvalbumin neurons comprise different sub-classes of GABAergic inhibitory interneurons, including chandelier and fast-spiking basket cells [47]. These sub-classes

Table 1
Results of the repeated measures ANOVA for the entopallium (top) and visual Wulst (bottom). (*$p < 0.05$; **$p < 0.01$).

Main effects and interactions - Entopallium	
sex	$F_{(1,32)} = 3.128$, $p = 0.087$
Incubation condition	$F_{(1,32)} = 3.332$, $p = 0.077$
hemisphere	$F_{(1,32)} = 0.616$, $p = 0.438$
sex*incubation condition	$F_{(1,32)} = 0.046$, $p = 0.832$
hemisphere*sex	$F_{(1,32)} = 1.732$, $p = 0.198$
hemisphere*incubation condition	$F_{(1,32)} = 8.251$, $p = 0.007$**
hemisphere*sex*incubation condition	$F_{(1,32)} = 0.976$, $p = 0.330$
Main effects and interactions – Visual Wulst	+
sex	$F_{(1,33)} = 0.001$, $p = 0.980$
Incubation condition	$F_{(1,33)} = 1.129$, $p = 0.296$
hemisphere	$F_{(1,33)} = 1.973$, $p = 0.170$
sex*incubation condition	$F_{(1,33)} = 0.392$, $p = 0.536$
hemisphere*sex	$F_{(1,33)} = 1.091$, $p = 0.304$
hemisphere*incubation condition	$F_{(1,33)} = 5.311$, $p = 0.028$*
hemisphere*sex*incubation condition	$F_{(1,33)} = 1.067$, $p = 0.309$

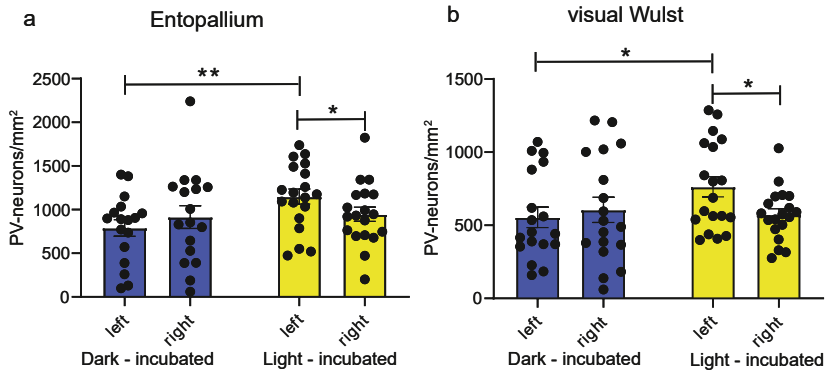

Fig. 3. Quantitative results. Parvalbumin neural densities in the entopallium (a) and visual Wulst (b). The barplots represent the mean and the standard error with individual values overlayed. Dark-incubated chicks are in blue, and light-incubated are in yellow. (*$p < 0.05$; **$p < 0.01$).

Table 2
Mean ± SEM values of parvalbumin-ir cell densities in the left and right hemispheres of the entopallium and visual Wulst in light- and dark-incubated groups.

	Entopallium		Visual Wulst	
	Left	Right	Left	Right
Light-incubated	1149.7 ± 86.7	947.1 ± 82	763.4 ± 68.9	572.5 ± 41.1
Dark-incubated	789.6 ± 94.3	915.3 ± 130.1	554.2 ± 70	605.7 ± 86.5

provide different functional contributions to visual processing in the mammalian cortex, including attention control and altering response gains in visual circuits [43–46]. Based on this, chicks' parvalbumin neurons might be involved in inhibitory functions and visual attention, which is a prominent function of the avian tectofugal system [14]. Light exposure acting on parvalbumin neurons could thus strengthen functions such as attention allocation and response inhibition in the left telencephalic visual recipients [51]. An increase in the density of inhibitory neurons in the left telencephalic visual recipients could also reflect a compensation mechanism. This could counterbalance the increased activity caused by light stimulation in the left than in the right hemisphere.

The fast-spiking property of parvalbumin neurons is consistent with our previous observation that light incubation often increased the rate and decreased the latency of single-unit responses in the left entopallium [17]. An increase in the density of fast-spiking parvalbumin neurons is a potential mechanism behind this effect. The situation, however, could be different in the visual Wulst, where our electrophysiological study revealed a more complex pattern of light effects [16]. Furthermore, much less is known about the functions and diversification of parvalbumin-expressing neurons in birds as opposed to mammals. Even in mammals, it has been suggested that not all mammalian parvalbumin-expressing neurons have an inhibitory function in cortical circuits [42]. We must therefore remain open to the possibility that some of the parvalbumin-expressing cells we stained could be excitatory in nature [50–53]. Lastly, in mammals, the maturation of parvalbumin neurons plays a crucial role in the neural plasticity caused by experience during critical periods [47,48]. Similarly, modulation by parvalbumin neurons has been reported during a sensitive period of birdsong learning in zebra finches [54]. In chicks, during the sensitive period for filial imprinting [55], the expression of immediate early genes like c-Fos can be used to visualise transitory plastic changes induced by learning in the intermediate medial mesopallium. Importantly, this transient increase in c-Fos expression is limited to parvalbumin expressing GABAergic neurons, as opposed to calbindin expressing ones [56]. Based on this, the parvalbumin neurons may be involved in the plasticity of neural circuits during the sensitive period when light causes asymmetries in behaviour and visual projections in chicks [1,8,29]. In the visual Wulst parvalbumin-neuron activation has also been reported during filial imprinting in chicks [57].

An increasing number of studies have reported behavioural and neuroanatomical lateralisation in chicks that had been dark-incubated [29–35,37–40]. Recent studies provide also evidence for spontaneous lateralisation of visual neurons' responses in both the Wulst and the entopallium [16,17]. Morandi-Raikova et al. [41] found a significant, although not very pronounced, rightward lateralisation in the distribution of entopallial parvalbumin neurons. In contrast, in the present study, chicks incubated in darkness did not exhibit significant asymmetry in the density of parvalbumin neurons in either brain region. Moreover, the absolute density of parvalbumin neurons in the entopallium was substantially higher here compared to our previous study. This could be due to a number of reasons, such as the use of animals hatched from different batches and born in different periods of the year. Being a developing model, domestic chickens are particularly sensitive to environmental occurring outside of the research lab (eggs are purchased from commercial hatcheries since chickens are not bred in our experimental facilities). Moreover, this discrepancy in absolute values could be due also to the specific threshold settings for automated counting, as well as to stainings of different intensity obtained through the use of different batched of antibodies and reagents. Notably, automated measurements using ImageJ are inherently approximations and not absolute measurements of cell densities. For all these reasons, it is generally not recommended to run direct numerical comparison of the values obtained in different independent experiments, across which confounding factors cannot be matched. Indeed,

absolute densities are not relevant for the question we are investigating, which pertains the comparison of relative densities between hemispheres and across conditions.

Overall, with regards to our research question, it is important to highlight that in the current study, dark-incubated chicks displayed a qualitatively similar pattern to that reported by Ref. [41]. While this difference was not statistically significant, at the qualitative level we find that more neurons expressing parvalbumin in the right hemisphere of dark incubated chicks, for both the visual Wulst and the entopallium (see Table 2). Our current data distribution, however shows much larger variability than in our previous study [41], which could account for the absence of statistically significant lateralisation effects (please note that also in our previous study, we reported a relatively small lateralisation effect compared to previous studies). The lack of significant lateralisation found in the current study for dark incubated chicks could also be due to a different quantification approach. Here, we conducted automated counting of stained neurons across the entire entopallium. Morandi-Raikova et al., [41] estimated the parvalbumin densities by counting cells at the locations with the highest density within various subregions of the entopallium. This might have led to discrepancies in the final estimations achieved in that and the present study. It is also worth noting that visual responses have lower latencies in the right than in the left entopallium of dark-incubated chicks [17]. As discussed above, this fits well with the hypothesis of a higher number of fast-spiking parvalbumin neurons in the right hemisphere. Overall, while based on the current results, we cannot confirm the lateralisation of parvalbumin neurons in dark-incubated chicks, we still consider the presence of neuroanatomical lateralisation in the entopallium of dark-incubated chicks to be very likely. Future studies should thus investigate this with a broader array of cell markers, to confirm whether anatomical lateralisation can be found in the entopallium of dark-incubated chicks.

Parvalbumin is often used as an anatomical marker in mammals and in birds [58–62]. In birds, it can stain the entopallium, being particularly abundant in this structure. In pigeons and zebra finches, parvalbumin staining allows the delineation of a border between the entopallial core and the surrounding belt region [52,63–66]. In chicks, we can clearly see a high density of parvalbumin neurons in the entopallium. However, we are not able to delineate the borders of the belt. Instead we observed a gradual decrease in parvalbumin cell density in the region surrounding the entopallium. Very low densities of parvalbumin neurons are also present in the nidopallium. This could reflect species-specific anatomical differences. However, it is more likely that the belt region is not fully formed in newly hatched chicks. In the visual Wulst, on the other hand, we observe a similar distribution of parvalbumin neurons across the different layers, as reported in zebra finches [52].

Paradoxically, the two main bird species used to study how light affects the development of lateralisation exhibit opposite asymmetries in the two visual pathways to the telencephalon, despite showing similar behavioural lateralisation profiles (for a review, see Ref. [6]). In pigeons, functional and neuroanatomical lateralisation caused by light exposure is well documented in the tectofugal pathway. This includes stronger recrossing projections from the right tectum to the left rotundus than from the left tectum to the right rotundus [66], providing a potential advantage to the left entopallium for integration of information from the two eyes [6]. In contrast, in pigeons thalamofugal projections do not seem to be lateralised [6,67]. Our current study helps resolve this apparent paradox by demonstrating light-dependent lateralisation of the entopallium in chicks. However, species-specific differences should not be overlooked. While chickens are a precocial species, pigeons are altricial. Consequently, there is a difference in the developmental stage of the embryos shortly before hatching, which affects how light incubation can influence their asymmetries. Indeed, pigeons exhibit a secondary, post-natal sensitive period during which monocular occlusion, and consequently asymmetric light exposure, can further influence asymmetries [6].

In conclusion, our results show that prenatal experience can influence neural compositions in the entopallium and the visual Wulst and underscore the significance of research in birds for understanding these mechanisms.

3.1. Limitations of the study

The main limitation of the current study is that we restricted our observation to only one cell type. While our results provide the first evidence of the effects of light incubation at the cellular level, this study only investigates a sub-population of GABAergic neurons. Since plasticity during neural development involves multiple cell types regulating the balance between inhibition and excitation (see, e.g. Ref. [68]), we expect the effects of prenatal sensory stimulation to extend to other cell types. Our current knowledge of the neural circuits and cell composition of the avian visual pallium is, however, still very limited. Another partial limitation is that we studied newly hatched chicks, precluding us to test whether the asymmetries we detected persist during subsequent postnatal development. However, the asymmetry of chicks' thalamofugal visual projections is known to be a transient trait, which can be observed in the first days after hatching and has disappeared by three weeks of age [69]. Thus, if we had conducted our study in older, visually-experienced animals, we would not have been able to investigate the selective role of pre-natal visual stimulation in the early development of brain lateralisation, which we could do here in visually naïve newborn animals. Despite these limitations, we believe that this first simple finding can pave the way to further investigation of the mechanisms that regulate neural circuit development in birds.

4. Methods

4.1. Subjects and experimental procedures

Overall, we used 40 (20 male) domestic chicks (*Gallus gallus*, Aviagen ROSS 308), obtained as fertilised eggs from a commercial hatchery (Crescenti Società Agricola S.r.l.—Allevamento Trepola—cod. Allevamento 127BS105/2) and incubated in standard conditions (37.7 °C, 60 % humidity). Chicks hatched all together in darkness and remained in the incubator until the first day after hatching, when they were perfused one at a time.

4.1.1. Experimental treatment

During incubation, the eggs were assigned to two groups that underwent different incubation conditions. The eggs of the 'dark-incubated' group (N = 20, M = 10) were maintained in darkness and never exposed to any light source from E0 to E21 (embryonic days). Eggs assigned to the 'light-incubated' group (N = 20, M = 10) were exposed to light from the morning of day E18 to the evening of E19 (corresponding to the sensitive period during which light-dependent lateralisation in chicks can be induced, Rogers, 1982). The eggs of this group were stimulated with a light intensity of 1036 lx, emitted by 15 LED lamps (270 lm) on a plastic panel (38 × 38 cm) suspended in the incubator above the eggs.

4.1.2. Histology and immunohistochemistry

On the first day after hatching, all animals were euthanised by an intramuscular injection of 0.04 ml ketamine/xylazine solution (1:1 ratio of ketamine 10 mg/ml and xylazine 2 mg/ml). After the injection, we performed transcardial perfusion through the left ventricle with phosphate-buffered saline (PBS; pH = 7.4, 0.1 mol, 0.9 % sodium chloride, 4 °C) and paraformaldehyde (4 % PFA in PBS, 4 °C). The severed heads were further fixated being put in a solution of 4 % PFA/PBS for a minimum of two days. To obtain consistent orientation, the brains were removed from the skulls using the method described in Kuenzel and Masson's chick's brain atlas [70] at an angle of 45 °C. Once all the brains were extracted, the left and the right hemisphere were separated. Each hemisphere was embedded in 7 % gelatine mixed with egg yolk, followed by at least 48 h of post-fixation in a solution of 4 % PFA with 20 % sucrose, plus additional 48 h in a solution of 0.4 % PFA/PBS with 30 % sucrose. We cut four sets of 40 μm thick coronal sections: one set for parvalbumin (PV) labelling and three as backups. Each of the following steps was preceded by cold PBS washes. To deplete endogenous peroxidase activity, we treated the free-floating brain sections for 20 min with 0.3 % H_2O_2 in PBS. We then incubated the sections at room temperature with 3 % normal horse serum (S-2000; Vector Laboratories, Burlingame, CA, USA) in PBS/Triton (0.3 % Triton in PBS) for 30 min, to block nonspecific binding sites. We then incubated the sections in a solution containing anti-parvalbumin antibody (produced in goat, 1:2000, SAB2500752-100UG, Sigma-Aldrich, St. Louis, MO, USA), with 0.1 % Bovine Serum Albumin (BSA, SP-5050, Vector Laboratories, Burlingame, CA, USA) in PBS, placed on a rotator at 5 °C for 48 h. A biotinylated anti-goat solution (BA-9500, Vector Laboratories, Burlingame, CA, USA) in PBS was used as the secondary antibody solution and applied for 60 min at room temperature. The signal was amplified using the ABC kit (Vectastain Elite ABC kit, PK 6100; Vector laboratories), and the visualization of parvalbumin was achieved with the VIP substrate kit for peroxidase (SK-4600; Vector Laboratories). To verify the specificity of the immunolabeling, standard controls omitting the primary antibody were conducted, demonstrating complete elimination of the signal. As a last step, the sections were mounted on gelatine-coated slides, all with the same orientation (regardless of the hemisphere), dried (at 50 °C), gradually dehydrated in ethanol, and coverslipped with Eukitt (FLUKA). This ensured that the following analyses could be conducted without knowledge of the hemisphere, which was not revealed by its orientation.

4.2. Brain analysis

All the analyses of the brain sections were conducted blind to the experimental condition and hemispheres with the use of a Zeiss microscope, equipped with a × 20 objective magnification and a numerical aperture of 0.5. A digital camera (Zeiss AxioCam MRc5) was connected to the microscope. The Zeiss imaging software ZEN was utilized for image acquisition. The camera's exposure time and the microscope's light conditions remained constant for all photographs. Parvalbumin immunoreactive (PV-ir) cells were quantified automatically using the software ImageJ [71]. A total of 10 brain sections for each region of interest (entopallium and visual Wulst) were chosen from each hemisphere of each subject to undergo the automated quantification. The selection of sections was based on the presence of anatomical landmarks and on the shape of the coronal sections, which were aligned with the Kuenzel and Masson atlas images [70]. For the entopallium we referred to the images at the A (anterior) coordinates of A9.6 to 11.8; for the visual Wulst, we referred to the images at A10.2 to A13.6 (Fig. 2). In the Kuenzel and Masson atlas [70], coordinates were estimated in chicks of two weeks of age (body weight 300–325 g). On the contrary, the chicks used in our study were newly-hatched and had an average weight of approximately 46 g, resulting in different anterior coordinates. For the automated quantification, the areas of the images corresponding to the regions of interest (entopallium and visual Wulst) were cropped out with the cropping tool in ImageJ. During this procedure, the overall area of each cropped region was quantified for the subsequent normalisation of the values. Counting of PV-ir cells was then performed using the "analyse particle" function of ImageJ. All photos were analysed with a predefined macro, where the image was transformed into 8bit, with the threshold set to 120, circularity of particles to 0.5–1.0 and particle size to 60–1200. The cell densities obtained from the 10 brain sections were averaged for each measured area and normalised to 1 mm^2. The individual bird means were employed for further statistical analysis.

4.3. Statistical analysis

A repeated measures ANOVA was performed on the density of PV-ir cells in each region of interest. The ANOVA included a within-subject factor (*hemisphere*, 2 levels: left, right) and two between-subject factors (*incubation condition*, 2 levels: dark-incubated, light-incubated; *sex*, 2 levels: males, females). For the post-hoc analyses, paired t-tests were performed to compare PV-ir cell densities between the two hemispheres. In contrast, independent samples t-tests were used to compare cell densities between the two incubation conditions. All statistical analyses were performed with the software R v.4.0.4 (R Core Team, 2021), while the graphs were created with the software GraphPad Prism 8.

CRediT authorship contribution statement

A. Morandi-Raikova: Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **R. Aydın:** Writing – review & editing, Investigation. **C.D. Corrales-Parada:** Writing – review & editing, Investigation, Formal analysis. **H.M. Rowland:** Writing – review & editing, Funding acquisition, Conceptualization. **G. Vallortigara:** Writing – review & editing, Funding acquisition, Conceptualization. **U. Mayer:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **O. Rosa-Salva:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Ethic statement

All procedures and experiments were carried out in accordance with the ethical guidelines current to European and Italian laws (including the European Communities Council Directive 2010/63/EU) and with the ethical standards of the University of Trento. The experiments and experimental procedures were licensed by Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanità Pubblica Veterinaria (Italian Ministry of Health) (permit number: 560/2018-PR).

Data availability statement

All data, as well as the script used for the analyses, are available in the supplementary materials.

Research data

All data used for the analyses can be found in the supplementary material S2.

Declaration of generative AI in scientific writing

We used Grammarly and ChatGPT (OpenAI) to improve the grammar and clarity of some sentences. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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