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Acknowledgements. The research programme was supported by the Nature Conservancy Council for England (English Nature) and the Plymouth Environmental Research Centre. We thank A. Fox, B. Broughton, D. Murphy, A. Giles, D. Uren, R. Harris, P. Ede and R. Hopgood for their assistance in research at sea; D. Merrett for help in zooplankton analysis; and the late F. Carey and D. Nelson, whose work helped to inspire the principal investigator (D.W.S.) to begin these studies. Satellite images of SST were supplied by the NERC Satellite Receiving Station.

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Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes

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The cyanobacterium *Prochlorococcus*[1,2] is the dominant oxygenic phototroph in the tropical and subtropical regions of the world's oceans[1,3,4]. It can grow at a range of depths over which light intensities can vary by up to 4 orders of magnitude. This broad depth distribution has been hypothesized to stem from the coexistence of genetically different populations adapted for growth at high- and low-light intensities[4–6]. Here we report direct evidence supporting this hypothesis, which has been generated by isolating and analysing distinct co-occurring populations of *Prochlorococcus* at two locations in the North Atlantic. Co-isolates from the same water sample have very different light-dependent physiologies, one growing maximally at light intensities at which the other is completely photoinhibited. Despite this ecotypic differentiation, the co-isolates have 97% similarity in their 16S ribosomal RNA sequences, demonstrating that molecular microdiversity, commonly observed in microbial systems[7–12], can be due to the coexistence of closely related, physiologically distinct populations. The coexistence and distribution of multiple ecotypes permits the survival of the population as a whole over a broader range of environmental conditions than would be possible for a homogeneous population.

Using sea-going flow cytometry for studying picoplankton populations, we and others[4,13,14] have observed multiple populations of *Prochlorococcus* in single water samples, as distinguished by their chlorophyll fluorescence intensities. These populations could be derived from the mixing together of genetically identical *Prochlorococcus* cells which have acclimated to different past light

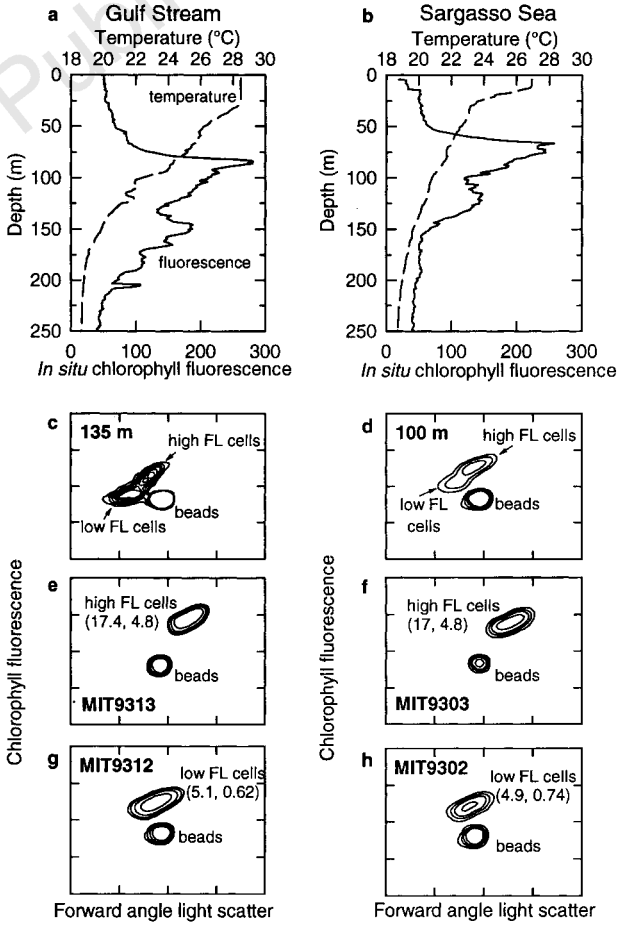


Figure 1 Properties of the euphotic zone and flow cytometric signatures of the *Prochlorococcus* populations and isolates. **a**, **b**, The physical features of the water columns were similar at the Gulf Stream station, 37° 30.8′N, 68° 14.4′W, and the Sargasso Sea station, 34° 45.5′N, 66° 11.1′W. **c**, **d**, Flow cytometry signatures of coexisting *Prochlorococcus* populations from 135 m in the Gulf Stream and 100 m in the Sargasso Sea from which the isolates were obtained. **e–h**, Flow cytometry signatures of the cultured isolates maintained at an irradiance of 9 μmol Q m^{-2} s^{-1}. Numbers in parentheses refer to the mean chlorophyll fluorescence per cell (FL) and FALS per cell. Differences in the absolute values of the flow cytometry parameters between natural populations and isolates result from unmatched growth conditions.

and/or nutrient regimes, or they could represent genetically distinct populations, adapted for optimal growth under different conditions but co-occurring in environments where conditions are favourable for both. The latter interpretation has been suggested by indirect evidence from field studies[4,13,14], from physiological diversity among *Prochlorococcus* isolates[6], and from studies of the molecular phylogenetic diversity of marine cyanobacteria, which have revealed a number of closely related gene sequences[7–9]. This high microdiversity has also been observed for marine bacterioplankton[7,8,10] and microorganisms in other environments[11,12], although the ecological significance has remained unclear. Differences in RNA polymerase gene sequences have been correlated with pigment content in marine *Synechococcus*[15]; however, molecular microdiversity observed in the more widely used 16S rRNA gene has not been connected directly to physiological function in any group of microorganisms from a particular habitat. The multiple *Prochlorococcus* populations observed by flow cytometry provided an excellent opportunity for studying these relationships.

In two locations of the North Atlantic Ocean at the junction of two subsurface chlorophyll fluorescence maximum layers, we used flow cytometry to sort the high- and low-fluorescence cells from coexisting *Prochlorococcus* populations and then brought them into culture (Fig. 1). After several years of growing under identical conditions, the cells sorted from a Gulf Stream water sample (isolates MIT9312 and MIT9313) and a Sargasso Sea water sample (isolates MIT9302 and MIT9303) have maintained their differences in chlorophyll fluorescence and forward-angle light scatter (FALS) per cell (Fig. 1e–h), indicating that the phenotypic differences originally observed in these coexisting populations were due to genetic differences.

We hypothesized that the high- and low-fluorescence *Prochlorococcus* are low- and high-light adapted, respectively, because differences in flow cytometry parameters are correlated with differences in photophysiology between *Prochlorococcus* SS120, isolated from the Sargasso Sea, and MED4, isolated from the Mediterranean Sea[6]. To test this hypothesis, we compared these four isolates with respect to their light-dependent growth response. The pattern of growth rate as a function of irradiance differs greatly between each coexisting pair (Fig. 2a, b). The most striking difference is that the high-fluorescence isolates, MIT9303 and MIT9313, show complete photoinhibition of growth at irradiances where the low-fluorescence isolates, MIT9302 and MIT9312, are growing at or close to maximal rates (Fig. 2a, b). Additionally, MIT9303 and MIT9313 grow better at lower light than their co-isolates (Fig. 2a, b).

To understand better the physiological underpinnings of the differential growth response of the isolates, we compared their pigment content, absorption properties and photosynthetic performance when grown at low irradiance. The high-fluorescence isolates, MIT9303 and MIT9313, contain higher concentrations of divinyl chlorophylls *a* and *b* (chl a_2 and chl b_2) relative to their low-fluorescence co-isolates MIT9302 and MIT9312 (Table 1). They also contain higher ratios of chl b_2/a_2, resulting in higher spectrally weighted average chlorophyll a_2-specific absorption coefficients, $\bar{a}^*_{chl}$

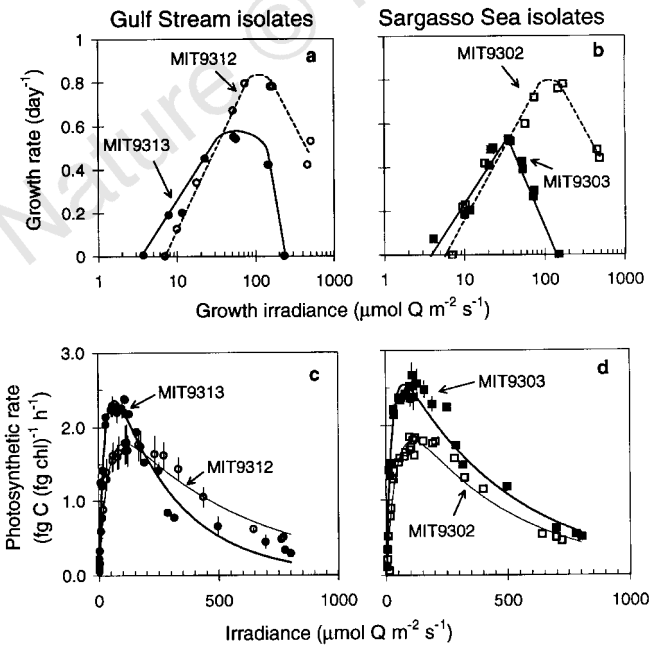
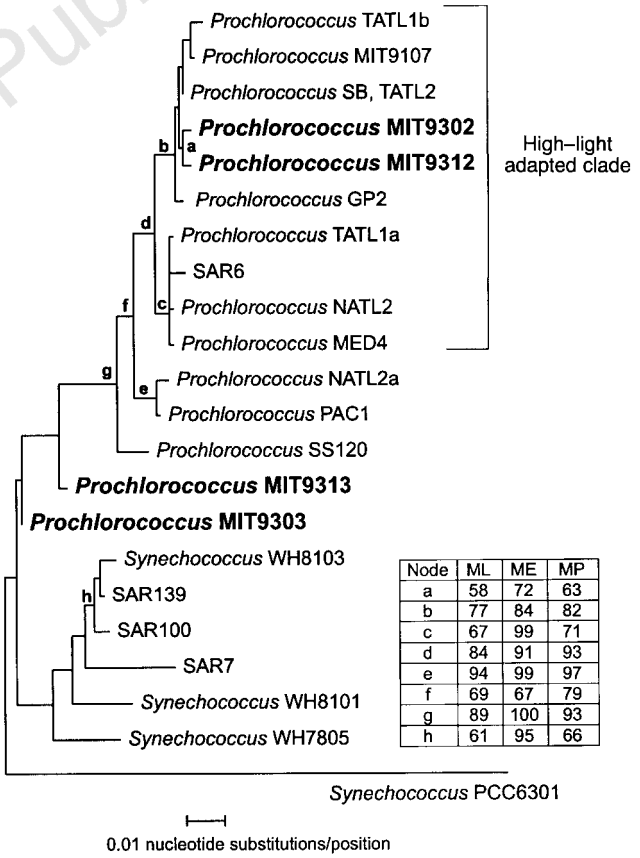


Figure 2 Growth and photosynthetic rate as a function of irradiance for the *Prochlorococcus* isolates shown in Fig. 1. **a**, **b**, Specific growth rate as a function of growth irradiance. **c**, **d**, Photosynthetic rate (normalized to chl a_2) as a function of irradiance for the four isolates when grown at an irradiance of 9 μm Q m^{-2} s^{-1}. Symbols and error bars correspond to the mean ± 1 s.e. of duplicate measurements.



Node	ML	ME	MP
a	58	72	63
b	77	84	82
c	67	99	71
d	84	91	93
e	94	99	97
f	69	67	79
g	89	100	93
h	61	95	66

Figure 3 Phylogenetic relationships of *Prochlorococcus* and *Synechococcus* isolates and environmental sequences from the Sargasso Sea ('SAR') inferred from 16S rRNA sequences. One of the three most probable trees found in a maximum likelihood analysis is presented. (Only the relative branching orders of *Prochlorococcus* isolates MED4, TATL1a and NATL2 and the environmental sequence SAR6 differ between the three trees.) Bootstrap proportions are percentages of 100 resampled data sets from analyses of maximum likelihood (ML), distance (using minimum evolution as the objective function (ME)) and maximum parsimony (MP); values below 50 are not shown. The freshwater cyanobacterium *Synechococcus* PCC 6301 was used to root the tree. Analyses with additional members of the cyanobacterial radiation resulted in essentially similar branching orders (data not shown).

Table 1 Physiological characteristics of *Prochlorococcus* isolates grown at a low growth irradiance of 9 μmol Q m^{-2} s^{-1}

	Gulf Stream		Sargasso Sea	
Parameter	MIT9312	MIT9313	MIT9302	MIT9303
Pigments				
chl a_2	3.15	5.1	3.2	4.2
chl b_2	2.08	6.0	2.1	5.0
chl b_2/a_2	0.661	1.175	0.65	1.19
Absorption				
$\bar{a}^*_{chl}$	0.0152	0.0213	0.0147	0.0232
Photosynthetic				
α_{chla}	0.057	0.118	0.055	0.125
P^{chla}_{max}	1.8	2.4	1.8	2.54
ϕ_{max}	0.086	0.128	0.086	0.125

Chl a_2 and chl b_2 are in units of fg per cell; chl b_2/a_2 are on a weight/weight basis. $\bar{a}^*_{chl}$ is presented in units of (m^2 (mg chl a_2)$^{-1}$). Photosynthetic parameters are in the following units: α_{chla} = fg C (fg chl a_2)$^{-1}$ h^{-1} (μmol Q m^{-2} s^{-1})$^{-1}$; P^{chla}_{max} = fg C (fg chl a_2)$^{-1}$ h^{-1}; and ϕ_{max} = mol carbon (mol Q)$^{-1}$. All values are means of duplicate cultures with coefficients of variance ≤ 10%.

(Table 1). Furthermore, the higher chl b_2/a_2 ratio of MIT9303 and MIT9313 drives a 2.1–2.3 times higher chl a_2-specific photosynthetic efficiency (α_{chla}) and results in higher maximum quantum yields (ϕ_{max}) relative to MIT9302 and MIT9312 (Fig. 2c, d; Table 1). Thus, we conclude that the isolates MIT9303 and MIT9313 are low-light adapted and MIT9302 and MIT9312 are high-light adapted.

We next explored whether the physiology of these four isolates is related to their phylogeny by sequencing their 16S rRNA genes. Although they were isolated from different waters, the two high-light adapted isolates, MIT9302 and MIT9312, have very high sequence similarity (99.7%), as do the two low-light adapted isolates, MIT9303 and MIT9313 (99.2%). The two co-isolates from each region are more disparate than the high- and low-light adapted pairs: the Sargasso Sea co-isolates MIT9302 and MIT9303 are only 97.3% similar, whereas the Gulf Stream co-isolates MIT9312 and MIT9313 are 97.7% similar. The divergence between the co-isolates is meaningful because their degrees of similarity are comparable to those between the low-light adapted *Prochlorococcus* isolates and marine isolates of phycobilisome-containing *Synechococcus* (96.9–98%). A phylogenetic tree constructed from the coexisting *Prochlorococcus* and other cyanobacterial sequences shows that the high-light adapted MIT9312 and MIT9302 cluster with the high-light adapted MED4 and two isolates from the Pacific, SB and GP2, which have low ratios of chl b/a_2 (ref. 16) (Fig. 3). The monophyly of this 'high-light adapted clade'[17] is well supported in all analyses. The low-light adapted MIT9303 and MIT9313 are on separate, more basal branches of the tree (Fig. 3). Thus, the phylogenetic relationship is correlated with the phenotypic relationship.

On the basis of the physiology and molecular phylogeny of these co-isolates, the previously characterized isolates, SS120 and MED4[6,17–19], and four additional isolates from the Pacific Ocean (ref. 20 and G.R. and S.W.C., unpublished results), it appears that at least two distinct ecotypes can be identified for isolates of the genus *Prochlorococcus*. This is analogous to two types of marine *Synechococcus*, which have different ratios of phycourobilin to phycoerythrobilin[21], and which appear to be distributed differently on the basis of their relative light harvesting abilities[22]. It has been suggested that low-light adapted, high chl b_2/a_2 *Prochlorococcus* predominate in the deeper portion of the euphotic zone where nutrients are abundant, and that high-light adapted, low chl b_2/a_2 *Prochlorococcus* predominate in the surface where nutrients are typically limiting[4–6]. This distribution of multiple *Prochlorococcus* ecotypes in the same water column would result in greater integrated production than could be achieved by a single ecotype. For example, if we estimate primary production ($P = \bar{a}^*_{chl}\phi_m C_c c_v E$) for the same number of cells (c_v) in the low light of the deep euphotic zone ($E = 10$ μmol Q m^{-2} s^{-1}) (see Methods), using the strain-specific values of $\bar{a}^*_{chla}$, ϕ_m and chl a_2 per cell (C_c), then the contribution of the low-light adapted MIT9313 to primary production would be 3.4 times higher than that of its high-light adapted coisolate MIT9312. Thus, the use of a single set of physiological parameters will result in errors in the estimates of *Prochlorococcus* primary production in the oceans.

Our results show that high phylogenetic microdiversity observed for coexisting marine cyanobacterial picoplankton[7–9] is due, in part, to physiological diversity within the *Prochlorococcus* population. Although studies using allozyme banding patterns have demonstrated ecotypic differentiation in eukaryotic phytoplankton[23,24], we have gone a step further in that we have specifically linked physiological diversity to small differences in 16S rRNA sequences. Thus, high microdiversity at the 16S rRNA locus observed in other microbial communities examined primarily by comparative sequence analysis may also reflect physiologically distinct microbial populations. The existence of multiple ecotypes in a particular environment may be a general phenomenon in the microbial world, allowing for survival over a broader range of conditions than could be achieved by a physiologically and genetically homogeneous population. □

Methods

Flow cytometry and culturing. Field populations were identified as *Prochlorococcus* on the basis of the characteristic low chlorophyll fluorescence and FALS signature on the flow cytometer[1]. Populations were designated as 'double' when bimodal signatures were visible, defined by different fluorescence and FALS signals (Fig. 1). Coexisting *Prochlorococcus* populations were sorted from each other into sterile test tubes of modified K/10-Cu media with 1.17 μM EDTA[6] using an EPICS 753 flow cytometer (Coulter Corp.). Cells were grown at 21 °C and 17 μmol quanta m^{-2} s^{-1} blue light (14:10 light:dark) immediately after isolation on the ship, and back in the laboratory at 24 °C and varying light intensities obtained using neutral density filters and cool-white fluorescent bulbs. For photosynthesis, pigment and absorption measurements, cells were grown at a low irradiance (9 μmol Q m^{-2} s^{-1}), where differences between SS120 (CCMP1375) and MED4 (CCMP1378) were apparent[6]. Cell counts for growth rate calculations and normalization of pigment data were obtained using a FACScan flow cytometer (Becton-Dickinson), and mean chlorophyll fluorescence per cell and FALS are presented relative to 0.474 μm yellow–green fluorescent beads (Polysciences). Flow cytometric data were analysed using 'CytoPC' software provided by D. Vaulot (Station Biologique, Roscoff, France).

Photosynthesis, pigment and absorption measurements. Photosynthesis–irradiance measurements were carried out on exponentially growing cells spiked with $NaH^{14}CO_3$ (0.1 μCi ml^{-1} culture; specific activity between 100,000 and 200,000 d.p.m.). The spiked culture was dispensed into glass scintillation vials (1 ml each) and incubated at 24 °C for 45 min over a range of irradiances obtained using very high output/daylight spectrum fluorescence bulbs attenuated with neutral density filters and measured with a 4π quantum light meter (model QSL-100, Biospherical Instruments, San Diego, CA). Photosynthesis was terminated and unincorporated ^{14}C removed by adding 2 M HCl (100 μl) and shaking for ~2 h under a fume hood. Samples were then analysed by scintillation spectrometry. The photosynthetic parameters, P_{max} and α, were obtained by fitting the results to the equation from ref. 25, using the curve-fitting program in SigmaPlot (Jandel Scientific), and normalized to chl a_2 and chl b_2 which were measured spectrophotometrically[26]. *In vivo* chlorophyll a_2-specific absorption, $a^*_{chl}(\lambda)$ (m^2 (mg chl a_2)$^{-1}$), was obtained using an opal diffuser on a Beckman DU-640 spectrophotometer, and the weighted average ($\bar{a}^*_{chl}$) was calculated over the photosynthetically available radiation range (400–700 nm). Maximum quantum yield, ϕ_{max}, is calculated as the ratio of α_{chla} to $\bar{a}^*_{chl}$.

Molecular phylogenetic analysis. Genomic DNA was isolated from cultures according to standard methods[27]. 16S rDNA was amplified using the general eubacterial primers 8-27f (AGAGTTTGATCCTGGCTCAG) and 1504-1486r (CTTGTTACGACTTCACCCC). Polymerase chain reactions (PCRs) were performed in quintuplicate, using the high-fidelity polymerase *Pfu* (Stratagene). Reactions were pooled and purified using QIA quick kit (QIAGEN,

Chatsworth, CA) and cloned using the pCR-Script kit (Stratagene). A minimum of 8 clones from each culture were sequenced on an automated sequencer (LI-COR, Lincoln, NE). Sequences were aligned manually with other marine cyanobacterial sequences available in the Ribosomal Database Project[28] using the Genetic Data Environment[29]. The sequence for MIT9303 obtained previously from a PCR product[17] is contained within the sequence we report here. A total of 1,094 unambiguously aligned and determined nucleotides were used in the analyses. Phylogenetic analyses used PAUP* (version 4.0d47, provided by D. Swofford). For both distance and maximum likelihood analyses the model of nucleotide substitution used was the Hasegawa Kishino Yana 1985 model. Nucleotide frequencies and the transition transversion ratio were estimated from the data.

Received 11 November 1997; accepted 26 March 1998.

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Acknowledgements. We thank M. Sogin for access to an automated DNA sequencer and helpful discussion; A. Shimada for the use of unpublished sequences of *Prochlorococcus* GP2 and SB; R. Olson and E. Zettler for cell sorting at sea; H. Hsu for technical assistance; and C. Cavanaugh, H. Sosik and S. Sathyendranath for helpful consultation. This work was supported by the US National Science Foundation and the US National Aeronautics and Space Administration.

Correspondence and requests for materials should be addressed to S.W.C. (e-mail: chisholm@mit.edu). Sequences have been deposited in GenBank under accession numbers AF053396, MIT9302; AF053397, MIT9303; AF053398, MIT9312; and AF053399, MIT9313.

Conscious and unconscious emotional learning in the human amygdala

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If subjects are shown an angry face as a target visual stimulus for less than forty milliseconds and are then immediately shown an expressionless mask, these subjects report seeing the mask but not the target. However, an aversively conditioned masked target can elicit an emotional response from subjects without being consciously perceived[1,2]. Here we study the mechanism of this unconsciously mediated emotional learning. We measured neural activity in volunteer subjects who were presented with two angry faces, one of which, through previous classical conditioning, was associated with a burst of white noise. In half of the trials, the subjects' awareness of the angry faces was prevented by backward masking with a neutral face. A significant neural response was elicited in the right, but not left, amygdala to masked presentations of the conditioned angry face. Unmasked presentations of the same face produced enhanced neural activity

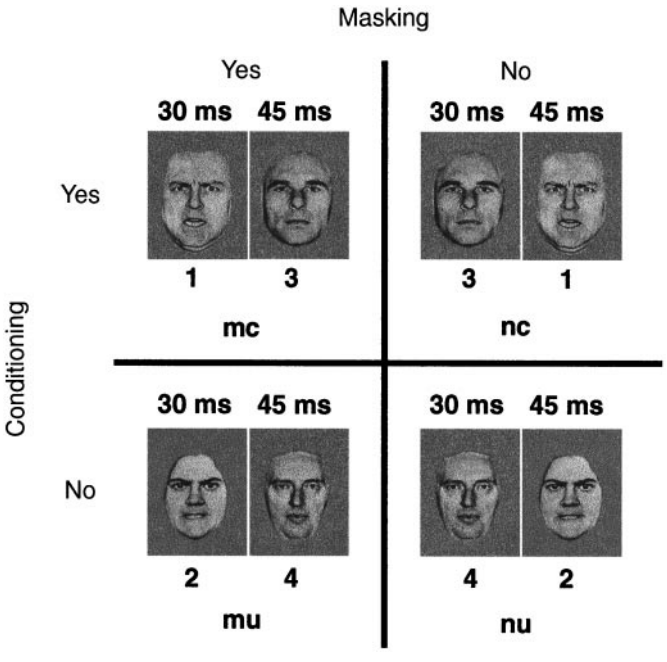

Figure 1 Stimulus parameters and experimental design. In the scanning window, pairs of target and masking faces were shown in four separate conditions, determined by the combination of masking and conditioning of the angry face. Mc, masked conditioned (the CS+ angry face was the target and the neutral face was the mask); nc, non-masked conditioned (a neutral face was the target and the CS+ face was the mask); mu, masked unconditioned (the CS– face was the target and the neutral face the mask); nu, non-masked unconditioned (the neutral face was the target and the CS– face the mask). Face 1, angry face paired with noise (CS+); face 2, angry face not paired with noise (CS–); faces 3 and 4, neutral faces. In all conditions, the target face was displayed for 30 ms and immediately followed by the mask for 45 ms.