Breaking the Balance: Ocular BDNF-Injections Induce Visual Asymmetry in Pigeons

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ABSTRACT: In pigeons, asymmetric photic stimulation around hatch induces functional visual asymmetries that are accompanied by left–right differences in tectal cell sizes. Different aspects of light-dependent neuronal differentiation are known to be mediated by the brain-derived neurotrophic factor (BDNF). Therefore, we investigated by means of single or triple BDNF- or saline-injections into the right eye of dark-incubated pigeon hatchlings if ocular BDNF enrichment mimics the effects of biased visual input. As adults, the birds were tested in a grit–grain discrimination task to estimate the degree and direction of visual lateralization followed by a morphometric analysis of retinal and tectal cells. The grit–grain discrimination task demonstrated that triple BDNF-injections enhanced visuoperceptual and visuomotor functioning of the left eye system. Morphometric analysis showed bilateral cell-type dependent effects within the optic tectum. While single-BDNF injections increased cell body sizes of calbindin-positive efferent neurons, triple-injections decreased cell sizes of parvalbumin-positive cells. Moreover, single BDNF-injections increased retinal cell sizes within the contralateral eye. Analysis of BDNF-induced intracellular signaling demonstrated enhanced downstream Ras activation for at least 24 h within both tectal halves whereas activity changes within the contralateral retina could not be detected. This points to primarily tectal effects of ocular BDNF. In sum, exogenous BDNF modulates the differentiation of retinotectal circuitries and dose-dependently shifts lateralized visuomotor processing towards the noninjected side. Since these effects are opposite to embryonic light stimulation, it is unlikely that the impact of light onto asymmetry formation is mediated by retinal BDNF. © 2008 Wiley Periodicals, Inc. Develop Neurobiol 00: 000–000, 2008

Keywords: **calbindin; neurotrophic factor; parvalbumin; Ras activation; retina; tectum**

INTRODUCTION

Birds – like chicks and pigeons develop a behavioral lateralization with a superiority of the right eye/left hemisphere for detailed visual feature analysis during an early ontogenetic period (Güntürkün, 2002a; Manns, 2006; Rogers, 2006). This functional lateralization can be associated with anatomical left–right differences in the ascending visual projections (Rogers, 1996; Güntürkün, 2002b). Chicks exhibit a transient left–right difference in the thalamofugal pathway (laterodorsal geniculate nucleus → Wulst; Rogers and Deng, 1999; Deng and Rogers, 2002; Koshiba et al., 2003). In pigeons, it is the tectofugal system (tectum → nucleus rotundus → entopallium) which is lateralized. Retinorecipient tectal and rotundal cells are enlarged on the left brain side (Güntürkün, 1997; Manns and Güntürkün, 1999a,b,

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2003; Skiba et al., 2002) and the left rotundus receives a stronger bilateral tectal input (Güntürkün et al., 1998).

Avian embryos take an asymmetrical prehatch position with the right eye next to the eggshell and the left eye covered by the body. Light traversing the semitranslucent eggshell therefore primarily activates the right eye system. Anatomical and behavioral asymmetries develop in response to this asymmetric light exposure shortly before hatching (Rogers, 1996; Güntürkün, 2002a), whereas dark incubation prevents their emergence (Rogers, 1982; Skiba et al., 2002). In the altricial pigeon, this pattern can be modified by monocular deprivation after hatching (Manns and Güntürkün 1999a,b). Ocular TTX-injections demonstrate that this plasticity is dependent on retinal activity (Prior et al., 2004) but the mediating neuronal mechanisms are still unclear. It is likely that an unbalanced visual stimulation primarily influences differentiation of the retinotectal system (Debski and Cline, 2002; Ruthazer and Cline, 2004). Many effects of light stimulation are mediated by the brain-derived neurotrophic factor (BDNF). Visual stimulation adjusts the expression and/or release of BDNF and hence, regulates the trophic support of target cells in retro- as well as anterograde manner (von Bartheld et al., 1996; Menna et al., 2003; Chytrova and Johnson, 2004). In turn, BDNF controls sprouting, branching, and maintenance of axo-dendritic trees (Vicario-Abejón et al., 2002; Cohen-Cory and Lom, 2004), regulates efficacy of synaptic transmission and synaptogenesis (Lessmann et al., 2003) and controls tectal neuropeptide expression (Tu and Debski, 2001). BNDF and its high-affinity receptor TrkB are present in the developing retinotectal system of pigeons (Theiss and Güntürkün, 2001) and the tectal TrkB signaling cascade is asymmetrically activated in response to embryonic light stimulation (Manns et al., 2005). Thus, it is conceivable that asymmetric light effects are mediated by BDNF.

Since BDNF is present within the retina as well as within the optic tectum, two primary sites of BDNF action are conceivable. The first possibility is that asymmetrical retinal BDNF expression shapes tectal differentiation by affecting retinotectal synaptogenesis or by anterograde trophic support of tectal cells. In fact, sensory experience alters not only synthesis but also trafficking of BDNF (Pollock and Frost, 2001; Chytrova and Johnson, 2004). The second possibility is that the retinal input regulates BDNF release of tectal neurons in an activity-dependent and hence, asymmetrical manner. According to the first hypothesis, ocular BDNF-injections should mimic light effects onto visual asymmetry formation. To investigate this possibility, we injected BDNF or saline for control into the right eye of dark-incubated freshly hatched pigeons which normally do not develop visual asymmetries (Skiba et al., 2002) and examined their adult behavioral and anatomical lateralization pattern. If activity-dependent higher BDNF release of the right retina indeed ignites the formation of visual asymmetry, these animals should develop left–right differences in morphology and behavior, similar to adult pigeons that were not incubated in the dark.

MATERIALS AND METHODS

For dark incubation, fertilized eggs from pigeons breeding within the lab were incubated in a still-air incubator kept in darkness at constant temperature (38, 3°C) throughout the entire period of incubation. Directly after hatching, animals received injections of either 50 ng BDNF (Alomone Labs, Munich, Germany) dissolved in 5 μL saline or 5 μL saline as a control into the right eye once or at three consecutive days (Table 1). It has been shown that ocular injections of 15–75 ng BDNF are effective within the isthmo-optic system (Primi and Clarke, 1996). Under local anesthesia of the eye with Xylocain (Astra Zeneca, Wedel, Germany), injections were performed by penetrating the needle of a 5 μL Hamilton syringe about 2.5 mm deep within the caudodorsal eye ball (Prior et al., 2004). Thereafter, the nestlings were swapped with the artificial eggs the breeding birds were sitting on (Skiba et al., 2002, Manns and Güntürkün, 2003). Five animals developed an inflammation of the injected eye and had to be discarded from the experiment.

As adults (at least 6 month after birth), the animals were tested within a grit–grain discrimination task to estimate the degree and direction of lateralization in visual object analysis (Güntürkün and Kesch, 1987; Manns and Güntürkün, 1999a). Under normal rearing conditions, adult pigeons reach higher levels of discrimination with the right eye (Güntürkün and Kesch, 1987). Afterwards, the animals were perfused to perform a morphometric analysis of retinal and tectal cell populations. The experiments were carried out according to the specifications of the German law for the prevention of cruelty to animals.

Grit–Grain Discrimination

Pigeons were tested bi- and monocularly seeing in a grit–grain discrimination task within their homecages (Güntürkün and Kesch, 1987; Manns and Güntürkün,

Table 1 Number of Experimental Animals

	Single injections	Triple injections
Saline	9	7 (9)
BDNF	8 (9)	7 (9)

1999a; Skiba et al., 2002). For monocular testing, one eye was occluded by a cardboard cap that was fixed around the eye with a Velcro strip. To perform the task, the animals are required to peck 30 white Dari-grains from a translucent trough filled with 30 g small pebbles of varying size but resembling the seeds in color and shape. The trough was positioned below the cage opening and the animals were allowed to peck the grains for 30 s, then the trough was immediately removed. The quotient of consumed grains and performed pecks multiplied by 100 (percentage of pecks leading to consumption) served as an index for the discrimination accuracy.

Each animal completed 20 sessions each under binocular, left-eye, and right-eye seeing conditions. Each seeing condition was tested twice a day in a balanced order so that every pigeon performed six sessions/day. During the first ten sessions, the animals were allowed to habituate to the procedure and the subsequent 10 tests were considered for the analysis of discrimination performance. During the complete testing period, the animals were food deprived and maintained at 80% of their normal weight.

Histology

For morphometric analysis, animals received an injection of 200 units sodium heparin (Ratiopharm). After 15 min, the animals were deeply anesthetized with an overdose of equitesin (0.45 mL/100 g body weight) and perfused through the heart with 0.9% saline (40°C) followed by 4% paraformaldehyde in 0.12 *M* phosphate buffered saline (PBS), pH 7.2, 4°C. Brains and eyes were postfixed in the fixative + 30% sucrose for 2 h and cryoprotected overnight in 0.12 *M* PBS +30% sucrose at 4°C. On the following day, the brains were cryosectioned in frontal plane at 40 μm and the slices were collected in 0.12 *M* PBS containing 0.1% sodium azide. The left or right brain side was marked by a hole stuck with a small needle. The slices were stored at 4°C until used for immunohistochemistry. Sections were immunolabeled with antibodies against calbindin (CB, monoclonal mouse IgG, SWant), or parvalbumin (PV, monoclonal mouse IgG, Sigma). Parallel series were stained with cresyl violet. Retinae were cut with a cryostat at 20 μm and stained with cresyl violet (Güntürkün, 1997).

Immunohistochemistry

Brain slices were reacted free-floating according to the ABC-technique (Manns and Güntürkün, 2003). All steps of the immunohistochemical detection were performed on a shaker table at room temperature unless otherwise stated. Three washes at 5 min each with PBS followed all incubation steps. After the first three washes, endogenous peroxidases were blocked with 0.3% H_2O_2 in distilled water. Unspecific binding sites were blocked with 10% normal horse serum (Vector) in 0.12 *M* PBS + 0.3% Triton X 100 (PBS-X).

Sections were incubated with primary antibody solution (PV 1/1000 in PBS-X; CB: 1/12,000 in PBS-X) for 72 h at 4°C. The secondary antibody reaction was carried out with biotinylated horse anti-mouse IgG (1/200 in PBS-X; Vectastain Elite kit, Vector) for 1 h. Afterwards, the sections were incubated in an avidin–biotin–peroxidase solution (Vectastain ABC-Elite kit, 1/75 in PBS-X). Peroxidase-activity was detected using a heavy metal intensified 3′3-diaminobenzidine (Sigma) reaction which was started by 1% β-d-glucose-oxidase (Sigma). The sections were mounted on gelatinized slides, dehydrated, and coverslipped with Permount (Fisher Scientific, NJ). Parallel series were counterstained with cresylviolet.

Soma Size Measurements

To ensure a blind analysis, slides were coded for experimental group and left and right brain side. In the immunolabeled sections corresponding to the stereotaxic level A 3.75 (Karten and Hodos, 1967), the cross sectional soma areas of 50 neurons in each layer containing immunopositive cells were measured in the left as well as right tectum by means of the image analyzing system "analySIS" (Soft Imaging System, Münster, Germany) with 40× objective of a Leica DMR microscope (Manns and Güntürkün, 2003). For retinal morphometry, cells within the area centralis which project onto the lateral tectum (Clarke and Whitteridge, 1976; Remy and Güntürkün, 1991) were analyzed.

An index for the extent of behavioral or morphometric asymmetries was calculated as the quotient of left- minus right- and left- plus right-sided results. Statistical analyses were performed with Statistica 7.1 (StatSoft, Tulsa, OK). Since according to Shapiro–Wilks-tests, data sets were normally distributed, we compared behavioral and anatomical data by using mixed analyses of variance (ANOVAs). Post-hoc comparisons were performed by paired or unpaired sample *t* tests, respectively.

Pulldown Assay and Western Blot Analysis

To analyze BDNF-induced intracellular signaling, we injected 50 ng BDNF dissolved in 5 μL saline ($n = 6$) or only 5 μL saline ($n = 6$) into the right eye of dark-incubated pigeons hatchlings, decapitated the animals after one or 24 h survival time and collected retinal and tectal tissue for quantitative analysis of Ras activation level.

Homogenized tissue (in 50 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 10% Glycerol, 1% Nonidet P-40) was centrifuged at 14,000 g, 4°C, and the supernatant was used for further experiments. The protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). For the detection of total Ras, equal amounts of proteins (15 μg/slot) were analyzed with sodium dodecyl sulfate-polyacrylamid gel electrophoresis and Western blot. The immunoreactive bands were detected using the primary antibody anti Ras IgG (Upstate, diluted 1:10,000 in Tris-buffered saline), anti mouse horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) and an enhanced chemiluminescence substrate detection kit (Amersham

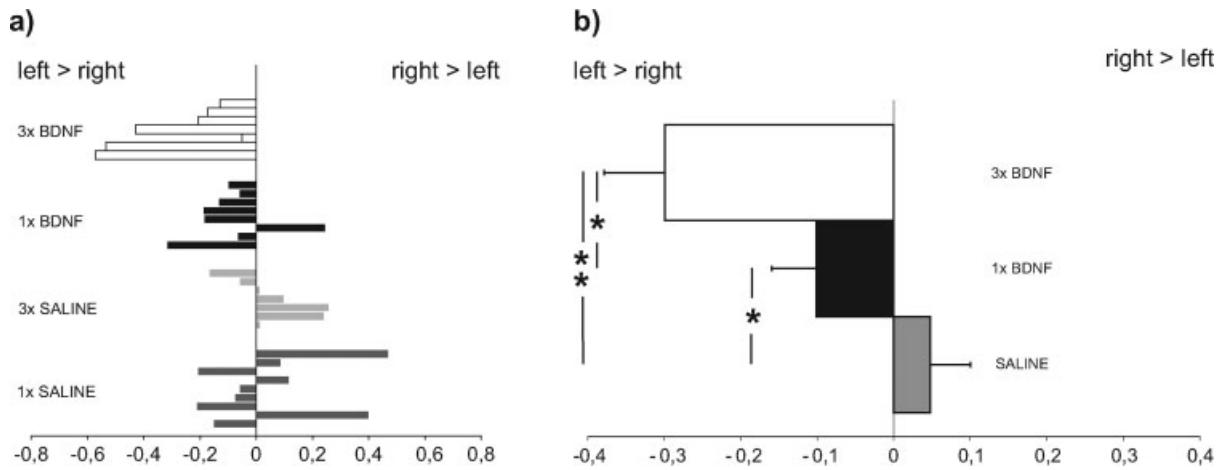

Figure 1 Asymmetry of discrimination accuracy within the different experimental groups at the individual (a) and group (b) level. Bars represent standard error (* $p < 0.05$; ** $p < 0.01$ according to post hoc t tests).

Pharmacia Biotech). Blots were analyzed by densitometric measurement and quantified using the program TINA 2.09 (Raytest, Germany). Two hundered microgram protein from lysates was used for Ras pull-down experiments, which have been performed as described in detail by de Rooij and Bos (1997). The ratio between optic densities of Ras-GTP- and total Ras-immunoblots served as an index for the relative levels of active Ras within the probes. Differences in Ras activation level were evaluated by nonparametric Kruskal Wallis ANOVA and posthoc Mann–Whitney U tests for independent samples.

RESULTS

Behavioral Analysis: Grit–grain-Discrimination

Since performance of the single or triple saline-injected (SAL) groups did not differ [Fig. 1(a)], we pooled their data to compare them with that of BDNF-injected animals. We considered the discrimination accuracy between the groups as revealed by the mean performance of the 11th–20th trials by a mixed 3 × 3 ANOVA with seeing condition (bin, left-eye, right-eye seeing) as repeated measure and experimental group (SAL, 1×BDNF, 3×BDNF) as between-subject factors (Fig. 2). Statistical analysis showed no difference in the mean performance between the experimental groups (F (2, 28) = 0.094, $p = 0.911$) but between the seeing conditions (F (2, 56) = 0.337 $p < 0.0001$). In general, the animals displayed their highest performances when seeing with both eyes (posthoc Tukey HSD Test: binocular versus left- or right-eye performance: $p < 0.001$). Additionally, there was an interaction between experimental groups and seeing conditions ("experimental group" × "seeing condition": F (4, 56) = 5.570, $p < 0.001$).

The SAL group did not show differences in discrimination accuracy when seeing with the left or right eye [$t = -0.572$, $p = 0.576$; Fig. 2(a)]. This pattern demonstrated that the ocular injections themselves did not impair performance with the injected right eye. In contrast, virtually all BDNF-injected animals (except one) displayed better results when seeing with the left eye (Figs. 1 and 2). However, the difference between the left- and right-eye performance was only significant after triple injections [1×BDNF: $t = 1.790$, $p = 0.117$; 3×BDNF: $t = 3.510$, $p < 0.05$; Fig. 2(a)]. Compared to SAL animals, BDNF tended to decrease performance with the injected right eye (planned comparison: F (1, 28) = 3,440, $p = 0.074$). More intriguingly, BDNF-injections increased the performance with the contralateral left eye so that its performance was as good as the binocular one in the 3×BDNF group ($t = 1.204$, $p = 0.274$). This effect was also observed as a trend in the 1×BDNF group ($t = 2.272$, $p = 0.057$). By contrast, discrimination accuracy under binocular seeing conditions outperformed performance when seeing with the left ($t = 3.026$, $p < 0.01$) or the right eye (t test: $t = 6.992$, $p < 0.0001$) in the SAL group, a result which was also observed in previous studies (e.g. Güntürkün and Kesch, 1987; Manns and Güntürkün, 1999a).

Because of the differential extent of performance differences between the left- and right-eye seeing condition, the experimental groups differed significantly in the asymmetry of discrimination accuracy [F (2, 28) = 7.586, $p < 0.01$; Fig. 1(b)]. While performance asymmetry only tended to be significant between SAL and 1×BDNF animals ($t = -1.766$,

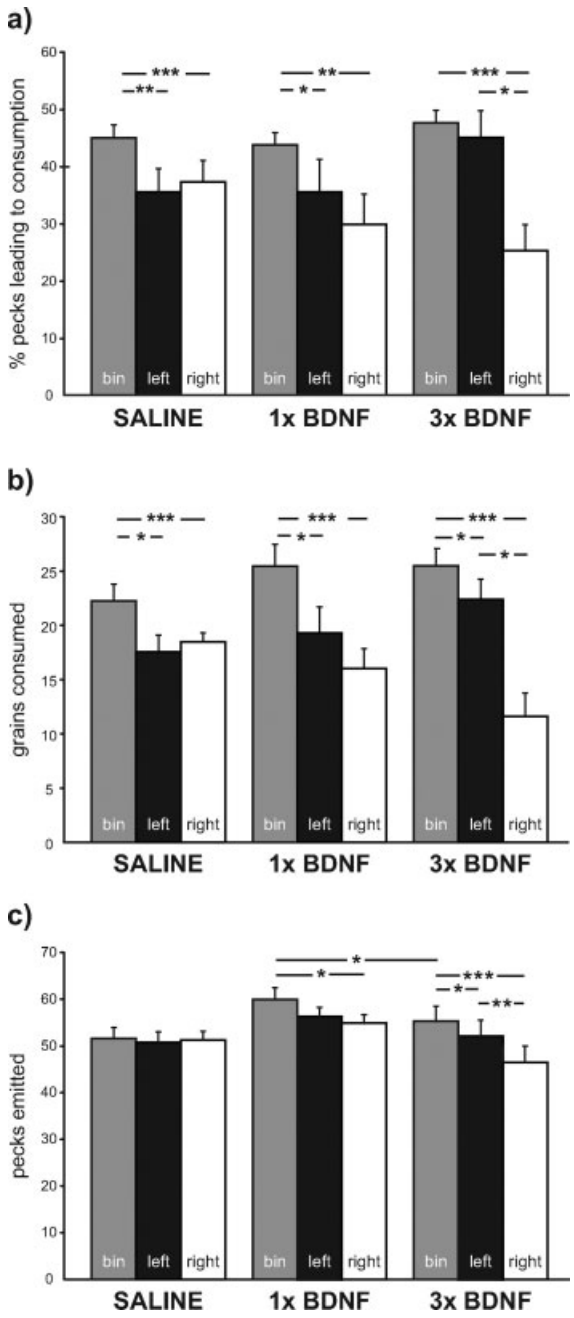

Figure 2 Performance of the experimental groups when seeing with the left, right or with both (bin) eyes as indicated by discrimination accuracy (a), number of consumed grains (b) and number of pecks emitted (c). Bars represent standard errors (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ according to post hoc *t* tests).

$p = 0.09$) or between 1×BDNF and 3×BDNF birds ($t = -2.056$, $p = 0.060$), it was significant between SAL and 3×BDNF birds ($t = -3.664$, $p < 0.01$).

Differences in discrimination accuracy could result from differences in the number of consumed grains and hence, due to differences of visual perception. However, they could also derive from differences in the number of performed pecks and hence, in visuomotor speed (Skiba et al., 2002). In line with previous studies (Güntürkün and Kesch, 1987; Manns and Güntürkün, 1999a), we found that only the number of consumed grains reflected the observed differences in discrimination success [Fig. 2(b)]. The experimental groups did not differ in the mean number of grains swallowed (F (2, 28) = 0.068, $p = 0.934$) but their actual number differed between the seeing conditions depending on the experimental group ("group" × "seeing condition": F (4, 56) = 7.390, $p < 0.0001$). A planned comparison showed that BDNF-injected animals consumed significantly fewer grains when the pigeons were seeing with their right eye compared to saline controls (F (1, 28) = 5.075, $p < 0.05$). Posthoc t-test demonstrated that triple ($t = 2.550$, $p < 0.05$) but not single ($t = 0.983$, $p = 0.336$) BDNF-injections reduced the number of consumed grains when seeing with the right-eye compared to the left one. Comparing bi- and monocular success in consuming grains, significantly highest numbers of consumed grains could be observed when seeing with both eyes in all groups. However, after triple injections left-eye performance only tended to differ from binocular success ($t = 2.338$, $p = 0.058$). Accordingly, only this groups displayed a significant difference in the number of consumed grains when seeing with the left or right eye ($t = 4.501$, $p = 0.01$).

Although the mean number of performed pecks did not differ between the experimental groups (F (2, 28) = 1.693, $p = 0.202$), the actual pecking number varied between the seeing conditions (F (2, 56) = 13.635, $p < 0.0001$) depending on the experimental manipulation ["group" × "seeing condition": F (4, 56) = 4.518, $p < 0.01$; Fig. 2(c)]. In general, the number of performed pecks tended to be higher after single BDNF-injections compared to the other groups (planned comparison: F (1, 28) = 3.211, $p = 0.084$). Whereas SAL animals did not display variation in pecking speed under different seeing conditions, BDNF-injected animals performed most pecks when seeing with both eyes and least pecks when seeing with the right eye. The difference in pecking speed under the binocular and right-eye seeing condition was significant in the 1×BDNF ($t = 3.200$, $p < 0.05$) as well as in the 3×BDNF ($t = 6.472$, $p < 0.001$) group. Moreover, the 3×BDNF group displayed significant differences in pecking speed between the binocular and left eye seeing ($t = 2.782$, $p < 0.05$) as

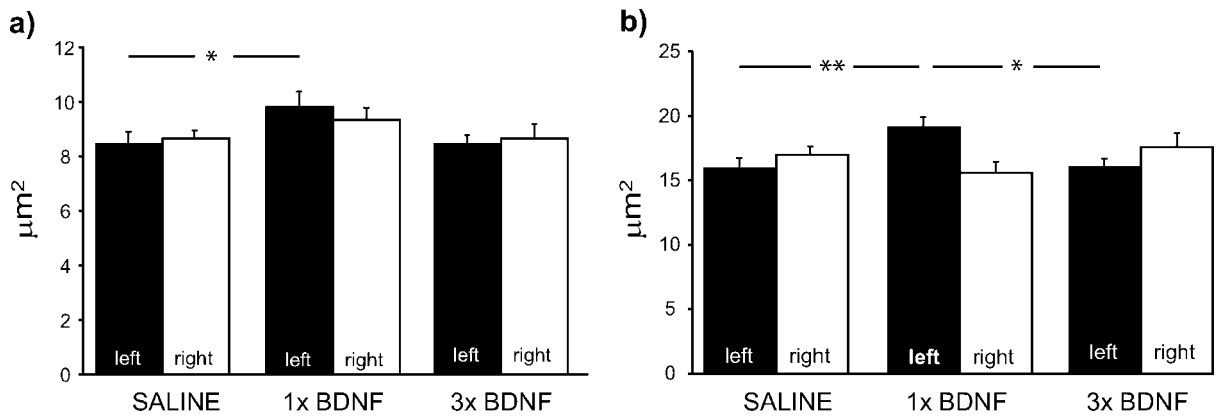

Figure 3 Retinal cell body sizes in the left and right inner granular (a), and ganglion cell layer (b). Bars represent standard error (* $p < 0.05$, ** $p < 0.01$ according to post hoc t tests).

well as between the left and right eye seeing condition ($t = 5.272$, $p < 0.01$).

Morphometric Analysis

Retina. Cell body sizes were analyzed within the inner nuclear (INL) and the ganglion cell layer (GCL) while the receptor cell layer was excluded from analysis because single cells could not be sufficiently separated from each other. Apart from glial Müller cells, the INL is composed of horizontal, bipolar, amacrine, and interplexiform neurons as well as displaced ganglion cells whereby the overwhelming majority is represented by bipolar cells (Dowling, 1987). Since these neurons are condensed in the middle of the INL, we tried to confine our morphometric analysis to this portion. Therefore, our measurements mainly represent cell sizes of the bipolar population. However, due to the overlap in location and size, we could not exclude measurements of the other cell types, too. The GCL is basically composed of retinal ganglion cells (RGLs) but also includes some displaced amacrine cells (Dowling, 1987). Accordingly, our analysis of GCL mainly describes RGCs. Differences in retinal cell body sizes were analyzed by performing a 3 × 2 × 2 ANOVA with experimental group as between-subject and retina (left, right) and layer (INL, GCL) as repeated measure factors (Fig. 3). This analysis showed that retinal cell body sizes did not differ between the experimental groups (F (2, 20) = 2.846, $p = 0.082$), or between the left and right retina (F (1, 20) = 1.104, $p = 0.306$) but depended on a significant interaction between both factors ("group" × "retina": F (2, 20) = 8.068, $p < 0.01$). Cells within the INL were significantly smaller than cells within the GCL (F (1, 20) = 371.417, $p < 0.0001$) in all experimental groups ("layer" × "group"-interaction: F (2, 20) = 0.186, $p = 0.831$). Additionally, cell sizes differed between retinal sides depending on the experimental group ("retina" × "layer" × "group" = : F (2, 20) = 4.259, $p < 0.05$). Post hoc t tests demonstrated that cell sizes within the injected right retina were not affected in any experimental group. Surprisingly, it was the contralateral left retina which displayed modified cell body sizes but only in the 1×BDNF group [Figs. 3(a,b)]. After single BDNF-injections, neurons within the left INL were enlarged compared to saline controls [$t = -2.348$, $p < 0.05$; Fig. 3(a)] and left RGCs were enlarged compared to saline- ($t = -3.193$, $p < 0.01$) and triple BDNF-injections [$t = 3.141$, $p < 0.05$; Fig. 3(b)]. Triple BDNF-injection obliterated this contralateral effect since cell sizes did not differ from that of saline controls (INL: $t = -1.800$, $p = 0.110$; CGL: $t = -0.078$, $p = 0.939$).

Tectum. At tectal level, the morphometric analysis was confined to distinct immunohistochemically characterized cell types. To this end, we selected neurons characterized by unique calcium-binding protein expressions because their differential expression is assumed to be linked to a modulation of neuronal firing pattern (Kawaguchi and Kubota, 1993). These neurons represent distinct nonoverlapping tectal cell populations which are known to respond differentially to photic stimulation (Manns and Güntürkün, 2003, 2005).

According to previous reports (Theiss et al., 1998; Manns and Güntürkün, 2003, 2005), immunoreactivity against parvalbumin (PV) and calbindin (CB) is present in distinct tectal layers. PV-ir cells can be detected within layers 2/3, layer 4, layers 6/7, and layer 10 while CB-ir cells are present within layers 3, 5, and 13. CB-ir layer 13 neurons represent a sub-

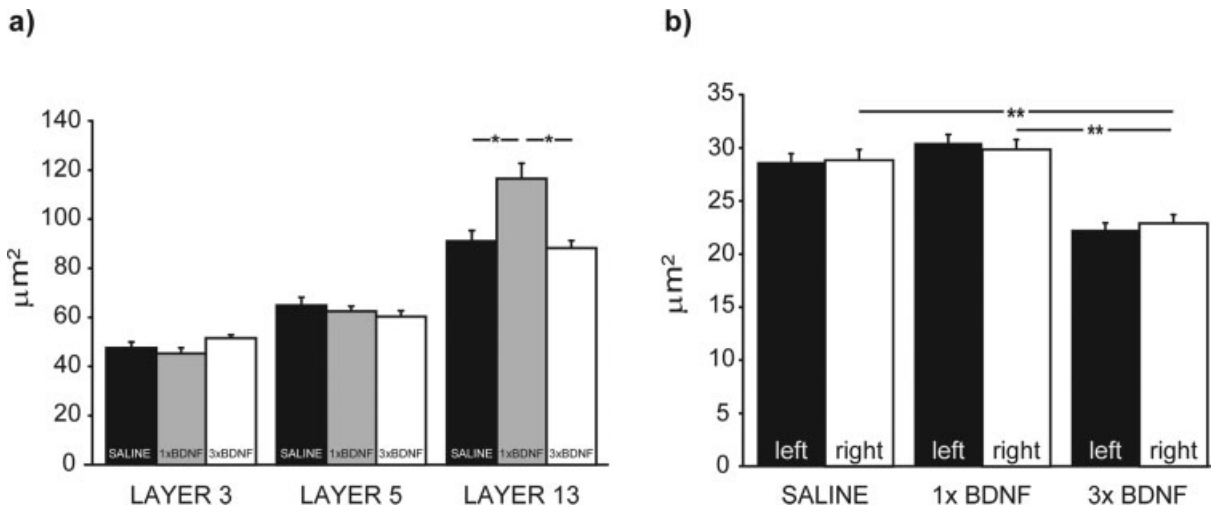

Figure 4 Tectal cell body sizes of CB-ir (a) and PV-ir (b) neurons. Bars represent standard error (* $p < 0.05$, ** $p < 0.01$ according to post hoc t tests).

population whose dendrites were confined to the deeper tectal layers. Hence, they presumably represent an efferent subtype, which does not receive direct retinal input and which ascends to the nucleus triangularis (Hellmann and Güntürkün, 2001) or which descends into the brainstem within the tectobulbar tract (Hellmann et al., 2004). The morphometric analysis of these CB- and PV-ir cells demonstrated differential modulations of cell body sizes within both hemispheres in a dose-dependent manner.

We analyzed these size differences by performing $3 \times 2 \times 3$ or $3 \times 2 \times 4$ ANOVAs with experimental group as between-subject and tectum (left, right) and layer (3 for CB, 4 for PV) as repeated measure factors (Fig. 4). We only included data from animals which were also considered within behavioral analysis.

The experimental groups displayed significantly different cell body sizes of CB-ir cells [F (1, 26) = 4.656, $p < 0.05$; Fig. 4(a)]. But there was no cell size differences between the left and the right tectum (F (1, 26) = 0.867, $p = 0.360$) in any group ("group" × "tectum": F (2, 26) = 0.786, $p = 0.466$). Cell sizes differed significantly between tectal laminae (F (2, 52) = 223.459, $p < 0.0001$) and were influenced by the experimental manipulation ("layer" × "group" F (4, 52) = 11.533, $p < 0.0001$). Post hoc t tests verified that group-dependent differences in cell sizes were confined to layer 13 whereby neurons on both sides were significantly enlarged after single BDNF-injections compared to saline-controls ($t = -4.936$, $p < 0.0001$) but not after triple ones ($t = 0.231$, $p = 0.820$). Thus, single BDNF-injections affected differentiation of a subset of efferent tectal neurons, an effect that was obliterated after triple injections.

Cell body sizes of PV-ir tectal cells likewise differed between the experimental groups [F (1, 27) = 22.590, $p < 0.0001$; Fig. 4(b)] with no differences between the tectal halves (F (1, 27) = 0.012, $p = 0.914$). Cell sizes varied between different layers (F (3, 81) = 12.922, $p < 0.0001$). No significant interactions could be verified between tectal side and experimental manipulation ("tectum" × "group": F (2, 27 = 0.512, $p = 0.605$) or between layer and experimental manipulation ("layer" × "group": F (6, 81) = 0.921, $p = 0.485$). According to post hoc t tests, cell sizes were significantly smaller after triple BDNF-injections compared to saline- ($t = 4.436$, $p < 0.001$) or single BDNF- ($t = 8.653$, $p < 0.001$) injections in all tectal laminae.

Ras Activation Pattern. In a first step to analyze primary neuronal effects of ocular BDNF injections, we investigated BDNF-induced intracellular signaling. BDNF binding to its specific TrkB receptor activates the intracellular membrane anchored GTPase Ras (Huang and Reichard, 2003), which plays an important morphogenetic role in neurons (Heumann, 1994; Kaplan and Miller, 2000) and which is activated in the developing tectum of pigeons in a light-dependent manner (Manns et al., 2005). Since pilot experiments could not reliably detect phospho-TrkB as an indicator for receptor activation, we confined our analysis to time-dependent downstream Ras activity level within the retinae and tecta of pigeon hatchlings in response to BDNF- or saline-injections.

Although retinae displayed a generally higher Ras activation level compared to the tectum a BDNF-dependent modulation of Ras activation could mainly

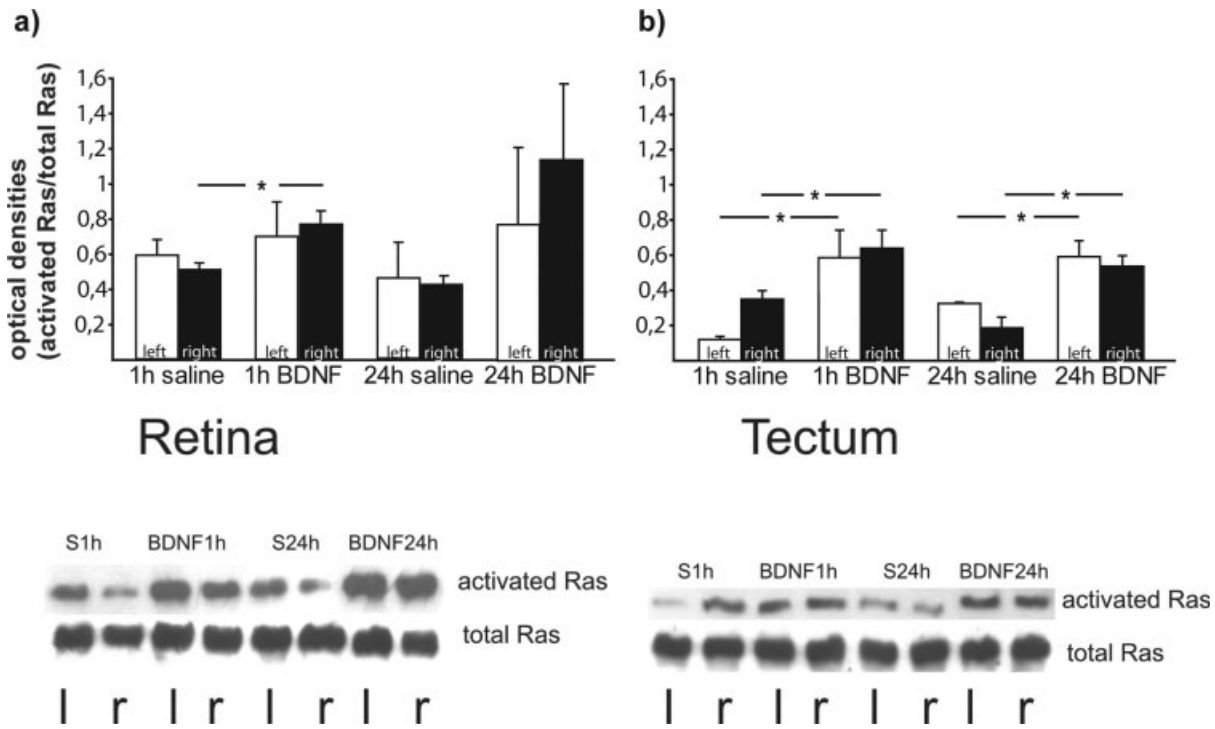

Figure 5 Ras activation level within the retina (a) and tectum (b). The relative amount of activated Ras to total Ras is analyzed by semiquantitative optico-densitometrical evaluation. Histograms depicting the means are underlayed with representative blots. Bars represent standard error (l = left, r = right, * $p < 0.05$ according to Mann-Whitney U tests).

be detected within the tectum (Fig. 5). A Kruskal Wallis ANOVA displayed a trend for differences within the right injected retina (H (3, $N = 12$) = 6.590, $p = 0.08$) but not for the left one (H (3, $N = 12$) = 1.051, $p = 789$). Posthoc analysis verified an enhanced Ras activation level within the right BDNF-injected retina compared to saline controls 1 h after injection [Mann–Whitney U: $Z = -1.964$, $p < 0.05$; Fig. 5(a)]. In contrast, Kruskal Wallis ANOVAs demonstrated significant differences in Ras activation level within the left (H (3, $N = 12$) = 9.359, $p < 0.05$) as well as the right tectum (H (3, $N = 12$) = 8.897, $p < 0.05$). Post hoc analysis verified a significant increase in Ras activation in BDNF- compared to saline- injected animals already 1 h after injections within both tectal halves [Mann–Whitney U: $Z = -1.964$, $p < 0.05$; Fig. 5(b)]. This increase was preserved for 24 h [Mann–Whitney U: $Z = -1.964$, $p < 0.05$; Fig. 5(b)].

DISCUSSION

The present results show that monocular BDNF-injections modify tectofugal morphology and visuomotor performance in both brain halves, indicating a modulation of the balance between left- and right-hemispheric visual circuits. Net effects differed substantially depending on the amount of exogenous BDNF and acted in a direction opposite to asymmetric embryonic light stimulation.

Ocular BDNF Adjusts Visuoperceptual and Visuomotor Circuits Opposing to Light

The performance of the experimental groups differed with respect to eye-condition. Because of the complete crossing of the optic nerves, this eye-specific effect provides evidence for differences in hemispheric-specific processing (Güntürkün, 2002b). As expected, no left–right group differences in discrimination accuracy of saline-injected and dark-incubated animals could be detected (Skiba et al., 2002). In contrast, BDNF-injections decreased slightly performance with the BDNF-enriched right eye and more intriguingly, increased performance with the contralateral left eye. After triple BDNF-injections, the left–right difference in discrimination accuracy was significant with the left-eye being on par with binocular performance. It is very rare that monocular and binocular performances are identical (Güntürkün and

Kesch, 1987; Manns and Güntürkün, 1999a). Possibly, the animals were mostly using this left eye, even under binocular conditions.

In principle, differences in grit–grain discrimination accuracy can result from differences in either visual perception or pecking speed. According to previous studies only visuoperceptual performance was higher with the right eye, while pecking speed (number of pecks within 30 s) was equal between both eyes (Güntürkün and Kesch, 1987; Manns and Güntürkün, 1999a; Skiba et al., 2002). This was usually interpreted as reflecting an asymmetry of ascending visual systems and not of descending visuomotor pathways (Skiba et al., 2002). The present results, however, show that both perceptual accuracy and visuomotor speed contributed to the final asymmetry. Hereby, BDNF enhanced binocular pecking speed and reduced it dose-dependently when seeing with the right eye. This profound reorganization of visuomotor circuits after BDNF-injections is unlike the pattern seen in normally hatched animals and thus indicates that ocular BDNF-injections do not mimic embryonic light stimulation. Consequently, retinal BDNF possibly does not mediate the embryonic signal which initiates visual asymmetry.

Contrasting Morphological Effects of Increasing BDNF Level

BDNF modified in a concentration-dependent manner cell body sizes of specific retinal and tectal cell populations. BDNF is known to influence axo-dendritic differentiations, synaptogenesis, and synaptic transmission (Huang and Reichardt, 2001; Vicario-Abejón et al., 2002; Cohen-Cory and Lom, 2004). Within the retinotectal system, BDNF enhances retinal axonal arbor complexity by expanding synaptic territories within a very short time scale (Alsina et al., 2001; Hu et al., 2005), while simultaneously coordinating synapse formation and stabilization with postsynaptic tectal cells (Cohen-Cory and Fraser, 1995; Lom et al., 2002). Dendritic arborisation is controlled in a contrasting manner by local and target-derived sources of BDNF as exemplified by retinal ganglion cells which display decreased arbor complexity after retinal but enhanced complexity after tectal BDNF applications (Lom and Cohen-Cory, 1999; Lom et al., 2002). Therefore, a decrease of ipsilateral retinal soma sizes could be expected after ocular BDNF-injections although the retinae of normal light-incubated animals do not show soma size asymmetries (Güntürkün, 1997). But surprisingly, it was not the BDNF-enriched retina which displayed modified cell sizes. Soma size enlargements were detected within the contralateral retina. This effect must be a secondary consequence of exogenous BDNF and in principle, three pathways can account for it.

First, embryonic chicken retinae give rise to a transient retinoretinal projection which disappears very quickly and whose terminal branching pattern can be modulated by ocular BDNF-injections (Thanos, 1999). If those retinoretinal fibers are likewise present in pigeon hatchlings, they might transport BDNF retrogradely into the contralateral retina. Since only target-derived BDNF increases dendritic branching of retinal ganglion cells (Lom et al., 2002), trophic effects were confined to the contralateral left retina. Second, the avian retina is innervated by the contralateral nucleus isthmo-opticus (ION) and from surrounding ectopic cells, which give rise to a small ipsilateral projection (Wolf-Oberhollenzer, 1987; Güntürkün, 1987; Woodson et al., 1995). Since during development, the portion of ipsilaterally projecting cells is higher (Clarke and Cowan, 1975; Péquignot and Clarke, 1992), contralateral BDNF effects might be mediated by these fibers. However, since retinoretinal as well as ipsilateral ION projections are rather small they might not convey substantial trophic influences onto the left retina. As outlined below, the morphometric results demonstrate bilateral tectal effects of monocular BDNF injections. Therefore as a third possibility, it is likely that contralateral retinal effects are consequences of interhemispheric interactions which affect tectal neurons (Woodson et al., 1991) that project onto the right ION and which in turn, innervates the left eye.

This conclusion is supported by our Ras activation analysis that did not show enhanced intracellular signaling in response to BDNF-injections within the contralateral left retina. This makes it very unlikely that anterogradely transported BDNF accounts for the observed cell size changes in the contralateral retina. In contrast, apart from a short term increase in the BDNF-injected retina that did not lead to ipsilateral retinal cell size changes a long lasting enhancement of Ras activation could be observed in both tectal halves. This suggests the tectum as a primary action site of ocular BDNF-injections. Since Ras activity plays an important morphogenetic role in neurons (Heumann et al., 2000) this pattern indicates long lasting changes of tectal differentiation processes. However, since light is shown to decrease Ras tectal activity (Manns et al., 2005), these results further support that ocular BDNF does not mimic an ontogenetic light pulse.

At tectal level, we detected changes of cells body sizes which were specific for different cell types.

CB-ir cells were present within the retinorecipient layers 3 and 5 as well as within a subclass of efferent neurons within layer 13 (Manns and Güntürkün, 2005). Only the efferent relay cells displayed enlarged cell sizes after single BDNF injections. Because of the restriction of their dendrites to layers 12–13, these cells presumably do not receive direct retinal input and either ascend onto the rotundus (Hellmann and Güntürkün, 2001) or descend onto brainstem premotor neurons (Hellmann et al., 2004). The enhanced pecking activity after single BDNF injections could be related to changes of this descending CB-ir cell system.

A completely different pattern could be observed for the PV-ir cell population. Only triple injections decreased cell body sizes of PV-ir cells in both tectal halves. Cell shrinkage provides evidence for an inhibitory influence of high BDNF doses onto the PV-ir cell population and is consistent with a suppressive effect of light onto PV-ir tectal neurons (Manns and Güntürkün, 2003). Inhibition was not confined to the BDNF enriched left tectum, but affected both tectal hemispheres. This supports that higher BDNF doses specifically influence interhemispheric interactions. Interhemispheric control onto cell type-specific differentiation is also demonstrated by the regulation of tectal substance P expression in frog tadpoles by a pathway that relays activity from one tectal lobe to the other (Tu et al., 2000). Our morphometric analysis provides indirect evidence for modifications of a tectal circuitry since soma size is seen as an estimate for axo-dendritic arbor complexity. However, BDNF does not directly affect tectal dendritic complexity (Sanchez et al., 2006; Marshak et al., 2007) and therefore presumably does not directly modulate cell body sizes of retinorecipient neurons. In fact in our study, the majority of neurons with altered soma sizes were not in direct receipt of input from the BDNF-injected retina. Thus, these cell size changes likely emerged as secondary consequences of the retinotectal differentiation. Moreover, the contrasting modulations of CB- and PV-ir cell populations suggest differential effects onto second order neurons depending on the amount of applied BDNF and hence, indicate divergent net effects onto tectal circuits.

Ocular BDNF Alters the Balance Between Left- and Right-Hemispheric Processing

Primarily, it might be that increased BDNF levels prevent activity-dependent pruning of exuberant connections (Isenmann et al., 1999). In this case, the performance decrease with the BDNF-injected eye might result from abolished activity-dependent fine tuning of visual circuits leading to reduced visual performance of this eye and its central target areas. Such impairment of primary visual processing entails an attenuation of visuomotor systems within the left hemisphere. However, this mechanism cannot directly explain the increase in discrimination accuracy with the contralateral left eye. Possibly an impaired differentiation of one eye system is counterbalanced by a parallel enhancement of the contralateral one. This would mean that it is the successfully pruned hemisphere which develops greater impact onto visuomotor control.

The relative importance of the left- and right eye systems are possibly regulated by the tectotectal commissures (Robert and Cuénod, 1969; Hardy et al., 1984; Keysers et al., 2000) which mediate the dynamic balance between left- and right tectal processing (Güntürkün and Böhringer, 1987). This is shown in experiments which temporarily block posthatch unilateral retinal activity with tetrodotoxin. This treatment shifts visual asymmetry towards the noninjected eye system without reducing visual accuracy of the deprived eye (Prior et al., 2004). However, since asymmetrical effects were only detected after triple-BDNF injections, only higher BDNF doses seem to facilitate the performance of contralateral visual circuits.

In sum, behavioral and morphological consequences of ocular BDNF-injections suggest substantial effects onto the functional architecture of the tectofugal system which are not confined to primary targets of retinal cells. They ultimately modify visuoperceptual as well as visuomotor systems, thereby affecting the interhemispheric balance of visuomotor processing in a dose-dependent manner.

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