AAV1 is the optimal viral vector for optogenetic experiments in pigeons (*Columbia livia*)

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Although optogenetics has revolutionized rodent neuroscience, it is still rarely used in other model organisms as the efficiencies of viral gene transfer differ between species and comprehensive viral transduction studies are rare. However, for comparative research, birds offer valuable model organisms as they have excellent visual and cognitive capabilities. Therefore, the following study establishes optogenetics in pigeons on histological, physiological, and behavioral levels. We show that AAV1 is the most efficient viral vector in various brain regions and leads to extensive anterograde and retrograde ChR2 expression when combined with the CAG promoter. Furthermore, transient optical stimulation of ChR2 expressing cells in the entopallium decreases pigeons’ contrast sensitivity during a grayscale discrimination task. This finding demonstrates causal evidence for the involvement of the entopallium in contrast perception as well as a proof of principle for optogenetics in pigeons and provides the groundwork for various other methods that rely on viral gene transfer in birds.

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Birds are valuable model organisms for comparative neuroscientific research as different avian species provide unique research opportunities. While crows have excellent cognitive abilities that are on par with primates, zebra finches and other songbirds are widely studied as a model for language. Furthermore, pigeons have outstanding visual capabilities, navigational skills, and represent classic animal models for research on learning and memory. Birds are capable of those behaviors although their brains are organized radically different than those of mammals. While the mammalian neocortex is organized in six layers, the pallium of birds is structured in a nuclear fashion. There is, however, cumulative evidence suggesting that, although the avian and mammalian brains differ on the macroscopic level, the local circuitry within their sensory systems is highly comparable indicating conserved principles in sensory systems organization. Finding those invariant properties can help to establish circuit–function relationships that highlight general principles of the brain. Thus, comparative research is indispensable to understand how brain functions emerge from structure.

Unfortunately, much of what we know about the function of the avian pallium, especially for the sensory system, comes from purely correlative methods or lesion studies that lack spatial and temporal precision. However, in order to study the function of neuronal networks, methods that are able to control neuronal activity precisely are mandatory. This ambitious goal was first achieved with optogenetics, allowing researchers to activate or silence specific networks with high temporal and spatial resolution through the integration of artificial light-sensitive ion channels into the cell membrane. Optogenetics brought a revolution to rodent research and has been established in other species such as primates, zebra finches and ferrets over the last years. However, the functional implementation of optogenetics in other species has been challenging. While several studies have been able to show the effects of electrical microstimulation during decision-making or perception in primates, studies using optogenetic stimulation have sometimes failed to find behavioral effects, despite reporting physiological changes. One explanation that has been provided for the absence of behavioral effects is insufficient viral efficiency resulting in low amounts of protein expression. As one key component in optogenetics is the expression of light-sensitive ion channels that are typically transferred into the brain via viral vectors, the efficiency of those constructs has to be carefully investigated prior to the application of optogenetics in vivo. Viral vector efficiency can vary considerably between brain areas and species highlighting the need for viral transfection studies in various model organisms. Especially in birds, viral transfection has proven to be difficult, possibly due to properties of the immune system. Although optogenetics has been already used in some areas of the zebra finch song system, the efficiency of different viral constructs was not compared within these and other brain areas such as the visual system. In this study, we compared the efficiency of six viral constructs in their ability to transduce neurons in the pigeon forebrain and found that AAV1 is the optimal viral construct for optogenetic experiments in birds. As it has been complicated to induce behavioral effects with optogenetics in primates due to insufficient protein expression, we furthermore confirmed that stimulation of channelrhodopsin (ChR2) leads to physiological as well as behavioral effects in pigeons. In our study, we have focused on the visual system, as birds are highly visual animals and recent studies have indicated that characteristic properties of sensory systems, such as a columnar and laminar organization, are conserved between birds and mammals. We targeted the entopallium, which is the most important primary visual area in the pigeon telencephalon and which has been associated with discrimination of form, pattern, color, motion, and luminance. We employed a grayscale visual discrimination task and found that optogenetic stimulation within this structure resulted in impaired contrast perception indicated by decreased discrimination accuracy. With this study, we provide a proof of principle for optogenetics in pigeons as well as further insights into the function of the entopallium.

Results

Comparative transduction analysis of adeno-associated viral vector serotypes 1, 5, and 9 in the avian forebrain. In a first step, we wanted to determine the most efficient adeno-associated viral vector (AAV) for optogenetic experiments in the visual system of pigeons. For the viral transfection study, we compared the efficiency of AAV serotype 2 pseudotyped with serotype 1 (here referred to as AAV1), pseudotyped with serotype 5 (here referred to as AAV5), and pseudotyped with AAV9 (here referred to as AAV9). All serotypes were combined with either the human synapsin 1 gene (hSyn) promoter or the chicken beta-actin (CAG) promoter and were injected into the entopallium of the pigeon brain (Fig. 1a, b). Each construct was injected into at least five separate hemispheres of three pigeons (for more information see Table 1). After 6 weeks of transfection, pigeons were sacrificed and immunohistochemical stainings against ChR2 were performed in all brain slices containing the entopallium. The counterstaining was performed to allow for an equal comparison between the serotypes, as the hSyn promoter were tagged with eYFP, whereas serotypes with the CAG promoter were tagged with mCherry. Moreover, the amount of transgene expression can be underestimated when analyzing native fluorescence, as the signal increases with counterstainings (see Supplementary Fig. 1 and Method section for more detail). The efficiency of all six constructs was assessed based on the number of ChR2 expressing somata and axons in relation to the size of the entopallium (Fig. 1b).

We found that the construct had a significant effect on the number of ChR2 expressing cells (one-way ANOVA with Welch correction, $F_{(4,9,511)} = 14.949, p < 0.001$, Fig. 1c). While there was no difference in the number of ChR2 expressing cells between injections of AAV1-hSyn-Chr2 and AAV1-CAG-Chr2 (Bonferroni corrected pairwise comparisons, $\alpha = 0.01$, Fig. 1c, e, h), both constructs were significantly more efficient than all other serotypes including AAV5-hSyn-Chr2 ($p \leq 0.001$, Fig. 1c, f), AAV5-CAG-Chr2 (AAV1-hSyn-Chr2: $p = 0.003$, AAV1-CAG-Chr2: $p = 0.025$, Fig. 1c, i) and AAV9-CAG-Chr2 ($p \leq 0.001$, Fig. 1c, j, for mean values and SEM see Table 1). Furthermore, the construct had a significant effect on the percentage of Chr2 expressing area within the entopallium (one-way ANOVA with Welch correction, $F_{(4,9,512)} = 12.791, p = 0.001$, Fig. 1d). There was no significant difference in the percentage of the transduced area between injections of AAV1-hSyn-Chr2 and AAV1-CAG-Chr2 (Bonferroni corrected pairwise comparisons, $p = 0.402$, Fig. 1d, e, h). However, AAV1-CAG-Chr2 resulted in a significantly greater area expressing Chr2 than all other serotypes including AAV5-hSyn-Chr2 ($p < 0.001$, Fig. 1d, f), AAV5-CAG-Chr2 ($p = 0.001$, Fig. 1d, i) and AAV9-CAG-Chr2 ($p < 0.001$, Fig. 1d, j, for mean values and SEM see Table 1). Moreover, the Chr2 expressing area was significantly greater for AAV1-hSyn compared to AAV5-hSyn ($p = 0.039$, Fig. 1d–f). Furthermore, transduction efficiencies of the serotypes followed a similar pattern when the Chr2 expressing area was compared to the size of the entopallium only in slices with transduction (see Supplementary Fig. 2). The expression pattern following injections of AAV9 differed from all the other serotypes, as for AAV9-hSyn-Chr2 only two Chr2 expressing...
Fig. 1 Comparative transduction analysis of AAV1, AAV5, and AAV9 in combination with the hSyn and CAG promoters. 

a Schematic illustration of the injection area and analysis type. For the first analysis, all somata were counted that displayed ChR2 expression. 
b Schematic illustration of the injection area and the analysis type. For the second analysis, the area of ChR2 expressing somata, dendrites, and axons was measured and compared to the total area of the entopallium. 
c Quantitative comparison of all tested constructs in their ability to drive transgene expression in somata of the entopallium. AAV1-hSyn-ChR2, as well as AAV1-CAG-ChR2, were significantly more efficient than all other tested constructs. 
d Percentage of ChR2 expressing entopallial area for all tested constructs. AAV1-CAG was significantly more efficient than all other tested constructs. All AAVs with hSyn promoter are depicted in gray and all AAVs with CAG promoter are depicted in blue. 
e–j Qualitative pictures of ChR2 expression following injections of e AAV1-hSyn-ChR2 (n = 5), f AAV5-hSyn-ChR2 (n = 5), h AAV1-CAG-ChR2 (n = 6), i AAV5-CAG-ChR2 (n = 5), and j AAV9-CAG-ChR2 (n = 5). All scale bars represent 500 µm. Error bars represent the standard error of the mean (SEM) and dots represent the raw data, ***p < 0.001, **p < 0.01, *p < 0.05. 

Abbreviations: AAV adeno-associated viral vector, hSyn human synapsin 1 gene promoter, CAG chicken beta-actin promoter.
cells were found in one of five cases (Fig. 1g), while AAV9-CAG-ChR2 led to reliable ChR2 expression in all five cases but also to neurotoxicity (Fig. 1j, will be discussed in detail later).

While AAV1-CAG-ChR2 and AAV1-hSyn-ChR2 did not differ in their efficiency to drive ChR2 expression within the entopallium, they differed in other properties such as anterograde and retrograde expression of ChR2 in target and input structures of the entopallium. We found that AAV1-CAG-ChR2 injections into the entopallium (Fig. 2a) resulted in extensive ChR2 expression in fibers projecting to target structures such as the ventrolateral mesopallium (MVL, Fig. 2b, d) and nidopallium intermedium (NI, Fig. 2b, d). In contrast to this, only little ChR2

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**Table 1: Comparative transduction analysis of AAV1, AAV5, and AAV9 in combination with the hSyn and CAG promoter.**

| Serotype      | Number of ChR2 expressing cells | Transduced entopallial area in % |
|---------------|---------------------------------|----------------------------------|
| AAV1-CAG      | 12405 ± 2230 SEM, n = 6         | 15.89 ± 3.17 SEM, n = 6          |
| AAV1-hSyn     | 15028 ± 3170 SEM, n = 5         | 9.73 ± 2.62 SEM, n = 5           |
| AAV5-CAG      | 3782 ± 690 SEM, n = 5           | 2.79 ± 0.81 SEM, n = 5           |
| AAV5-hSyn     | 406 ± 125 SEM, n = 5            | 0.18 ± 0.05 SEM, n = 5           |
| AAV9-CAG      | 574 ± 83 SEM, n = 5             | 1.67 ± 0.38 SEM, n = 5           |

For all serotypes, the mean number of ChR2 expressing cells and the mean ChR2 expressing entopallial area in % was assessed for at least five injections into separate hemispheres of at least three pigeons.

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Fig. 2: Anterograde and retrograde ChR2 expression in target and input structures of the entopallium. 

a. Schematic illustration of the injection area in the entopallium. 
b. Anterograde ChR2 expression in fibers projecting to the NI/MVL following injections of AAV1-CAG-ChR2. 
c. Little anterograde ChR2 expression following injections of AAV1-hSyn-ChR2. 
d. Schematic illustration of anterograde labeling in fibers projecting to the NI/MVL. 
e. Schematic illustration of anterograde labeling in fibers projecting to the striatum. 
f. Anterograde ChR2 expression in fibers projecting to the striatum following injections of AAV1-CAG-ChR2. 
g. Little anterograde ChR2 expression in the striatum following injections of AAV1-hSyn-ChR2. 
h. Schematic illustration of ChR2 expression in the nucleus rotundus, which is the main input region of the entopallium. 
i. Extensive retrograde ChR2 expression in the nucleus rotundus following injections of AAV1-CAG-ChR2 into the entopallium. 
j. Scarce retrograde ChR2 expression in the nucleus rotundus following injections of AAV1-hSyn-ChR2. All scale bars represent 200 µm. Note that images f, g, i, j have been rotated 90° to the left. Abbreviations: Arco arcopallium, E entopallium, GP globus pallidus, LSt lateral striatum, MSt medial striatum, MVL ventrolateral mesopallium, NI nidopallium intermedium, nRT nucleus rotundus, TrO tractus opticus.
expression could be detected in NI and MVL following injections of AAV1-hSyn-ChR2 (Fig. 2c, Supplementary Table 1, Supplementary Figs. 3 and 4). A similar pattern of expression could be seen in the striatum (Fig. 2e), which is another target structure of the entopallium. We found extensive ChR2 expression in fibers projecting to the striatum following injections of AAV1-CAG-ChR2 into the entopallium (Fig. 2f), which was weaker for AAV1-hSyn-ChR2 injections (Fig. 2g, Supplementary Table 1, Supplementary Figs. 5 and 6). The main input region to the entopallium is the diencephalic nucleus rotundus (Fig. 2h). Injections of both AAV1-hSyn-ChR2 and AAV1-CAG-ChR2 into the entopallium resulted in ChR2 expression in neurons in the nucleus rotundus after 6 weeks of expression time (Supplementary Table 1, Supplementary Figs. 7 and 8). However, retrograde ChR2 expression was more extensive after AAV1-CAG-ChR2 injections (Fig. 2i) than after AAV1-hSyn-ChR2 injections (Fig. 2j), especially after longer expression times of 6 months (Fig. 2i, j, Supplementary Table 1, Supplementary Figs. 7 and 8).

The efficiency of AAV1-CAG-ChR2 was furthermore investigated with single injections of 5 µl each in other regions of the avian pallium to assess its brain-wide usefulness for optogenetic experiments in birds. AAV1-CAG-ChR2 was able to drive ChR2 expression in the hippocampus (Fig. 3a, b), the nidopallium caudolaterale (Fig. 3a, c), the entopallium (Fig. 3d, e), the globus pallidus (Fig. 3d, f), the hyperpallium apicale (Fig. 3g, h), and the medial striatum (Fig. 3g, i).

Since the CAG promoter can drive transgene expression in all cell types, the extent to which ChR2 expressing cells were also co-localized with a neuronal marker was investigated. Therefore, we performed combined immunohistochemical stainings against ChR2 and NeuN to visualize neurons (Fig. 4a–d). We found that AAV1-CAG-ChR2 led to significantly more transgene expression in neurons than in glial cells (neurons: 92.19% ± 1.99 SEM, n = 5; glial cells: 7.81% ± 1.99 SEM, n = 5; Z = −2.023, p = 0.043, Fig. 4a–d). This quantification could not be performed for AAV9-CAG-ChR2 injections, as this serotype resulted in a severe reduction of NeuN expression within the ChR2 expressing area (AAV1-CAG: 1871 NeuN+ cells per mm² ± 41 SEM, n = 6; AAV9-CAG: 1102 NeuN+ cells per mm² ± 77 SEM, n = 4; Z = −2.558, p = 0.01, Fig. 4e–h), suggesting neurotoxicity of this serotype. To further investigate the possible neurotoxicity of this serotype, we performed combined stainings against ChR2 and glial fibrillary acidic protein (GFAP) for AAV9-CAG-ChR2 and AAV1-CAG-ChR2, as GFAP is a marker for astrocyte activation after stress or injury to the brain45,46. We found that injections of AAV1-CAG-ChR2 led to extensive ChR2 expression in the injection area, while GFAP expression was low and occurred mainly around blood vessels (ChR2 expression: 8.5% ± 1.6 SEM, 200 µm).

Fig. 3 AAV1-CAG is efficient in driving transgene expression in various regions of the pigeon forebrain. a Schematic illustration of the hippocampus (HC) and nidopallium caudolaterale (NCL). b ChR2 expression in HC. c ChR2 expression in NCL. d Schematic illustration of the entopallium (E) and globus pallidus (GP). e ChR2 expression in E. f ChR2 expression in GP. g Schematic illustration of the hyperpallium apicale (HA) and the medial striatum (MSt). h ChR2 expression in HA. i ChR2 expression in MSt. All scale bars represent 200 µm. Abbreviations: APH area parahippocampalis, E entopallium, GP globus pallidus, HA hyperpallium apicale, HC hippocampus, LFB lateral forebrain bundle, LSt lateral striatum, MSt medial striatum.
n = 3; GFAP expression: 1.5% ± 0.5 SEM, n = 3; Fig. 4i–l, Supplementary Fig. 9). In contrast to this, injections of AAV9-CAG led to weak ChR2 expression, while GFAP expression was strong and occurred throughout the injection site (ChR2 expression: 1.5% ± 0.6 SEM, n = 3; GFAP expression: 3.7% ± 0.9 SEM, n = 3; Fig. 4m–p, Supplementary Fig. 9). This supports the idea of neurotoxicity for AAV9-CAG-ChR2.

**Physiology of ChR2 expressing cells during optical stimulation investigated with in vivo electrophysiology and immediate early gene expression.** The physiology of ChR2 was assessed in two experiments. In the first experiment, pigeons were anesthetized, and extracellular single-unit recordings and optical stimulation were performed simultaneously within the entopallium of two pigeons in four hemispheres. The goal of these specific experiments was to preselect cells that were responsive to light and assess their characteristics. Therefore, a constantly repeated light pulse of 1 s duration was presented to evoke spikes during the advancement of the electrode. When a responsive cell was encountered, we used a sequence of blue light pulses (465 nm) of different durations (1, 10, 100, 200, and 500 ms; see Supplementary Table 2) for optical stimulation and repeated this sequence (sweeps, Fig. 5a(i)) three times. In total, the neuronal responses of nine cells were recorded during optical stimulation (Fig. 5, Supplementary Figs. 10–17). In all preselected cells that were recorded, a significant number of action potentials could be evoked by optical stimulation and all cells showed a significant response when the duration of the stimulation was at least 10 ms (one-sided Wilcoxon rank-sum test, all p values < 0.05, for details, see...
Supplementary Table 3). To assess the variability of the evoked neuronal responses, we compared the spikes evoked during these sweeps using a nonparametric analysis of variance (Kruskal–Wallis test). If we found significant differences between the sweeps a Bonferroni-corrected multiple comparison test was conducted. In only two out of the nine cells, significant differences between sweeps in some conditions were detected (see Supplementary Table 3). The differences were found in stimulation trials of longer duration (>100 ms). For stimulation durations below 100 ms no significant differences could be detected (the significance is indicated in Supplementary Table 3 and in the single-cell raster plots). Overall, the evoked responses were robust.

The recorded cells differed in their overall response properties (Supplementary Figs. 10–18). We found cells that responded throughout the entire optical stimulation period with a constant amount of spikes, albeit showing a pronounced peak of activation at the onset of light stimulation (Fig. 5, Supplementary Figs. 15 and 17, Supplementary Fig. 18 cells 4, 7, and 9). Further, we found cells that weakened their responses over the course of prolonged stimulation (Supplementary Figs. 10–12, Supplementary Fig. 18 cells 1–3). Another response pattern that was found showed a sharp peak only during the onset of the stimulus (Supplementary Figs. 13, 14, and 16, Supplementary Fig. 18 cells 5, 6, and 8). After the electrophysiological experiments were conducted...
finished, histology was performed to check for ChR2 expression in the entopallium (Supplementary Fig. 19).

We decided to use a pulsed optical stimulation protocol of 40 Hz in our behavioral experiments, as some cells exclusively showed a sharp onset peak and pulses of 10 ms durations reliably evoked spikes in all recorded cells (pulse duration: 15 ms; inter-pulse interval: 10 ms). The physiological validity of the applied protocol was further verified in an additional experiment. Here, we investigated the functionality of ChR2 with immediate-early gene expression in awake pigeons (Fig. 6).

Following a sensory deprivation phase of one hour, pigeons were stimulated for a period of 30 min with alternating intervals of 5 min 40 Hz stimulation and 5 min no stimulation with orange light in one hemisphere and blue light in the other hemisphere (Fig. 6a). After that, the pigeons were sensory deprived for a further 60 min to allow for adequate c-Fos expression and subsequently transcardially perfused with PFA. Sensory deprivation before and after the experiment was performed to reduce stimulation unrelated c-Fos expression. Subsequently, stainings against the immediate early gene c-Fos were performed. The cellular activation was assessed at two stimulation sites in each hemisphere of three pigeons within the stimulated entopallium and in a control area within the visual wulst to make sure that both hemispheres show comparable levels of c-Fos expression in general. Blue light stimulation resulted in increased c-Fos expression beneath the cannula within the entopallium (Fig. 6b, c, f, i) compared to orange light stimulation (blue light stimulation: 515 cells ± 59 SEM, n = 6; orange light stimulation: 12 cells ± 3 SEM, n = 5; Z = 2.739, p = 0.004, Fig. 6b, g, h), although both hemispheres showed reliable ChR2 expression (Fig. 6d–f). In the control area within the visual wulst, there was no difference between orange and blue light stimulation, indicating that staining intensities were similar between the two hemispheres (blue light stimulation: 464 cells ± 107 SEM, n = 6; orange light stimulation: 585 cells ± 133 SEM, n = 5, Z = −0.548, p = 0.662, Fig. 6b).

Transient activation of ChR2 expressing cells in the entopallium reduces contrast sensitivity indicated by impaired
performance in a grayscale visual discrimination task. For all behavioral experiments, AAV1-CAG was used to deliver the ion channel ChR2 or the fluorescent protein tdTomato into neurons of the entopallium. To investigate the behavioral effect of optogenetic stimulation, an experimental group expressing ChR2 and a control group expressing tdTomato were bilaterally stimulated in the entopallium during the whole stimulus presentation phase or until the pigeon responded. Stimulation took place in 50% of the trials of a forced-choice visual discrimination task. The remaining 50% of the trials were void of optical stimulation (Fig. 7a).

In every trial of a session, pigeons were confronted with two grayscale pictures that varied in their luminance. The grayscale picture pairs could belong to five different stimulus classes (SC), depending on their luminance difference. SC1 consisted of grayscale pictures with the lowest luminance difference (mean luminance difference 5.81 cd/m², Supplementary Fig. 20). Thus, these stimuli were similar to each other and therefore hard to discriminate. In contrast, grayscale picture pairs of the SC5 showed the highest luminance difference (mean luminance difference 30.39 cd/m², Supplementary Fig. 20). Consequently, these stimuli were easier to discriminate (see more details in the
Fig. 7 Transient 40 Hz activation of ChR2 expressing cells in the avian entopallium reduces contrast sensitivity. a Schematic illustration of the experimental procedure. Pigeons were conditioned to discriminate grayscales of different stimulus classes (SC). SC1 consisted of grayscale pictures that were difficult to discriminate and SC5 consisted of grayscale pictures that were easy to discriminate. Pigeons were bilaterally stimulated in half of the trials in a given session and stimulation took place during the whole stimulus presentation phase or until the animal responded. b Visual discrimination performance of the control (n = 6) and experimental group (n = 6) displayed in one graph. Experimental group data can be seen in more detail in panel (c) and control group data can be seen in more detail in panel (d). The control and experimental group had comparable performances and a significant drop in performance could only be seen for optogenetic stimulation in the experimental group in SC3 and SC5. c Visual discrimination performance of the experimental group expressing ChR2 in the entopallium. Optogenetic stimulation reduced contrast sensitivity as indicated by a significant reduction of discrimination accuracy for stimuli in SC3, SC4, and SC5, but unimpaired discrimination performance in SC2 and SC1. d Visual discrimination performance of the control group expressing tdTomato in the entopallium. Optogenetic stimulation had no effect on contrast sensitivity as discrimination performance was comparable between stimulated and unstimulated trials in all stimulus classes. e, f Reaction times for stimulated and unstimulated trials of the experimental (n = 6) and control group (n = 6). There was no difference in reaction times between the different stimuli classes or between stimulated and unstimulated trials for both groups. e, f Mean performances of all sessions are plotted for all pigeons in individual colors. Stimulated and unstimulated performances within each pigeon have been connected with lines. Error bars represent the standard error of the mean (SEM), **p < 0.01, ***p < 0.001.
Discussion

In the current study, we investigated the efficiency of three AAVs (AAV1, AAV5, and AAV9) in combination with two different promoter systems (hSyn and CAG) in their ability to drive ChR2 expression in neurons within the entopallium, the first visual input structure of the avian forebrain. This comparison was performed to determine the optimal viral construct for optogenetic experiments in the visual system of birds. We found that AAV1 was the most efficient viral vector regardless of the promoter system as this vector transduced the greatest number of cells and showed the highest transfection density compared to the other AAV constructs. When AAV1 was used in combination with the CAG promoter, we observed extensive anterograde as well as retrograde labeling that was weaker when AAV1 was combined with the hSyn promoter. Since several studies have reported difficulties in producing behavioral effects with optogenetic stimulation in primates, we furthermore wanted to confirm that stimulation of ChR2 could produce physiological as well as behavioral effects in pigeons. The physiological effect of ChR2 was verified with combined optical stimulation and electro-physiological recordings as well as with immediate-early gene expression. The behavioral effect was investigated in a grayscale visual discrimination task. We could show that contrast sensitivity decreased when neural activity was temporarily increased in the visual discrimination task. We could show that contrast sensitivity decreased when neural activity was temporarily increased in the visual discrimination task. We could show that contrast sensitivity decreased when neural activity was temporarily increased in the visual discrimination task. We could show that contrast sensitivity decreased when neural activity was temporarily increased in the visual discrimination task.

The finding that AAV1 was the most efficient construct in transducing cells in the entopallium of pigeons is in line with several studies showing that this construct is also highly efficient in primates, cats, mice, and rats. Nevertheless, in primates, cats, mice, and rats, AAV5 seems to be even more efficient in driving transgene expression in cells of the visual wulst, the entopallium, the globus pallidus, the hippocampus, the striatum, and the nidopallium caudolaterale. Importantly, ChR2 expression following injections of AAV1 combined with the unspecific CAG promoter occurred mainly in neurons indicating that AAV1 has a natural tropism for this cell type. That AAV1 transfects mainly neurons has already been reported in other studies supporting the idea that this serotype is a useful tool for optogenetic experiments.

In birds, AAV1 has not been used in the visual system so far. However, AAV1 has recently been used in zebra finches in the song system to study how optogenetic stimulation of ventral tegmental area (VTA) axon terminals can guide learned changes in song. This study reported anterograde labeling in Area X following injections of AAV1-CAG into the VTA. This is in line with our finding that injections of AAV1-CAG result in extensive anterograde ChR2 expression in well-described target structures of the entopallium such as the NI, MVL, and the striatum. In addition, our study observed extensive retrograde ChR2 expression in input structures of the entopallium such as the nucleus rotundus. The projection from the diencephalic nucleus rotundus into the telencephalic entopallium is well known and ideal to test the retrograde properties of a viral vector as those two structures are located in different parts of the brain. Thus, it is impossible that the retrogradely labeled cells that have been observed in the nucleus rotundus are the result of injection leakage from the entopallium. The finding that AAV1 in combination with the CAG promoter leads to retrograde ChR2 expression is in line with transduction studies performed in mice and rats that have reported similar properties for this viral vector. Retrograde properties of AAV1 in birds have, to the best of our knowledge, so far not been reported. However, in the song system of zebra finches, AAV9 has been used to transduce cells retrogradely. Our findings indicate that AAV1 in combination with the CAG promoter provides a useful alternative for those who depend on commercially available products. Identifying anterograde as well as retrograde properties of AAVs in birds is crucial since this is a way of gaining specificity, which can be used for detailed circuit analyses.

We found that 20 Hz as well as 40 Hz blue light optical stimulation of ChR2 expressing neurons within the entopallium responses in rodents as well as in primates. Those studies concluded that AAV9 is able to transduce antigen-presenting cells (APC) and can trigger a cell-mediated immune response dependent on the immunogenicity as well as the expression level of the transgene. Our finding that the inflammatory response only occurred when AAV9 was used in combination with the CAG promoter, but not when used with the hSyn promoter, supports this idea. This is the case as the CAG promoter drives strong transgene expression in various cell types, whereas the hSyn promoter has been shown to be neuron-specific, making it unlikely that transgene expression occurred in APC.

Thus, it is still possible that AAV9 combined with other promoters provides a useful optogenetic tool. Indeed, several studies conducted in zebra finches use scAAV9 in combination with neurexin or CMV promoter in areas of the zebra finch song system. However, our results cannot easily be compared to these studies, as they used custom-built self-complementary AAVs (scAAVs), which differed not only in the integrated promoters but also in other aspects, as scAAVs do not require second strand synthesis but are immediately ready for replication and transcription.

For our purposes, AAV1-CAG was the most efficient construct and was therefore tested in other areas of the pigeon brain to investigate its brain-wide usefulness for optogenetic experiments. We found that AAV1-CAG was effective in driving transgene expression in cells of the visual wulst, the entopallium, the globus pallidus, the hippocampus, the striatum, and the nidopallium caudolaterale. Importantly, ChR2 expression following injections of AAV1 combined with the unspecific CAG promoter occurred mainly in neurons indicating that AAV1 has a natural tropism for this cell type. That AAV1 transfects mainly neurons has already been reported in other studies supporting the idea that this serotype is a useful tool for optogenetic experiments.

In birds, AAV1 has not been used in the visual system so far. However, AAV1 has recently been used in zebra finches in the song system to study how optogenetic stimulation of ventral tegmental area (VTA) axon terminals can guide learned changes in song. This study reported anterograde labeling in Area X following injections of AAV1-CAG into the VTA. This is in line with our finding that injections of AAV1-CAG result in extensive anterograde ChR2 expression in well-described target structures of the entopallium such as the NI, MVL, and the striatum. In addition, our study observed extensive retrograde ChR2 expression in input structures of the entopallium such as the nucleus rotundus. The projection from the diencephalic nucleus rotundus into the telencephalic entopallium is well known and ideal to test the retrograde properties of a viral vector as those two structures are located in different parts of the brain. Thus, it is impossible that the retrogradely labeled cells that have been observed in the nucleus rotundus are the result of injection leakage from the entopallium. The finding that AAV1 in combination with the CAG promoter leads to retrograde ChR2 expression is in line with transduction studies performed in mice and rats that have reported similar properties for this viral vector. Retrograde properties of AAV1 in birds have, to the best of our knowledge, so far not been reported. However, in the song system of zebra finches, AAV9 has been used to transduce cells retrogradely. Our findings indicate that AAV1 in combination with the CAG promoter provides a useful alternative for those who depend on commercially available products. Identifying anterograde as well as retrograde properties of AAVs in birds is crucial since this is a way of gaining specificity, which can be used for detailed circuit analyses.

We found that 20 Hz as well as 40 Hz blue light optical stimulation of ChR2 expressing neurons within the entopallium responses in rodents as well as in primates. Those studies concluded that AAV9 is able to transduce antigen-presenting cells (APC) and can trigger a cell-mediated immune response depending on the immunogenicity as well as the expression level of the transgene. Our finding that the inflammatory response only occurred when AAV9 was used in combination with the CAG promoter, but not when used with the hSyn promoter, supports this idea. This is the case as the CAG promoter drives strong transgene expression in various cell types, whereas the hSyn promoter has been shown to be neuron-specific, making it unlikely that transgene expression occurred in APC. Thus, it is still possible that AAV9 combined with other promoters provides a useful optogenetic tool. Indeed, several studies conducted in zebra finches use scAAV9 in combination with neurexin or CMV promoter in areas of the zebra finch song system. However, our results cannot easily be compared to these studies, as they used custom-built self-complementary AAVs (scAAVs), which differed not only in the integrated promoters but also in other aspects, as scAAVs do not require second strand synthesis but are immediately ready for replication and transcription.

For our purposes, AAV1-CAG was the most efficient construct and was therefore tested in other areas of the pigeon brain to investigate its brain-wide usefulness for optogenetic experiments. We found that AAV1-CAG was effective in driving transgene expression in cells of the visual wulst, the entopallium, the globus pallidus, the hippocampus, the striatum, and the nidopallium caudolaterale. Importantly, ChR2 expression following injections of AAV1 combined with the unspecific CAG promoter occurred mainly in neurons indicating that AAV1 has a natural tropism for this cell type. That AAV1 transfects mainly neurons has already been reported in other studies supporting the idea that this serotype is a useful tool for optogenetic experiments. In birds, AAV1 has not been used in the visual system so far. However, AAV1 has recently been used in zebra finches in the song system to study how optogenetic stimulation of ventral tegmental area (VTA) axon terminals can guide learned changes in song. This study reported anterograde labeling in Area X following injections of AAV1-CAG into the VTA. This is in line with our finding that injections of AAV1-CAG result in extensive anterograde ChR2 expression in well-described target structures of the entopallium such as the NI, MVL, and the striatum. In addition, our study observed extensive retrograde ChR2 expression in input structures of the entopallium such as the nucleus rotundus. The projection from the diencephalic nucleus rotundus into the telencephalic entopallium is well known and ideal to test the retrograde properties of a viral vector as those two structures are located in different parts of the brain. Thus, it is impossible that the retrogradely labeled cells that have been observed in the nucleus rotundus are the result of injection leakage from the entopallium. The finding that AAV1 in combination with the CAG promoter leads to retrograde ChR2 expression is in line with transduction studies performed in mice and rats that have reported similar properties for this viral vector. Retrograde properties of AAV1 in birds have, to the best of our knowledge, so far not been reported. However, in the song system of zebra finches, AAV9 has been used to transduce cells retrogradely. Our findings indicate that AAV1 in combination with the CAG promoter provides a useful alternative for those who depend on commercially available products. Identifying anterograde as well as retrograde properties of AAVs in birds is crucial since this is a way of gaining specificity, which can be used for detailed circuit analyses.
resulted in impaired grayscale visual discrimination, while the same manipulations in control birds expressing tdTomato did not result in any behavioral effects. This indicates that the blue light stimulation itself had no effect on visual discrimination accuracy and that the discrimination deficit in the experimental group can be traced back to the changed physiology of cells within the entopallium. Furthermore, these findings suggest that the entopallium is involved in contrast perception/visual discrimination of luminance, which is well in line with lesion studies that have been performed in the entopallium and other areas of the avian collothalamus pathway17.39,20.57. For example, lesions of the entopallium have been shown to reduce the ability to categorize stimuli into bright and dim19 or to discriminate between pictures of varying patterns and luminance20. However, in most cases, the lesions were not confined to the entopallium, complicating the attribution of the observed perceptual and behavioral deficits to the functionality of the entopallium alone. Using optogenetics, a stronger claim for the role of the entopallium in contrast perception can now be made, as we selectively increased the firing rates of entopallial cells. This procedure has been shown to be a suitable approach for disrupting behaviors that rely on heterogeneous as well as time-varying population coding5,58,59. Visual processing depends on such adaptive coding mechanisms since neurons within the sensory system vary their responses dynamically according to the input60. In visual discrimination tasks, where one stimulus needs to be selected over the other, cells in higher visual areas code for this discrimination by selectively increasing and decreasing their firing rates for the selected and nonselected stimuli, respectively. The difference in firing rates of these cells represents the discriminative information, which is greater for simple compared to complicated discriminations61. Since the optogenetic manipulation within the entopallium was not cell-type-specific, the discriminative information of these cells was probably disrupted by elevating the firing rates of all cells, thereby reducing their contrasts. This reduced contrast in neural representations might have led to impaired discrimination accuracy. Thus, for cell type-specific optogenetics during visual discrimination, excitatory optogenetic tools might offer advantages over inhibitory optogenetic tools, as excitation creates a new signal that can disturb the population dynamics more intensely than inhibition, which might result in floor effects62. Although excitatory optogenetic tools offer the above-mentioned advantages and might yield comparable effects to temporal lesions63, it needs to be noted that these tools might have knock-on effects on connected circuit areas of the entopallium such as the NFL, NI, and MVI64. Thus, to further substantiate the role of contrast perception to the entopallium, similar experiments using inhibitory tools or pharmacology could be performed. Moreover, future optogenetic studies could investigate the effect of optogenetic stimulation on other entopallial functions such as motion, pattern, and color discrimination. In these tasks, functional segregation has been described as lesions within the anterior entopallium affect the pattern, color, and form discrimination, whereas lesions of the posterior entopallium impair motion processing18,21. Based on these findings and comparable topographic projection patterns within the avian and mammalian tectofugal pathways, it has been proposed that the motion-sensitive posterior entopallium is comparable to area MT, whereas the color/form/pattern sensitive anterior entopallium is comparable to V2, V3, and IT in mammals21. As brightness sensitive neurons of the nucleus rotundus primarily project to the anterior entopallium, brightness processing has also been linked to this subdivision21. Although not showing a functional segregation, our study confirms that the anterior entopallium is involved in brightness perception as our neuronal manipulation was primarily focussed on this region.

The finding that this area governs brightness perception furthermore suggests a functional similarity of the entopallium to mammalian V165.

With our finding, we furthermore provided, to the best of our knowledge, the first proof of principle for the functionality of optogenetics in pigeons. Since the functional implementation of optogenetics in other species, especially on a behavioral level, has been challenging24,27, the establishment of this method in a novel species is interesting in itself. While attempts to drive behavior in primates with optical stimulation of the motor cortex have failed28, the production of saccadic eye movements with stimulation of V1 was successful66. Based on this, it has been suggested that, especially for unspecified optogenetic stimulation of both inhibitory and excitatory cell types, manipulations within sensory areas are more effective in driving behavior than manipulations within motor structures66. This is the case as unspecified stimulation is not able to produce finely tuned action plans that are necessary to drive motor behavior58, whereas unspecified stimulation of visual areas can produce artificial percepts that attract attention, therefore, changing behavior58. The idea of artificial percepts and their attentional effects might also apply to our finding that stimulation of the primary visual area in the pigeon brain impaired contrast perception in a grayscale discrimination task. However, as the reaction times in this task did not differ between stimulated and unstimulated trials it is unlikely that our findings are simply the results of averted attention but rather of deficient contrast coding within the entopallium.

However, the fact that unspecified stimulation is not able to produce finely tuned action plans that are necessary to drive motor behavior58 also indicates the importance of cell type or projection specific approaches when investigating behaviors that rely on tightly regulated mechanisms. In order to investigate the function of the columnar and laminar organization of the bird brain that has been proposed by several studies13,67,68, projection specific approaches are needed. With projection specific optogenetics, the role of specific visual circuits between layers can be investigated to establish circuit–function relationships that highlight general principles of brain organization. With our finding that AAV1 in combination with the CAG promoter is able to drive ChR2 expression anterogradely as well as retrogradely, we provided a useful tool for future studies that can use this serotype to investigate whether the conserved principle of a columnar and laminar organization in avian sensory areas also translates to similar functions. With the viral comparison study, we furthermore provided the groundwork for various other methods that rely on viral gene transfer, such as DREADDS24, genetic ablating69, calcium imaging69, or local genetic knock-in/out studies5, that can from now on be applied in the pigeon brain and can vastly improve comparative research. Overall, we created the foundations for a mechanistic understanding of the avian pallium and demonstrated conserved principles of avian and mammalian visual systems encouraging the use of avian model organisms for comparative and vision research in the future.

**Methods**

**Experimental subjects.** For this study, \(N = 35\) adult homing pigeons (Columbia livia) of undetermined sex were obtained from local breeders. The pigeons were between 1 and 4 years of age. They were individually caged and placed on a 12-h light–dark cycle. During the time period of training and testing, the birds were maintained at approximately 85% of their free-feeding weight. All experiments were performed according to the principles regarding the care and use of animals adopted by the German Animal Welfare Law for the prevention of cruelty to animals as suggested by the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were approved by the animal ethics committee of the Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany. All efforts were made to minimize the number of animals used and to minimize their suffering.
Viral vector injections/canula implantations. The anesthesia was initiated with a 7:3 mixture of Ketamine (Ketavet 100 mg/ml, Zoetis GmbH, Berlin Germany) and xylazine (Rompun 100 mg/ml, Bayer Vital GmbH, Leverkusen, Germany) and Pigeons received intramuscular injections of 0.075 ml for each 100 g body weight. This translates to 52.5 mg Ketamine per kg body weight and 4.5 mg Xylazine per kg body weight. Following that, the anesthesia was sustained with a consistent flow of Isoflurane (Forane 100%, Abbott GmbH & Co. KG, Wiesbaden, Germany). Prior to the surgery on the heads, they were shaved and the ears were cut. As soon as the pigeons no longer showed any pain reflexes, they were positioned in a stereotactic apparatus. At first, the skin covering the head was incised to expose the cranial bone. Then, craniotomies were performed to uncover the brain tissue. Cannulotomies were performed at different locations depending on the target structure (entopallium: A + 9.5, L ± 5.5, DV − 4.5; hippocampus: A + 6.0, L ± 1.0, DV − 1; wulst: A + 1, L ± 10, DV − 1.5; globus pallidus: A ± 9.0, L ± 4.5, DV − 6; NCL: A − 5.5, L ± 7.0, DV − 2.5; medial striatum: A + 11.0, L ± 1.5, DV − 6). After the removal of the dura mater injections were made at the specific locations. For the viral transfection study 5 µl of each viral vector (AAV1.hSyn.CAG.hChR2(H134R)-eYFP, AAV1.CAG.hChR2(H134R)-mCherry.WPRE.SV40, AAV5.hSyn.hChR2(H134R)-eYFP, AAV5.CAG.hChR2(H134R)-mCherry, WPRE.SV40, AAV9.hSyn.hChR2(H134R)-eYFP, AAV9.CAG.hChR2(H134R)-mCherry.WPRE.SV40 (all vectors were obtained from Addgene, Watertown, USA, all titer ± 1×10^13 vg/ml) were pressure injected into at least five separate hemispheres of three pigeons. Therefore, 5 µl were pipetted on parafolium and drawn into a glass pipette with a 25 µm tip. The whole volume was distributed over a range of 500 µm dorsal/ventral. For the behavioral and electrophysiological experiments, 10 µl of AAV1.CAG.hChR2(H134R)-mCherry.WPRE.SV40 (Addgene, Watertown, USA, all titer ± 1×10^13 vg/ml) was injected bilaterally into the entopallium (5 µl at A + 9.5, L ± 5.5, DV − 4.5, 4% PFA), A + 10.5, L ± 5.5, DV − 4.5. The brains were received of AAV1.CAG.tTomato (Addgene, Watertown, USA) and embedded with DAPI Fluoromount-G (MP Biomedicals, Santa Ana, USA). For the combined ChR2 and c-Fos stainings, slices were incubated in a mixture of a goat anti-mouse AlexaFluor594 antibody (1:500 in PBST, Invitrogen, Darmstadt, Germany) and a goat anti-rabbit AlexaFluor488 antibody (1:200 in PBST, Invitrogen, Darmstadt, Germany). The slices for the combined ChR2 and GFAP stainings were incubated in a mixture of a goat anti-mouse AlexaFluor594 antibody (1:500 in PBST, Invitrogen, Darmstadt, Germany) and a goat anti-rabbit AlexaFluor488 antibody (1:200 in PBST, Invitrogen, Darmstadt, Germany). The slices for the combined ChR2 and NeuN stainings were incubated in a mixture of an anti-mouse AlexaFluor488 antibody (1:200 in PBST, Invitrogen, Darmstadt, Germany) and a goat anti-neuron antibody AlexaFluor594 antibody (1:500 in PBST, Invitrogen, Darmstadt, Germany). The slices for the combined ChR2 and NeuN stainings were incubated in a mixture of an anti-mouse and anti-rabbit AlexaFluor594 antibodies (1:500 in PBST, Invitrogen, Darmstadt, Germany) and a goat anti-rat AlexaFluor488 antibody (1:200 in PBST, Invitrogen, Darmstadt, Germany). All secondary antibodies were incubated for 1 h at room temperature. After further rinsing (3 × 10 min), the slices were mounted onto glass slides (Superfrost® Plus, Thermo Scientific) in PBS, and embedded with DAPI Fluoromount-G (SouthernBiotech, Birmingham, USA), with minimal light exposure to preserve as much fluorescence as possible. Primary antibody specificity was either evaluated with western blots, based on previously published literature or investigated with immunohistochemical stainings in negative control samples. All stainings were performed twice with separate brain series to qualitatively confirm the reproducibility of the stainings.

Microscopic analysis. For the quantitative analysis of ChR2 expression, all slices that contained ChR2 expressing cells were imaged bilaterally with 200x magnification using a Leica Fluvis AXIO Imager M1 with a camera (Leica DFC5000 660N- C 2/3’0.63x). All sections containing the entopallium between A + 8.0 and A + 11.25 of one brain series (every tenth slice) were analyzed. For the quantification of somata, every ChR2 expressing soma within the whole entopallium was highlighted in ZEN 2.3 lite and later automatically counted by the program. For all viral vector injection analysis was performed in one-tenth of the number of all ChR2 expressing soma was summed up for further statistical analysis. Furthermore, the density of the stained area was determined with ZEN 2.3 pro Image analysis tool wizard. For this, the whole entopallium was selected as an analysis frame and parameters for segmentation were adjusted (smooth: none, sharpen: none, minimum area 1 pixel, threshold: Otsu threshold, separate: none). Following this, the size of all stained particles within the analysis frame was automatically measured in µm². In the same sections, the size of the entopallium was measured in µm² by delineating the borders. The same analysis was performed in the middle of the 100x magnification and the determined size of the ChR2/ GFAP expression within the injection site for all cases used in the tropism experiment. For the quantification of NeuN signals in the neuron loss analysis upper and lower thresholds were also adjusted with Otsu’s method. However, parameters for segmentation were adjusted and set to (smooth: none, sharpen: delineate, threshold: 0, size: 6, minimum area: 96 pixels, separate: morphology, count: 20). Likewise, the c-Fos signal was quantified in the entopallium and wulst, with the only difference being that the minimum area was adjusted to 24 pixels. For the quantification of anterograde and retrograde transport, the target area was delineated and intensity values were determined. For anterograde transport cases we calculated the Fos+ ratio into ++, +, ++, ++ and +++ (60% darker than the background, +++ signal was >12% darker than the background). For retrograde transport cases we calculated into ++, +, ++ and +++ (signal was <2% darker than the background, +++ signal was 2−4% darker than the background). All quantifications were performed blinded with the experimenter not being aware of the serotype investigated.
Electrophysiology. For extracellular recordings, insulated tungsten wire electrodes with an impedance of ~4 MΩ were used (Product No. 926-05-3; FHC, Bowdoinham, ME, USA). At the tip of each recording electrode, an optical fiber for light stimulation (Doric lenses Inc, Quebec, Canada) was glued. The electrodes were mounted on a micromanipulator (Narishige, MO-8, Tokyo, Japan) and advanced into the brain tissue. During the advancement of the electrode, a constantly repeated light pulse of 1 s duration was presented to evoke spikes. When a responsive cell was encountered, a fixed stimulation protocol was applied. The protocol consisted of repeated light pulses of variable duration (1, 10, 100, 200, and 500 ms) and was repeated three times (sweeps, Fig. 5a) and Supplementary Table 2). The resulting neuronal activity was amplified (10,000×) and band-pass filtered (32 Hz–10 kHz). The output for extracellular recordings was digitized using an analog-digital converter (Cambridge Electronic Design, Micro 1401 mkII). Spikes were sorted offline and analyzed using Spike 2 software (Cambridge Electronic Design, V.8) and Matlab (The Mathworks, Natick, MA, USA). For optical stimulation, we employed LED modules (Plexon Compact modules, 465 nm, Plexon, TX, USA) driven by a control unit (PlexBright 4 Channel Optogenetic Controller, Plexon, TX, USA) using Radiant Software (Plexon, TX, USA). The time course of the light stimulation was synchronously digitized at a rate of 41 kHz using an analog-digital converter (Cambridge Electronic Design, Micro 1401 mkII). To compare the number of spikes evoked by optical stimulation with the baseline activity of each cell, a one-sided Wilcoxon rank-sum test was performed in all conditions tested. To assess the variability of the evoked neuronal responses, we compared the spikes evoked during single sweeps using a non-parametric analysis of variance (Kruskal–Wallis test). If we found significant differences between the sweeps Bonferroni-corrected multiple comparison tests were conducted.

Skinner boxes. All training and testing were conducted in two operant chambers (32 cm (w) × 34 cm (d) × 32 cm (h)). The operant chambers were equipped with cameras for monitoring purposes, two white house lights on either side on the ceiling, and a touch screen monitor in the front panel. Furthermore, a feeder was mounted on a micromanipulator (Narishige, MO-8) positioned in the front panel directly beneath the touch screen where the birds received mixed grains as a food reward for correct responses. A light above the feeder indicated when food was available and served as a second reinforcer. All behavioral paradigms for this experiment were programmed in MATLAB with help of the Biopsychology Toolbox 2.3.

Stimuli. The stimulus set that was used in this study consisted of an orange initialization stimulus and grayscale pairs with varying luminance differences that were divided into five different SC (SC1 mean luminance difference 5.81 cd/m²; SC2 mean luminance difference 12.56 cd/m²; SC3 mean luminance difference 17.96 cd/m²; SC4 mean luminance difference 23.91 cd/m²; SC5 mean luminance difference 30.32 cd/m²; see Supplementary Fig. 20). A stimulus pair of one SC was always displayed at the center of the touchscreen computer monitor. The image with the same luminance as the grayscale pair was assigned to the participant and the monitor position in a randomized order. An example SC1 stimulus pair and an example SC5 stimulus pair is depicted in Fig. 7a. All stimuli measured 3.5 cm × 3.5 cm and the grayscale pairs were displayed 5 cm left and right of the center of the screen.

Grayscale visual discrimination task. The first training phase was an autoshaping procedure, where the birds learned to peck stimuli from all SC as well as the orange initialization stimulus. After the pigeons had successfully learned to peck all stimuli, they were trained in a grayscale visual discrimination task. During this phase, stimulus pairs with a high luminance difference (SC3) were presented and the pigeon had to learn to peck on the stimulus with the lower luminance. Once the discrimination performance reached 75% pigeons were trained in the final paradigm to all five SC. Once pigeons showed a stable performance (at least 50% initializations and 75% correct discrimination performance in one SC) they underwent surgery. After 14 days and full recovery, pigeons were trained in the final paradigm again to get accustomed to the patch chords. Behavioral testing was started when 6 weeks since the surgery had elapsed and behavioral performance was back to the criteria. The 6 weeks between surgery and behavioral testing allowed for stable transgene expression.

Every test session contained 600 trials in total. An individual trial started with the illumination of an orange initialization stimulus in the center of the front panel together with a tone indicating the beginning of the trial (Fig. 7a). After pecking the orange stimulus, a grayscale pair of one of the SC was displayed on the touch screen monitor for 8 s or until a pecking response was detected (Fig. 7a). Pigeons were conditioned to peck on the darker of the two grayscale images. When responding correctly, pigeons received a food reward for 2 s. When responding incorrectly pigeons were punished by turning off the house lights for 2 s. At the end of each trial, a 2 s intertrial interval was employed before the next trial started with the presentation of the orange initialization stimulus and a tone. Pigeons were stimulated with blue light (465 nm, Plexon) in a randomized order during the stimulus presentation phase in half of the trials (Fig. 7a). Thus, stimulation was stopped at the end of the stimulus presentation (either after 8 s or when pigeons responded to the grayscale images). Furthermore, in every session, an equal number of stimuli belonging to each SC was displayed in a randomized order. Pigeons were assigned to the experimental group (expressing ChR2) and to the control group (expressing tdTomato) in a randomized order. The behavioral performance of both experimental and control pigeons was recorded in MATLAB and was thus not subject to observation. Furthermore, the experimenter that trained and tested the pigeons was not aware of the group allocation.

Optogenetic manipulations. A 20 Hz (pulse duration: 15 ms; inter-pulse interval: 35 ms) and 40 Hz (pulse duration: 15 ms; inter-pulse interval: 10 ms) light stimulation were produced with the Radiant software (Plexon, TX, USA). For the behavioral experiments, the entopallium was illuminated with blue light (blue LED, 465 nm, Plexon). For the c-Fos experiment, the entopallium was illuminated with blue light (blue LED, 465 nm, Plexon) in one hemisphere and orange light (orange LED, 620 nm, Plexon) in the other hemisphere for a period of 30 min with alternating stimulation intervals of 5 min with no stimulation and 5 min of stimulation. The LED was mounted on a rotary joint placed in a fixed location above the behavior chamber. Lightweight optical patch chords (BFP(2)_200/220/LMW-0.53_1.5m_LC_G50.7, Doric Lenses Inc, Quebec, Canada) were used to connect the dual fiber optic cannulas with the light source. The light output was controlled with the PlexBright4-channel controller (Plexon, TX, USA). For the behavioral experiments, the light output at the cannula tip was verified using a power meter. Before each experiment, the light output of the patch chord was also checked (range of light intensity cannula: 3.5–4.5 mW, range of light intensity at patch chord: 4.5–6 mW, Thorlabs, Newton, USA).

Statistics and reproducibility. For the viral transfection study, the sample size was determined based on previously published viral transfection studies that have been published in other species such as rats14, mice38, primates39, and cats40. For all viral vectors, a minimum of five injections was performed in separate hemispheres of at least three pigeons. The total amount of ChR2 expressing cells was determined for every injection in one brain series (one-tenth of all brain sections) and then multiplied by ten to estimate the actual amount of ChR2 expressing cells. For the analysis of the transduced area, the total ChR2 expressing area within the entopallium was determined in the same brain series. Furthermore, the size of the entopallium was measured in all sections of one series. The transduced area was then put into relation with the size of the whole entopallium and with the size of the entopallium in sections with ChR2 expression. All serotypes were included in this analysis except for AAV9-hSyn-ChR2, as this serotype did not result in reliable ChR2 expression. We used the Shapiro–Wilk test to test for normal distribution of the data and the Levene’s test to test for the homogeneity of the variance. A one-way ANOVA with Welch correction was calculated to investigate the effect of serotype on the number of ChR2 expressing cells and on the ChR2 expressing area. Post hoc tests were Bonferroni corrected. For the NeuN overlay experiments, ChR2 expressing soma co-localized with NeuN, as well as ChR2 cells without co-localization, were counted in one series per injection. The percentage of cells with and without co-localization was determined and compared with a Wilcoxon signed-rank test. For the neuron loss experiment, NeuN signal was quantified in the injection site of AAV9-CAG and AAV1-CAG injections of one brain series per injection. Mean neuron numbers of both serotypes were compared with a Mann–Whitney U test. For the c-Fos experiment, c-Fos signals were quantified at two points in two brain areas in the blue and orange light stimulated hemispheres in three animals. One stimulated site in the orange stimulated hemisphere could not be reconstructed leading to n = 6 for the blue light stimulation and n = 5 for the orange light stimulation. Mean c-Fos signals for the blue and orange stimulated hemispheres within the entopallium and within the wulst were compared with a Mann–Whitney U test.

For the behavioral analysis, six control pigeons and six experimental pigeons were tested in five sessions. The sample size was determined on previously published entopallium lesion studies that investigated visual discrimination in birds17–19. For the behavioral data analysis, five sessions that matched the criterion of at least 50% trial participation and 75% correct answers in at least one SC were considered. The mean reaction times and the mean performance in stimulated and unstimulated trials for all SC were extracted using MATLAB. We used the Shapiro–Wilk test to test for normal distribution of the data and the Levene’s test to test for the homogeneity of the variance. A repeated-measures ANOVA with the within-subject factors session, stimulation, and SC and the between-subject factor group was calculated to investigate the effects on reaction times (in four sessions) and performance (in five sessions). Reproducibility of the behavioral findings was verified by comparing the performance of all tested sessions. Post hoc tests were Bonferroni corrected. Alpha was set at 0.05 for all analyses. All statistical analyses were performed with the software IBM SPSS Statistics (v. 20).
Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are included in the Article and its Supplementary Information or available from the corresponding author upon request. The source data of the main text figures is available in Supplementary Data 1.

Code availability
All codes were written in Matlab using Biopsychology Toolbox (available at http://biopspytoolbox.sourceforge.net/). Custom codes will be made available upon request. Please contact the corresponding author.

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