Creating colored letters: Familial markers of grapheme–color synesthesia in parietal lobe activation and structure

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Creating Colored Letters: Familial Markers of Grapheme–Color Synesthesia in Parietal Lobe Activation and Structure

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Abstract

■ Perception is inherently subjective, and individual differences in phenomenology are well illustrated by the phenomenon of synesthesia (highly specific, consistent, and automatic cross-modal experiences, in which the external stimulus corresponding to the additional sensation is absent). It is unknown why some people develop synesthesia and others do not. In the current study, we tested whether neural markers related to having synesthesia in the family were evident in brain function and structure. Relatives of synesthetes (who did not have any type of synesthesia themselves) and matched controls read specially prepared books with colored letters for several weeks and were scanned before and after reading using magnetic resonance imaging. Effects of acquired letter–color associations were evident in brain activation. Training-related activation (while viewing black letters) in the right angular gyrus of the parietal lobe was directly related to the strength of the learned letter–color associations (behavioral Stroop effect). Within this obtained angular gyrus ROI, the familial trait of synesthesia related to brain activation differences while participants viewed both black and colored letters. Finally, we compared brain structure using voxel-based morphometry and diffusion tensor imaging to test for group differences and training effects. One cluster in the left superior parietal lobe had significantly more coherent white matter in the relatives compared with controls. No evidence for experience-dependent plasticity was obtained. For the first time, we present evidence suggesting that the (nonsynesthete) relatives of grapheme–color synesthetes show atypical grapheme processing as well as increased brain connectivity. ■

INTRODUCTION

A fundamental question in the philosophy of mind is how experience arises from sensory information. Investigation into the subjective experience of color, especially illusory color, can elucidate how the brain constructs the first-person perceptual experience from objective external information. Some individuals, synesthetes, experience highly specific, consistent, and automatic cross-modal experiences, for which the external stimulus corresponding to the additional sensation is not physically present (Rothen, Tsakanikos, Meier, & Ward, 2013; Eagleman, Kagan, Nelson, Sagaram, & Sarma, 2007; Asher, Aitken, Farooqi, Kurmani, & Baron-Cohen, 2006; Baron-Cohen, Wyke, & Binnie, 1987). For example, the (black) letter "a" evokes the experience of red (Baron-Cohen, Harrison, Goldstein, & Wyke, 1993; Baron-Cohen et al., 1987; Cytowic & Wood, 1982). Synesthesia is informative because it provides an opportunity to understand how an additional experience like color arises in the absence of the external input corresponding to wavelengths of light. Synesthetic experiences are distinct from hallucinations (Sagiv, Ilbeigi, & Ben-Tal, 2011; Cytowic, 2002), and developmental synesthesia is normally present in the

absence of pathology (Cytowic, 2002; Baron-Cohen & Harrison, 1997).

The role of the environment in shaping perception is well illustrated in the development of synesthesia (Novich, Cheng, & Eagleman, 2011; Barnett et al., 2008; Beeli, Esslen, & Jäncke, 2007; Smilek, Carriere, Dixon, & Merikle, 2007; Rich, Bradshaw, & Mattingley, 2005; Simner et al., 2005). Two studies have shown that synesthetic color experiences can be acquired by associative training methods (Bor, Rothen, Schwartzman, Clayton, & Seth, 2014; Howells, 1944). The studies of Howells and Bor et al. involved intensive training periods (~30,000 trials and 9 weeks of training, respectively). Additionally, Bor et al. administered a battery of diverse and adaptive tasks. Other studies designed to train synesthetic experiences did not result in (strong) evidence for acquired synesthetic color phenomenology after training but were notably less intensive or nonadaptive (for reviews see Rothen & Meier, 2014; Deroy & Spence, 2013). Synesthesia-like behavior—without experiencing color—is commonly found after synesthetic training paradigms (Rothen & Meier, 2014).

If our fundamental perceptual experiences are determined by the environment to a significant extent, why does not everyone become a synesthete? Synesthesia tends to run in the family (Barnett et al., 2008; Baron-Cohen, Burtlf, Smith-Laittan, Harrison, & Bolton, 1996; Galton,

1880), and there is evidence for a genetic predisposition (Tomson et al., 2011; Asher et al., 2009). The predisposition to having the trait of synesthesia does not, however, determine which type of synesthesia a person will develop (Rouw, Scholte, & Colizoli, 2011). It seems to be the case that an interaction between environmental and genetic factors determines the specifics of an individual's perceptual experiences.

Synesthetic Color in the Brain

An extended network of brain regions was implicated in synesthetic color activation across multiple fMRI studies, which employed the contrast of synesthetic color experience versus no synesthetic experience (Rouw et al., 2011). Brain regions implicated in the experience of synesthetic colors were the following: occipitotemporal cortex (not restricted to color area V4), posterior parietal cortex (including superior and inferior regions), insular cortex, precentral gyrus, and (right) dorsolateral pFC. This review noted that "The most compelling area of common activation across studies was the inferior parietal lobule" and that "All locations of activation in inferior parietal lobule, however, are best summarized as either near the intraparietal sulcus or in the angular gyrus." Causal evidence for the role of the parietal lobe in synesthesia was obtained in TMS studies targeting the angular gyrus at the junction of the posterior intraparietal sulcus and transverse occipital sulcus (Rothen, Nyffeler, von Wartburg, Müri, & Meier, 2010; Muggleton, Tsakanikos, Walsh, & Ward, 2007; Esterman, Verstynen, Ivry, & Robertson, 2006). The parieto-occipital region is associated with color–form binding in normal perception (Donner et al., 2002). The role of parietal cortex in synesthesia is generally proposed to be the "hyperbinding" of form to color in synesthesia (Weiss & Fink, 2009; Hubbard, 2007; Esterman et al., 2006; Robertson, 2003). We hypothesized that potential effects of additional color acquired by training would be evident in occipitotemporal (see Colizoli et al., 2016) and parietal brain regions, as it is for developmental synesthesia.

Studies investigating conflict between veridical and synesthetic color in the brain activation of synesthetes measured with fMRI have employed a variety of tasks and conditions and have yielded mixed results (van der Veen, Aben, Smits, & Röder, 2014; Laeng, Hugdahl, & Specht, 2011; van Leeuwen, Petersson, & Hagoort, 2010; Cohen Kadosh, Cohen Kadosh, & Henik, 2007; Weiss, Zilles, & Fink, 2005). Networks of activation across the brain related to congruency effects in synesthetes are evident from studies employing Stroop-like comparisons (van der Veen et al., 2014; Laeng et al., 2011; van Leeuwen et al., 2010; Cohen Kadosh et al., 2007). This is not surprising considering that the synesthetic Stroop effect can arise due to either perceptual or semantic conflict (or both) and therefore related brain activation likely reflects both sensory and semantic processes in addition to motor and general cognitive control factors. We therefore did not have a priori predictions of specific brain regions involved in interference between veridical and associated colors after cross-modal training of letters to colors, but instead expected to find a widespread network of activation for contrasts of letter–color congruency.

Familial Markers of Synesthesia

The brains of developmental synesthetes differ from nonsynesthetes in both functional and structural measures (Rouw & Scholte, 2010; Jäncke, Beeli, Eulig, & Hänggi, 2009; Weiss & Fink, 2009; Hänggi, Beeli, Oechslin, & Jäncke, 2008; Rouw & Scholte, 2007). By comparing nonsynesthetic relatives of synesthetes to (nonsynesthete and nonrelative) matched controls, differences may point toward underlying familial traits of synesthesia. Determining familial markers of the presence of synesthesia running in the family may help to unravel the complex genetic versus environmental interaction determining the range of an individual's perceptual experience. We hypothesized that the familial trait of synesthesia "running in the family" might be evident in relatives of synesthetes who do not report having any types of synesthesia themselves. As this is the first study to test for neurological markers of synesthesia in the nonsynesthetic relatives of synesthetes, we consider group comparisons to be exploratory in nature.

Goals of the Current Study

We used a reading-in-color paradigm (Figure 1) to train letter–color associations (Colizoli, Murre, & Rouw, 2012).
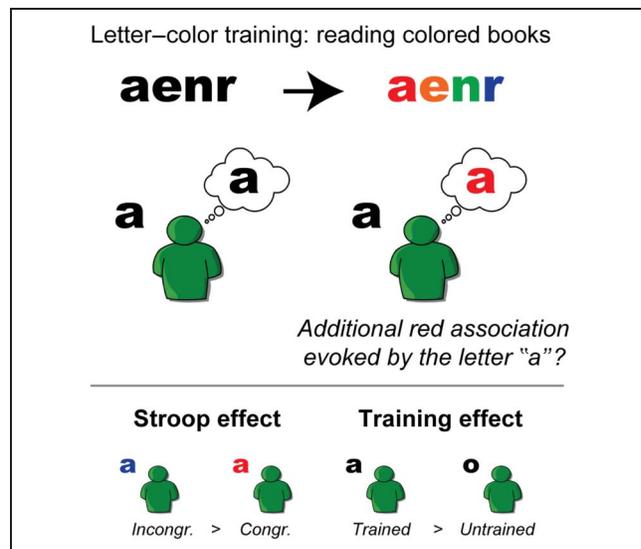
Figure 1. A schematic of the reading in color paradigm. Reading books with consistently colored letters over several weeks can influence behavior and experience. Comparisons of interest are incongruently versus congruently colored letters (Stroop effect) and achromatic trained versus untrained letters.

We have previously shown that behavioral congruency effects can be acquired by reading books with consistently colored letters (Colizoli et al., 2012, 2016). We also examined visual cortex activation related to acquired letter–color associations (Colizoli et al., 2016). The current study uses the same subject sample but, in contrast with the previous study, examines which regions are related to learning synesthesia in whole-brain analyses (rather than within visual cortex) and looks at structural as well as functional brain measurements. For the first time, fMRI, voxel-based morphometry (VBM), and diffusion tensor imaging (DTI) are used to investigate the neural underpinnings of acquired letter–color associations in relatives of synesthetes and matched controls.

First, we verified that interference between the acquired letter–color associations and veridical color (incongruently vs. congruently colored letters) was evident in whole-brain activation. Second, we investigated the relationship between achromatic training-related activation and letter–color congruency in whole-brain activation. As expected, effects of acquired letter–color associations were evident in training-related brain activation in the right parietal lobe. Third, within the parietal ROI, we examined if brain activation for letter conditions depended on group (relative vs. control). This analysis showed that the obtained region did indeed differ in activation depending on this familial factor. Finally, we tested for main effects of training (pre vs. post), group differences (relative vs. control), and their interaction in both gray and white matter structure. Relatives of synesthetes showed increased structural connectivity (compared with controls) in one cluster along the white matter skeleton near the postcentral gyrus of the left superior parietal lobe.

METHODS

Some of the methods have been published (Colizoli et al., 2016), as the same sample of participants is used in the current study. We report here all methods and materials directly related to the current study.

Participants

Relatives (first, second, and third degree) of synesthetes were recruited by contacting synesthetes from our participant database and asking them if they had any relatives who would be interested in participating in a study about the effects of "reading in color." The grapheme–color synesthesia of the synesthetes was verified with a standardized synesthesia battery (Eagleman et al., 2007) or with a questionnaire test–retest paradigm (Asher et al., 2006). Sample size was not predetermined; all relatives interested in participating who did not report having dyslexia, attention-deficit disorder, or synesthesia (by interview) and passed the standard MRI screening were included in the study. All participants were tested for color blindness (Ishihara, 1936). Eleven adult relatives of synesthetes (8 women, $M = 24.73$ years, $SD = 2.32$) and 11 controls matched for age, sex, handedness, and education (8 women, $M = 25.18$ years, $SD = 2.64$) took part in the study (entire sample: $M = 24.95$ years, $SD = 2.43$, range = 22–30 years). The familial relationships of the participants to their synesthetic relatives are given in Table 1. Three pairs of brothers and sisters were in the relative group. None of the control participants reported being aware of anyone with any type of synesthesia in their families, although this could not be objectively verified. All participants in the training study

Table 1. Familial Relationships of the Participants to Their Synesthete Relatives

Sex	Relationship to Synesthete(s)	% Genes	Side of Family
F*	Half sister	25	Father
M*	Half brother	25	Father
F**	Sister	50	Mother and father
M**	Brother	50	Mother and father
F***	First cousin	12.5	Father
M***	First cousin	12.5	Father
F	Sister; daughter	50	Mother and father; mother
F	Sister	50	Mother and father
F	Sister; daughter	50	Mother and father; father
F	Sister	50	Mother and father
F	Daughter	50	Mother

The side of the family refers to the relation shared between the participant and the synesthetic relative(s) listed. The estimated percentage of shared genes (% Genes) between the participant and synesthetic relative(s) is given. Three brother–sister pairs participated in the study and are marked with matching asterisk pairs (*, **, and ***).

were given a synthetic test of consistency (both the relatives and controls; Colizoli et al., 2016). None of the participants were deemed to have grapheme–color associations when tested objectively using a consistency test–retest procedure (cutoff at 70% for 43 items and scores ranged from 9.3% to 55.81%). All participants (including the synesthete relatives) were compensated financially for their participation and gave written informed consent. The Ethical Committee of the Department of Psychology at the University of Amsterdam approved this experiment. All participants were included in the final analyses. One of two runs of the Stroop task of one participant (relative group) and both behavioral log files of another participant (control group) were lost because of technical error during scanning.

Experimental Design

Participants read specially prepared books that contained four high-frequency lowercase letters ("a," "e," "n," and "r") in four high-frequency colors (red, orange, green, and blue). The combination of the letter–color pairs differed between participants. Before any testing began, participants reported their preferences for letter–color pairs (a 5-point Likert scale). These data were used to counterbalance letter–color preference with relative letter frequency (in Dutch) to ensure that, for example, the high-frequency letters did not receive only the most preferred colors (Colizoli et al., 2012). Half of the participants received their preferred letter–color pairs for the two higher-frequency letters ("e," "n") and their nonpreferred letter–color pairs for the two lower-frequency letters ("a," "r"). The other half of the participants received the opposite preference-to-frequency mapping. Each control participant was assigned to the same preference group as their counterpart in the relative group but received unique letter–color pairs based on their individual preferences. Books (in Dutch) were obtained from the Publisher Nijgh & Van Ditmar (www.nijghenvanditmar. nl). The content of the books was not altered in any way. The procedure for formatting the books and instructions for reading has been described in detail (Colizoli, Murre, & Rouw, 2014).

Participants completed a behavioral and MRI testing session before and after training. The order of the MRI and behavioral measurements as well as the order of the behavioral tasks (within each session) were counterbalanced across participants. The order of the MRI and behavioral measurements were kept constant between the pre- and posttraining measurements when possible. Each control participant received the same procedure as the relative he or she was matched to. Participants completed a Stroop task, a crowding task, and were asked to read while eye movements were recorded in the behavioral sessions (crowding and reading data not reported here). Each behavioral session took approximately 1 hr to complete. Participants were seated 50 cm in front of a

computer monitor. This distance remained constant by the use of a chin rest. All stimuli were presented on a PC with Presentation (version 14; www.neurobs.com) on a 20-in. VGA monitor. The screen resolution was 1280 × 1024 pixels. All responses were recorded with a USB keyboard. In the MRI scanner, the functional tasks differed between testing sessions. A visual word form area localizer and a retinotopic mapper were administered during the pretraining MRI session (these data are not reported here). The Stroop task and crowding task (crowding data not reported) were administered in addition to a color localizer during the posttraining MRI session. Functional and structural data were collected using a fixed order of runs across participants optimized to minimize fatigue. All participants were monitored with an eye tracker to ensure that no one fell asleep. Each MRI session took about 1.5 hr.

At the end of each session, participants filled out several questionnaires. Participants completed a general screening form, visual imagery questionnaires, and the first part of the test of synthetic consistency in the pretraining session. Participants completed the second part of the synthetic consistency test and a general questionnaire about their reading experience in the posttraining session (Colizoli et al., 2014). One representative question from the reading experience questionnaire was chosen to test the reliability of a correlation between Stroop effect sizes and self-reported color experience previously obtained (Colizoli et al., 2012). The question "Whenever I see or think about certain letters, I have no color experience," was expected to negatively correlate with Stroop effect sizes.

Stroop Task

Participants were shown one of eight letters ("a," "b," "e," "g," "k," "n," "r," or "t") consisting of three letter conditions: (1) the four trained letters ("a," "e," "n," and "r") presented in colors congruent with the colors within the books, (2) the four trained letters ("a," "e," "n," and "r") presented in colors incongruent with the colors within the books, and (3) the neutral condition consisted of four untrained letters ("g," "t," "k," and "b") that were always in black text within the colored books. Instructions were to name the veridical color of the letter as fast and accurately as possible. A total of 288 trials (96 congruent, 96 incongruent, and 96 neutral trials) were presented in random order in the behavioral sessions. The neutral letter condition was used as an exploratory measure. Two runs of the Stroop task were presented in the postreading MRI testing session. Each run consisted of 225 volumes and lasted 7.5 min. In each MRI run, 72 trials were presented in random order (24 trials per letter condition). Participants responded with their right hands. RTs that were greater than 2.5 times the standard deviation per participant and condition were removed for analysis.

Color Localizer

A color localizer was used to investigate effects of veridical and "synesthetic" color (van Leeuwen et al., 2010). The color localizer was a blocked design with 16-sec stimuli blocks and 16-sec periods of rest between blocks and consisted of three stimulus conditions: (1) trained letters presented in black ("a," "e," "n," and "r"), (2) untrained letters presented in black ("o," "z," "u," and "w"), and (3) untrained colored letters ("c," "m," "v," and "s") presented in eight distinct colors (red, orange, brown, yellow, green, blue, purple and pink). The order of the colored letters colors remained constant, whereas the four letters were randomly assigned (with four repetitions) to each color. Conditions were presented in pseudorandomized blocks, and each condition was presented six times in a run. Within each block, 16 stimuli were randomly presented (four letters with four repetitions) for 500 msec with an intertrial interval of 500 msec. The color localizer consisted of 330 volumes, lasted 11 min. Participants passively viewed the stimuli.

fMRI Acquisition and Data Analysis

fMRI scans were acquired on a Philips (Amsterdam, The Netherlands) 3-T Achieva TX scanner, located at the Spinoza Center for Neuroimaging, Amsterdam, the Netherlands. Whole-brain gradient-echo EPI measurements (voxel size = $3 \times 3 \times 3$ mm, repetition time [TR] = 2000 msec, echo time [TE] = 27.63 msec, flip angle = 76.1°, field of view [FOV] = 240×240, matrix = 80×80, slice thickness = 3 mm, slice gap = 0.3 mm, 38 slices per volume, sensitivity encoding factor of 2) were acquired to measure BOLD magnetic resonance images with a 32-channel SENSE head coil. Analyses of the MRI images were carried out using FMRIB Software Library (FSL) version 5.0.4 (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012; Woolrich et al., 2009; Smith et al., 2004). Statistical analyses were conducted using FSL's fMRI Expert Analysis Tool (FEAT version 6.00). Preprocessing steps included prewhitening (FILM algorithm), spatial smoothing (Stroop task: 5 mm; color localizer: 3 mm), grand-mean intensity normalization of the entire 4-D data set by a single multiplicative factor, motion correction, and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with $\sigma = 50$ sec). Voxels belonging to brain tissue were extracted from non-brain tissue voxels using the brain extraction tool (Smith, 2002). No runs were discarded because of motion or other artifacts. In the first-level analysis, the time course of each run was convolved with the double gamma hemodynamic response function and tested with an uncorrected voxel threshold of $p < .05$. For the Stroop task (event-related), the temporal derivatives of each explanatory variable were included as confound regressors. Resulting contrast images were linearly registered to the anatomical structure using FLIRT with 7 degrees of freedom and the full search space (Greve & Fischl, 2009;

Jenkinson, Bannister, Brady, & Smith, 2002; Jenkinson & Smith, 2001), then spatially normalized to the T1-weighted MNI-152 stereotaxic space template (2 mm) using FNIRT with 12 degrees of freedom and the full search space (Andersson, Jenkinson, & Smith, 2007; Andersson, Jenkinson, Smith, & Andersson, 2007). The two runs of the Stroop task were averaged per participant before being entered into the final high-level analysis. The high-level analysis was carried out using FLAME (FMRIB's local analysis of mixed effects) Stages 1 and 2 with automatic outlier detection (Woolrich, 2008; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004; Beckmann, Jenkinson, & Smith, 2003). Z-statistic (Gaussianised T/F) images were thresholded using clusters determined by $Z > 2.3$ and a corrected cluster significance threshold of $p < .05$.

VBM Acquisition and Data Analysis

MRI scans were acquired on a Philips 3-T Achieva TX scanner, located at the Spinoza Center for Neuroimaging, Amsterdam, the Netherlands. In the first and last run of each MRI session, a T1-weighted anatomical scan was acquired (four per participant, voxel size = $1 \times 1 \times 1$ mm, TR = 8229 msec, TE = 3.77 msec, flip angle = 8°, FOV = 256×256, matrix = 256×256, slice thickness = 1 mm, no slice gap, 160 slices per volume, 1 volume was acquired in 5 min). T1-weighted images were used for the VBM analysis in addition to registration of functional images from native anatomical space into the standard space (MNI). T1-weighted structural data were analyzed using an optimized VBM protocol (Douaud et al., 2007; Good et al., 2001a, 2001b) as part of FSL. First, structural images were brain-extracted and gray matter-segmented before being registered to the T1-weighted MNI-152 standard space using the nonlinear registration tool FNIRT. The resulting images were averaged and flipped along the x axis to create a left–right symmetric, study-specific gray matter template. Second, all native gray matter images were nonlinearly registered to this study-specific template and "modulated" to correct for local expansion (or contraction) because of the nonlinear component of the spatial transformation. This study-specific template was made separately for each session (only voxels in both sessions' templates were included in the final analysis). The modulated gray matter images were then smoothed with an isotropic Gaussian kernel with a sigma of 4 mm. Statistical analysis was implemented using the threshold-free cluster enhancement (Smith & Nichols, 2009) option in Randomise (Nichols & Holmes, 2002) and a permutation-based nonparametric test (Anderson & Robinson, 2001) with 25,000 permutations. Multiple comparisons across space were corrected (family-wise error rate = 5%).

DTI Acquisition and Data Analysis

MRI scans were acquired on a Philips 3-T Achieva TX scanner, located at the Spinoza Center for Neuroimaging,

Amsterdam, the Netherlands. Fractional anisotropy (FA) was calculated on the basis of the acquisition of diffusion-weighted spin-echo EPI measurements (voxel size = 2 × 2 × 2 mm, TR = 6310 msec, TE = 73.36 msec, flip angle = 90°, FOV = 224 × 224, matrix = 112 × 112, slice thickness = 2 mm, no slice gap, 60 slices per volume, sensitivity encoding factor of 2). Diffusion was measured in 32 noncollinear directions. The start of each run was preceded by the acquisition of a non-diffusion-weighted volume for purposes of registration for motion correction. Four runs of diffusion-weighted images were acquired in each MRI session (8 per participant). Each DTI run consisted of 34 volumes and lasted 4 min. A voxel-wise statistical analysis of FA data was carried out using the Tract-Based Spatial Statistics (Smith et al., 2006), a part of FSL. First, FA images were created by fitting a tensor model to the raw diffusion data using FDT and then brain-extracted using the brain extraction tool. All participants' FA data were then aligned into a common space using the nonlinear registration tool FNIRT, which uses a b-spline representation of the registration warp field (Rueckert et al., 1999). Next, the mean FA image was created and thinned to create a mean FA skeleton that represents the centers of all tracts common to the whole sample. Each participant's aligned FA data were then projected onto this skeleton. Statistical analysis was implemented using the Threshold-Free Cluster Enhancement option in Randomise and a permutation-based nonparametric test with 25,000 permutations. Multiple comparisons across space were corrected (family-wise error rate = 5%).

RESULTS

Grapheme–Color Consistency Did Not Differ between Groups before Training

The sample as a whole was deemed not to have grapheme–color synesthesia based on results of the consistency test (Colizoli et al., 2016). The consistency scores of the relatives (M = 31.74%, SE = 4.25) and matched controls (M = 32.98%, SE = 3.29) did not differ, t(20) = −0.24, p = .82.

Acquired Letter–Color Associations by Reading in Color

As previously reported (Colizoli et al., 2016), within an average of 20 days (SD = 11), participants read 80,397.50 words (SD = 38,278.92) and 381,008.00 characters (SD = 182,383.01). An acquired Stroop effect due to training is evidenced by a significant interaction between testing session (pretraining vs. posttraining) and congruency conditions (congruent vs. incongruent letter–color trials). For the behavioral results (outside the scanner), a significant interaction was found for RTs between testing session (pre vs. post) and congruency (congruent vs. incongruent) across the entire sample, F(1, 21) = 4.75, p = .041, η_p^2 = 0.184. The interaction showed that participants ac-

quired letter–color associations from reading the colored books to the point that they interfered with RT during color naming after training, but not before training. A posttraining Stroop effect in RT (incongruent > congruent) was also found while participants performed the Stroop task in the scanner, t(20) = 2.67, p = .015, d = 0.584. We concluded that these participants acquired letter–color associations from reading specially prepared books with colored letters. Despite instructions to ignore the trained color, automatic interference effects were measured in the Stroop task. The relatives and matched controls did not differ in terms of the amount of words (p = .29) or characters (p = .34) read, and therefore, the groups were considered to have received equal amounts of training.

The Stroop Effect Acquired after Training Is Reflected in Brain Activation

The acquired single-letter Stroop effect due to training was evident in brain activation (Table 2) in addition to behavior (e.g., Bor et al., 2014; Colizoli et al., 2012; Meier & Rothen, 2007; Elias, Saucier, Hardie, & Sarty, 2003). We found that the training was sufficient to evoke increased activation in incongruent trials compared with congruent trials in six clusters, including visual and memory structures. The Stroop effect in behavior is defined as the

Table 2. Whole-brain Results of the Stroop Task

| Cluster	Brain Region	x	y	z	Z-max	Voxels
Incongruent > Congruent						
1	L precuneus cortex	−14	−60	42	3.73	5442
2	L occipital fusiform gyrus	−36	−82	−20	3.5	1568
3	L hippocampus	−32	−14	−12	3.52	1473
4	R cerebellum	38	−60	−38	3.09	697
5	L precentral gyrus	−34	−4	44	3.57	541
6	L precentral gyrus	−56	6	30	3.58	425
Congruent > Incongruent						
	No significant clusters					
Group × Congruency (F test)						
	No significant clusters					

The Stroop task was administered in the scanner after participants had read the specially prepared colored books. Incongruent and congruent conditions were contrasted across the entire sample (n = 22). In addition, an interaction between congruency conditions and group (n = 11) at the level of the whole brain was investigated with an F test. Significant clusters of activation are reported (Z > 2.3, p < .05). Brain regions refer to the atlas-based location of the peak voxel of the normalized activation (Z-max) of each cluster. Coordinates are in MNI space.

increase in RTs for incongruent compared with congruent trials. Therefore, whole-brain contrasts of congruency necessarily include differences in RTs between the congruency conditions. Differential activation found in certain brain regions related to motor functions, such as that found in the left precentral gyrus (participants gave right-hand responses), may reflect RT differences between congruency conditions. Relatives of synesthetes did not differ from controls for the contrasts tested (*F* test: congruent vs. incongruent) at the level of the whole brain.

Training-related Parietal Activation Scales with Stroop Effect Size

At the level of the whole brain, we were interested in neural correlates of an interaction between congruency effects measured in behavior and training effects in brain activation. We therefore investigated whether any brain region could predict the "strength" of the acquired letter–color associations (measured as the posttraining Stroop effect in RTs) while participants viewed achromatic letters. During the color localizer, participants viewed trained and untrained black letters. The trained > untrained contrast of black letters is akin to localizing the experience of "synesthesia," in which color-inducing graphemes are contrasted with noninducing graphemes (van Leeuwen et al., 2010). Note that because each participant is trained on a particular set of letters in the current paradigm, we did not use "trained versus untrained" as main contrast, because low-level letter properties would have a mediating (confounding) effect on activation patterns. Instead, we investigated where in the brain an increased activation to trained (compared with untrained) letters is related to the size of the behavioral Stroop effect. Thus, we aimed to find brain areas reflecting the aspect that we were particularly interested in: the degree of association as measured in the acquired Stroop effect in trained letters.

In a whole-brain analysis, we tested for a (positive and negative) correlation between the acquired Stroop effect sizes (in the behavioral data) and relative brain activation for the trained > untrained contrast of achromatic letters. One region was obtained for the positive direction (trained > untrained), located in the right angular gyrus of the parietal lobe (*Z*-max = 3.13 at xyz = [50, −54, 50] MNI, 208 voxels; Figure 2A). In this angular gyrus region, the effect in brain activation of training on viewing achromatic letters was correlated with the strength of the acquired letter–color associations measured as the behavioral Stroop effect size, $s(20)$ = 0.63, p = .002, 95% CI = [0.24, 0.91] (Figure 2B). Increased brain activity for the trained > untrained letters was related to a larger behavioral acquired Stroop effect. Note that this right angular gyrus region did not overlap with any significant cluster obtained for the incongruent > congruent contrast while participants performed the Stroop task in the scanner
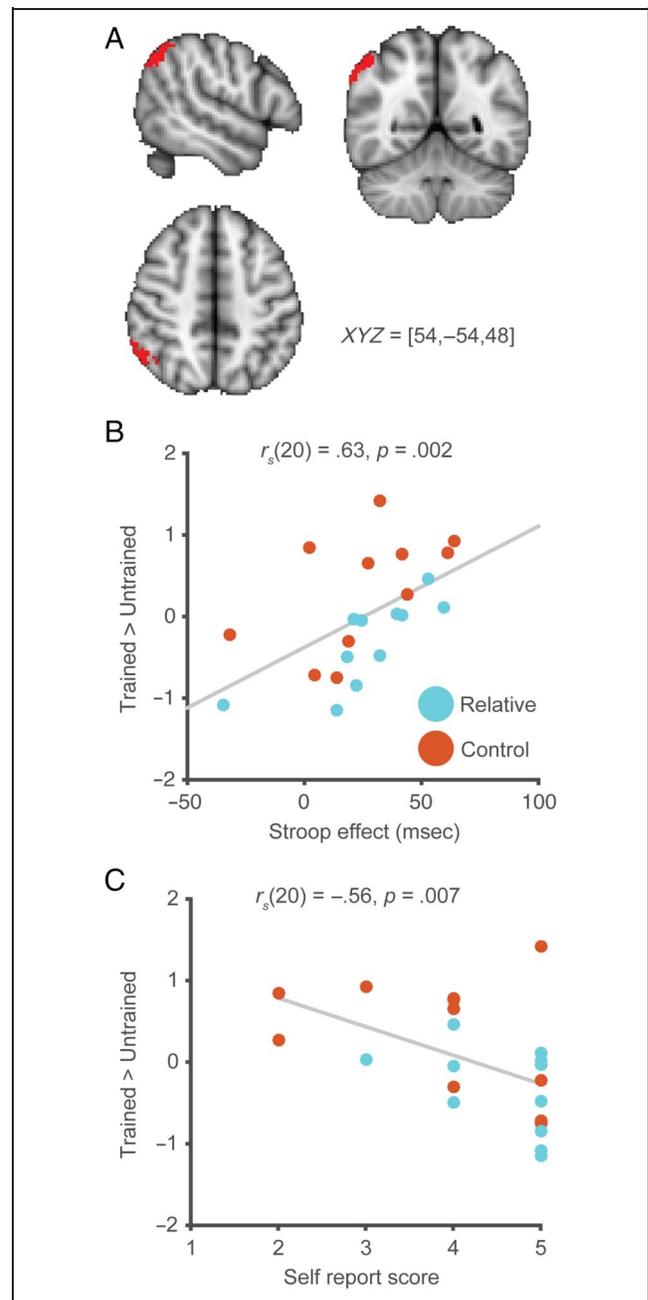
Figure 2. Training-related activation in the right angular gyrus of the parietal lobe scales with Stroop effect size. (A) In a whole-brain analysis, one region in the right angular gyrus showed increased activation for trained (compared with untrained) achromatic letters that was directly related to the strength of the acquired letter–color associations (measured as the posttraining Stroop effect). (B) The correlation between activation for the contrast trained > untrained letters and the posttraining Stroop effect in RTs is plotted for illustration. Participants who showed a greater difference in brain activity for the trained > untrained contrast also showed a greater acquired Stroop effect. (C) Within this angular gyrus ROI, trained > untrained activation was also negatively correlated to self-report scores for the question: "Whenever I see or think about certain letters, I have no color experience." (1 = *strongly disagree*, 5 = *strongly agree*). Participants who showed a greater difference in brain activity for the trained > untrained contrast also reported disagreeing with the statement to a larger degree. Coordinates are in MNI space. Note that activation within this localized region showed a difference between relatives of synesthetes and matched controls.

(see Table 2). No significant clusters were obtained for the same correlation with brain activation (untrained > trained) and the Stroop effect in the negative direction.

We previously found (in an independent sample) that participants with larger Stroop effect sizes after training also tended to agree more with the statement "I am experiencing color when thinking about certain letters" (Colizoli et al., 2012). The reading experience questionnaire has since been expanded to better probe overall color experience by adding several new statements (Colizoli et al., 2014). To minimize the number of comparisons made here, one representative question of overall color experience (out of five possible questions) was chosen from the reading experience questionnaire to test for a relationship in the expected direction with the Stroop effect: "Whenever I see or think about certain letters, I have no color experience" (5-point Likert scale). We expected participants who had larger Stroop effect sizes to tend to disagree with this statement. The correlation between responses to this statement and behavioral Stroop effect sizes was in the expected direction but did not reach significance, $r_s(20) = -0.34, p = .059$ (one-tailed), 95% CI = [−0.72, 0.15]. Finally, we tested whether activation in the brain region directly related to individual differences in the acquired Stroop effect reflected the overall reported color experience. We did not have a hypothesis regarding the direction of this relationship. Training-related activation in the angular gyrus ROI was negatively correlated with the responses to the statement, $r_s(20) = -0.56, p = .007$, 95% CI = [−0.84, −0.11]. Interpreted in the positive direction, participants who indicated having more of a color experience also showed greater activation for the trained > untrained contrast within this ROI defined by the Stroop effect (Figure 2C).

Familial Markers of Synesthesia in Parietal ROI

The angular gyrus region was deemed an appropriate candidate for an ROI analysis of being related to synesthetic associations. As explained in the Introduction, the parietal lobe is implicated in attentional binding mechanisms in synesthesia (Rothen & Meier, 2009; Weiss & Fink, 2009; Hubbard, 2007; Muggleton et al., 2007; Esterman et al., 2006; Robertson, 2003). Group differences obtained in this region might thus reflect the predisposition for developing the trait of synesthesia. We investigated differential group activation for contrasts of interest related to letter–color training and congruency within this ROI. Note that groups did not differ behaviorally (Colizoli et al., 2016). However, (small) neural effects can be observed in the absence of overt behavior (e.g., Kok, Failing, & de Lange, 2014; De Gardelle, Stokes, Johnen, Wyart, & Summerfield, 2013).

The readout of right angular gyrus activation was performed for the achromatic conditions of the color localizer. The within-subject factor Training (trained vs. untrained) and the between-subject factor of Group

(relatives vs. controls) were compared using a two-way mixed ANOVA. The interaction between groups and training levels was significant, $F(1, 20) = 5.87, p = .025, \eta_p^2 = 0.227$ (Figure 3A). Groups also differed on average,
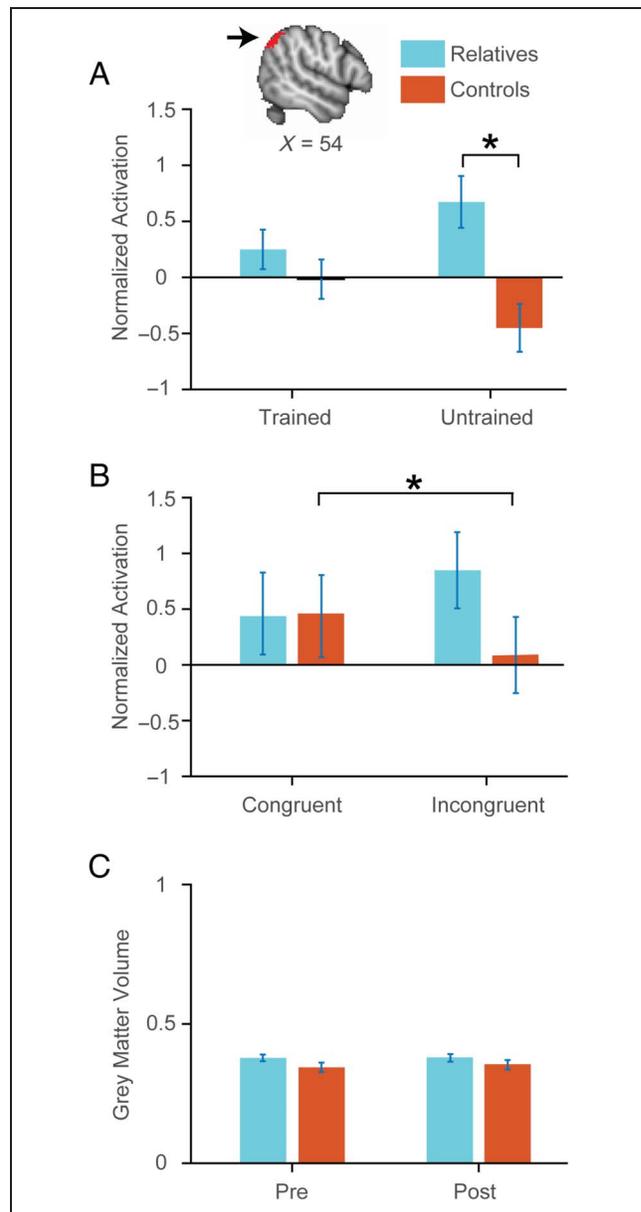
Figure 3. Functional and structural comparisons of the angular gyrus ROI. (A) Brain activation (Z-stat) during the color localizer. Conditions of interest were trained and untrained achromatic letters (during passive viewing) and are shown relative to baseline (fixation cross). The significant interaction between training condition (trained vs. untrained) and group (relatives vs. controls) is illustrated. (B) Brain activation (Z-stat) during the single-letter Stroop task (instructions were to identify the color of the letter presented). Conditions of interest were incongruent and congruent chromatic letters and are shown relative to baseline (fixation cross). The significant interaction between congruency condition (congruent vs. incongruent) and group (relatives vs. controls) is illustrated. (C) Normalized gray matter volume of T1-weighted images (obtained using VBM methods) acquired both before and after training. There was no interaction between training (pre vs. post) and groups (relatives vs. controls) for gray matter volume in this ROI. Bars represent the *SEM* (*n* = 11 per group). Coordinates are in MNI space.

$F(1, 20) = 10.76, p = .004, \eta_p^2 = 0.350$. There was no main effect of Training, $F(1, 20) < 0.01, p = .975$. Post hoc t tests showed that activation of the groups differed significantly for the untrained letters, $t(20) = 3.73, p = .001, d = 1.622$, but not for the trained letters, $t(20) = 1.06, p = .300$. The trained and untrained letters did not differ significantly for the relatives, $t(10) = -2.02, p = .071$, or controls, $t(10) = 1.52, p = .158$. The direction of the difference between trained versus untrained letters differed for the relatives ($M = -0.42, SE = 0.21$) as compared with controls ($M = 0.44, SE = 0.29$), driving the obtained interaction.

For the readout of right angular gyrus activation for the chromatic conditions of the Stroop task, the within-subject factor of Congruency (congruent vs. incongruent) and the between-subject factor of Group (relatives vs. controls) were compared using a two-way mixed ANOVA. The interaction between Group and Congruency levels was significant, $F(1, 20) = 9.35, p = .006, \eta_p^2 = 0.319$ (Figure 3B). There was no main effect of Congruency, $F(1, 20) = 0.02, p = .878$, or group, $F(1, 20) = 0.574, p = .458$. Post hoc t tests showed that activation for controls differed between the congruent and incongruent conditions, $t(10) = 2.59, p = .027, d = 0.780$, but not for the relatives, $t(10) = -1.94, p = .081$. Activation

of the two groups did not differ for the congruent condition, $t(20) = -0.04, p = .965$, or incongruent condition, $t(20) = 1.57, p = .132$. The direction of the difference between congruent versus incongruent conditions differed for the relatives ($M = -0.41, SE = 0.21$) as compared with controls ($M = 0.37, SE = 0.14$), driving the obtained interaction.

Comparisons of Gray and White Matter Structure

It was possible to directly test for changes in brain structure due to potential training effects in addition to group differences, because T1-weighted and diffusion-weighted images were obtained before and after training. At the level of the whole brain, structural differences were investigated in two-way mixed ANOVAs using a permutation-based analysis, testing main effects of Training and Group and the interaction between the two factors in one model.

For gray matter structure, investigated as VBM, no differences were obtained at the level of the whole brain for either main effect or the interaction term. For white matter structure, investigated as FA, effects of Training were not significant. The effect of Group was significant; a difference between groups along the average white matter skeleton was obtained at the level of the whole brain in 9 voxels. The cluster was located in the left superior parietal lobe near the postcentral gyrus (maximum voxel at $[-36, -42, 46]$ MNI; Figure 4A). Post hoc analysis of this cluster revealed that relatives showed increased structural connectivity in the ROI as compared with controls, $t(20) = 2.82, p = .010, d = 1.25$ (Figure 4B). The interaction between training and group was not significant.

We additionally investigated underlying gray matter differences within the right angular gyrus ROI (defined functionally, based on our previously obtained effects in the BOLD signal in gray matter). Average gray matter structure underlying the ROI was obtained with VBM methods (Figure 3C). The within-subject factor of Session (pre- vs. posttraining) and the between-subject factor of Group (relatives vs. controls) were compared in a two-way mixed ANOVA. No effects were significant (group: $F(1, 20) = 2.64, p = .120$; session: $F(1, 20) = 0.57, p = .458$; Group × Session: $F(1, 20) = 0.32, p = .577$).

DISCUSSION

In this study, we show for the first time that certain functional brain properties are related to the interference between acquired letter–color associations. Participants acquired these associations by reading books with colored letters. In a whole-brain analysis, one region in the right angular gyrus showed a direct relationship between the strength of the acquired associations, which was measured in an increased activation to trained letters that correlated with the behavioral acquired Stroop effect. Within this region of the angular gyrus, activation differed
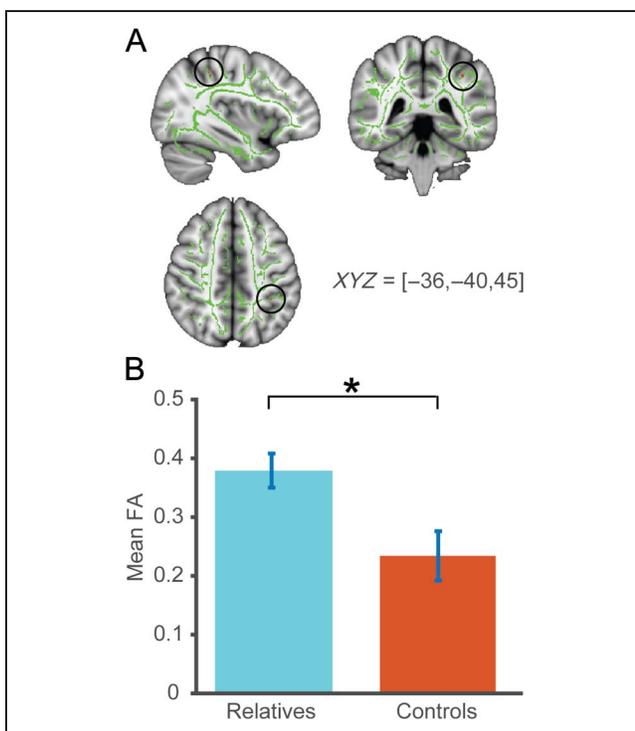
Figure 4. Increased structural connectivity in relatives of synesthetes compared with controls. (A) Familial markers of synesthesia were present in the left superior parietal lobe near the postcentral gyrus in a whole-brain analysis of white matter measured as FA. (B) Increased structural connectivity for the relatives as compared with controls was revealed in a post hoc analysis. Bars represent the *SEM* ($n = 11$ per group). Coordinates are in MNI space.

for relatives of synesthetes compared with matched controls. We also show for the first time that the effects of cross-modal training may elucidate markers related to having synesthesia in the family in brain activation. In line with previous research (e.g., Rouw et al., 2011), the location found was in the right parietal lobe, more specifically in the angular gyrus. Furthermore, we present the first evidence of increased structural connectivity at the level of the whole brain in nonsynesthetic relatives of synesthetes in white matter fibers near the left postcentral gyrus. This exploratory research suggests that the brains of the nonsynesthetic relatives of synesthetes show atypical connectivity when compared with matched controls, as do synesthetes (Rouw & Scholte, 2007).

Our findings are in line with mounting evidence on the importance of the parietal cortex in understanding synesthesia. The parietal lobe is implicated in binding associations between form and color. It is involved in feature binding (e.g., between color and form) when a stimulus is attended (Donner et al., 2002; Shafritz, Gore, & Marois, 2002; Friedman-Hill, Robertson, & Treisman, 1995) and is further shown to be crucial for binding between form and synesthetic color in developmental synesthesia (Rothen et al., 2010; Muggleton et al., 2007; Esterman et al., 2006). The angular gyrus region of the parietal lobe is considered to be a multisensory hub (Seghier, 2013), involved in multisensory integration of nonsynesthetic experience. The angular gyrus has been proposed to be of particular importance in understanding the mechanisms underlying synesthesia (Rouw et al., 2011; Hubbard, 2007) possibly specifically in "higher" or "associator" synesthetes (Rouw & Scholte, 2010; Brang & Ramachandran, 2008; Ramachandran & Hubbard, 2001).

The region in the right angular gyrus implicated in the trained letter–color binding is near to contralateral homologue regions previously reported in the synesthesia literature (Weiss et al., 2005; Nunn et al., 2002). Functional imaging studies of synesthesia have implicated mostly left-hemisphere activation of the parietal lobe (Rouw et al., 2011), whereas previous studies with TMS and our current findings show right hemisphere correlations (with trained associations). The location of the cluster in the angular gyrus obtained in the current study is more lateral, superior, and anterior to the regions in the TMS studies on synesthesia (Rothen et al., 2010; Muggleton et al., 2007; Esterman et al., 2006). The right parietal location in these studies was targeted based on the same Talairach coordinates. This location was found necessary for the synesthetic Stroop effect to arise (Muggleton et al., 2007; Esterman et al., 2006) and for bidirectional binding from color to grapheme measured in a conditioning paradigm (Rothen et al., 2010). Although it seems apparent that the parietal cortex is mediating acquired letter–color associations, as it is known to do for synesthetic letter–color associations, additional research is needed to untangle the roles of parietal subregions as well as the functional roles of the left and right hemispheres.

The bilateral angular gyri are involved in a wide variety of cognitive tasks in addition to being consistently deactivated during the absence of task-related processing as part of the "default-mode network" (Seghier, 2013; Smith et al., 2009). Hemispheric differences in task-related processing of the angular gyrus have been consistently found in the literature (for a review, see Seghier, 2013). For instance, the right angular gyrus is critical for inhibiting the inappropriate response and conflict resolution during go/no-go tasks (Nee, Wager, & Jonides, 2007; Wager, Jonides, Smith, & Nichols, 2005). The "synesthetic" version of the Stroop task taps into cognitive control mechanisms, because there is (supposed) conflict between veridical color and color associations during incongruent as compared with congruent trials (Rouw, van Driel, Knip, & Ridderinkhof, 2013). Our results are consistent with the hypothesis that the right angular gyrus is critical for the allocation of attention to task-relevant information (Taylor, Muggleton, Kalla, Walsh, & Eimer, 2011; Singh-Curry & Husain, 2009; Ciaramelli, Grady, & Moscovitch, 2008). The left angular gyrus in contrast is primarily related to semantic processing, as it is part of the mainly left-lateralized language network (Binder, Desai, Graves, & Conant, 2009).

Relatives of developmental grapheme–color synesthetes, who were exposed to thousands of consistently colored letters over several weeks, showed differential brain activation upon viewing these letters compared with controls in the right angular gyrus. This is the first evidence to suggest that the angular gyrus of the parietal lobe is a potential locus of atypical grapheme processing present in the relatives of synesthetes (who do not report having synesthetic experiences themselves). The interpretation of the direction of the neural markers of the familial trait could be that for the relatives of synesthetes, the right angular gyrus brain region needed to be driven more during the interference of incongruent letter–color combinations compared with congruent ones to arrive a indistinguishable behavior in the control group. These results suggest that there are underlying differences in visual feature binding processes for relatives under directed attention toward salient stimuli. Interestingly, while passively viewing the achromatic letters of the color localizer, brain activation for the two groups was in opposite directions for the untrained letters but was similar for the trained letters. In other words, the data imply that there are baseline differences while passively viewing untrained letters between the two groups that would be expected to diminish for the trained letters. Note that relatives did not differ from controls generally, at the level of the whole-brain analyses, when comparing the trained to untrained letters (Colizoli et al., 2016). The familial markers within the right angular gyrus were only apparent after functionally localizing effects related to the cross-modal training paradigm. All functional measurements were taken after training; we therefore cannot exclude the presence of training effects on untrained letters. It is possible that the untrained letters were affected by the cross-modal

training paradigm in a different way for the relatives as compared with controls. Grapheme-processing differences may be related to the expectation of color upon viewing graphemes (Kok, Jehee, & de Lange, 2012; Friston, 2010). Further research is required to replicate and extend these findings of atypical grapheme processing in relatives of synesthetes. Measurements should be made for the untrained and trained letters both before and after training to make better conclusions regarding differences in grapheme processing between the groups.

Although the relationship between brain function and structure has yet to be fully characterized (Kanai & Rees, 2011), the effects of genes on brain structure are measurable in neuroimaging phenotypes (Blokland, de Zubicaray, McMahon, & Wright, 2012). Specific neural loci of the familial trait in the nonsynesthetic relatives may help to further elucidate whether certain genes are the cause or result (or both) of the life-long experiences of a developmental synesthete. The general trait of having synesthesia—but not the specific subtype—seems to be inherited (Rouw et al., 2011; Barnett et al., 2008; Rich et al., 2005). Increased structural connectivity in white matter fibers has been related to developmental synesthesia in several brain regions within temporal, parietal, and frontal lobes (Rouw & Scholte, 2007). In addition to differences in brain function, we show here initial evidence suggesting increased structural connectivity (measured as FA) in relatives of synesthetes. At the level of the whole brain, one cluster in the left superior parietal lobe had significantly more coherent white matter for the relatives of synesthetes compared with controls. Although this again points at the relevance of parietal cortex in synesthesia, we did not have a priori predictions concerning an exact location of brain structure differences in the relatives. The parietal cluster previously found to be related to increased structural connectivity in synesthetes was located close to the left lateral occipital complex (Rouw & Scholte, 2007), whereas the cluster in the current study was found along the left postcentral gyrus. We therefore interpret this result with caution and stress the importance of replication for appropriate interpretation.

We were furthermore interested in whether experience-dependent plasticity in gray matter and white matter structure could be attributed to effects related to training. Previous studies have shown measurable differences in brain structure following relatively short periods of training (Kwok et al., 2011; Tang et al., 2010). In the current study, we found no evidence of measurable changes in brain structure within the weeks of training. We are unable to determine here if there was in fact no effect of training on brain structure or if the duration of training was insufficient.

A limitation of the current study is the lack of power given the small sample size (Button et al., 2013). Although we did obtain significant effects on certain measures, we are unable to conclude whether null effects are due to a limitation of measurement. Furthermore, note that there were three sibling pairs included in the current study (Table 1). Members of the same family would be expected to have less variance on measurements compared with random controls. However, there were in fact not many relatives of synesthetes available to be tested. For this reason, we included all relatives who meet the screening criteria. Future studies with relatives of synesthetes should aim for larger sample sizes than the current study. We recommend treating relatives of synesthetes as a special population for recruitment purposes.

Conclusion

Reading books with colored letters is sufficient to induce changes in behavior that resemble grapheme–color synesthesia. Using a letter–color training paradigm, atypical grapheme processing may be observed in relatives of synesthetes. Acquiring letter–color associations through high-frequency exposure led to differences in neural activations for relatives as compared with controls in a brain region known to be crucial for multisensory processing as well as synesthesia. Our results are consistent with the literature on visual feature binding and grapheme–color synesthesia. We propose that the angular gyrus critically mediates associations acquired (temporarily or permanently) by cross-modal training methods.

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