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Dose-dependent effect of donepezil administration on long-term enhancement of visually evoked potentials and cholinergic receptor overexpression in rat visual cortex

Mira Chamoun [1], Marianne Groleau [1], Menakshi Bhat, Elvire Vaucher *

Laboratoire de Neurobiologie de la Cognition Visuelle, École d'optométrie, Université de Montréal, Montréal, Québec, Canada

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ABSTRACT

Stimulation of the cholinergic system tightly coupled with periods of visual stimulation boosts the processing of specific visual stimuli via muscarinic and nicotinic receptors in terms of intensity, priority and long-term effect. However, it is not known whether more diffuse pharmacological stimulation with donepezil, a cholinesterase inhibitor, is an efficient tool for enhancing visual processing and perception. The goal of the present study was to potentiate cholinergic transmission with donepezil treatment (0.5 and 1 mg/kg) during a 2-week visual training to examine the effect on visually evoked potentials and to profile the expression of cholinergic receptor subtypes. The visual training was performed daily, 10 min a day, for 2 weeks. One week after the last training session, visual evoked potentials were recorded, or the mRNA expression level of muscarinic (M1-5) and nicotinic (α/β) receptors subunits was determined by quantitative RT-PCR. The visual stimulation coupled with any of the two doses of donepezil produced significant amplitude enhancement of cortical evoked potentials compared to pre-training values. The enhancement induced by the 1 mg/kg dose of donepezil was spread to neighboring spatial frequencies, suggesting a better sensitivity near the visual detection threshold. The M3, M4, M5 and α7 receptors mRNA were upregulated in the visual cortex for the higher dose of donepezil but not the lower one, and the receptors expression was stable in the somatosensory (non-visual control) cortex. Therefore, higher levels of acetylcholine within the cortex sustain the increased intensity of the cortical response and trigger the upregulation of cholinergic receptors.

1. Introduction

Acetylcholine (ACh) influences visual processing as early as in the primary visual cortex (V1) through muscarinic (mAChRs) and nicotinic (nAChRs) ACh receptors (Kirkwood et al., 1999; Zinke et al., 2006; Disney et al., 2007; Bhattacharyya et al., 2012; Chubykin et al., 2013; Groleau et al., 2015). The different AChR subtypes induce a variety of effects that determine the weight of the visual stimulus for further processing to high-level visual areas. Thus, early ACh modulation of visual responses shapes conscious visual perception.

ACh is spontaneously released in V1 by visual stimulation (Collier and Mitchell, 1966b; Fournier et al., 2004; Laplante et al., 2005), but ACh release might be evoked by pharmacological treatment or electrical stimulation of the basalo-cortical cholinergic projections. Recent studies showed that electrically boosting the ACh release synchronized with visual stimulation enhanced visual responses (Kang and Vaucher, 2009; Bhattacharyya et al., 2013; Pinto et al., 2013). Moreover, the repeated cholinergic potentiation of visual training by electrical stimulation induced long-term changes in behaviorally assessed visual perception (Kang et al., 2014a). However, boosting the cholinergic system by pharmacological means is less invasive than electrical stimulation and could be better translated to clinics as a novel approach for vision rehabilitation, for example. Therefore, combining the pharmacological cholinergic potentiation with visual training would be an interesting avenue toward improving vision. Pharmacological stimulation has, however, a more diffuse spatiotemporal distribution than the timely synchronized visual/electrical stimulation coupling and it is not known if it would be as efficient in enhancing the visual responses.

Acetylcholinesterase inhibitors (AChEIs), such as donepezil (DPZ), have been demonstrated to enhance visual memory performance in rats in radial water maze and visuospatial recognition

* Corresponding author at: Laboratoire de Neurobiologie de la Cognition Visuelle, École d'optométrie, Université de Montréal, CP 6128, succ. Centre-ville, Montréal, Québec H3C 3J7, Canada.
E-mail address: elvire.vaucher@umontreal.ca (E. Vaucher).
[1] MC and MG contributed equally to this work.

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tasks (Cutuli et al., 2008) and to increase contrast sensitivity to a grating stimulus in a two-alternative forced choice-task (Wise et al., 2007; Soma et al., 2013b). These effects are also observed in rats with a cholinergic deficit where AChEIs improve cognitive performance on an avoidance task, therefore decreasing learning impairments on procedural abilities in the water maze (Cutuli et al., 2008) and working memory (Itoh et al., 1997; Wang and Tang, 1998; Ogura et al., 2000; Cutuli et al., 2009). AChEIs also change evoked potentials in humans (Leroy et al., 2015) and rats (Bringmann, 1994; Lewandowski and Zmuda, 1995), but it is not known by which mechanism V1 reactivity is affected.

Thus, the aim of the present study was to determine whether DPZ administration during repeated visual stimulation in rats could enhance visual evoked potentials (VEPs). DPZ was chosen because it is the most current and efficient AChEI drug used in clinics (Cacabelos, 2007). Two different doses of DPZ were tested, 0.5 and 1 mg/kg, to estimate a possible effect of different ACh extracellular concentrations, which might differentially alter cortical responses (Hasselmo and McGaughy, 2004) and determine the most efficient dose for future studies. We used a paradigm similar to our previous studies (Kang et al., 2014a), i.e., a 2-week daily visual stimulation with a specific patterned stimulus paired with DPZ i.p. administration. The VEPs were recorded before and one week after the 2-week training. Moreover, we were interested to know whether the expression of the cholinergic receptor subtypes was differentially affected by DPZ treatment. The expression of the five mAChR subtypes and 4 of the main nAChR subunits were investigated because their contribution to different aspects of the modulation of the V1 neurons has been demonstrated (for review see (Disney et al., 2007; Thiele, 2013; Groleau et al., 2015)). The cholinergic receptor subtypes expression profile was evaluated by RT-PCR and compared to the basal mRNA expression of naïve animals. The results show a dose-dependent long-term enhancement of the visual cortical activity after the training and an upregulation of M3, M4 mAChR and α7 nAChR subtypes at the higher dose of DPZ.

2. Materials and methods

2.1. Animal preparation

All procedures were performed in accordance with the guidelines of the Canadian Council for the Protection of Animals and were accepted by the Ethics Committee of the Université de Montréal (#14–164). A total of 49 adult male Long Evans rats (200–225 g) were used in this study. The male rats were used in order to avoid any impact of estrogen on cholinergic activity. The animals were maintained in a 12 h light/dark normal daylight cycle with *ad libitum* access to food and water. The animals were separated in groups; visual stimulation with vehicle injection, saline i.p. (VS, $n = 7$ (VEP), $n = 7$ (PCR)), visual stimulation with 0.5 mg/kg DPZ i.p. injection (DPZ0.5/VS, $n = 6$ (VEP), $n = 6$ (PCR)) and visual stimulation with 1 mg/kg DPZ i.p. injection (DPZ1/VS, $n = 7$ (VEP), $n = 10$ (PCR)). Previous experiments did not show any effect of handling the animals (i.e., possible stress) during the sham visual training (Kang et al., 2014a, 2015) on VEPs, thus a sham-VS group was not added. VEP and PCR experiments were performed in different sets of animals, the cortical sampling being compromised by the electrophysiological recording in V1. In addition, naive animals (no treatment, no visual stimulation; $n = 6$) were used in RT-PCR experiments to determine the basal level of expression of the cholinergic receptors genes at rest.

2.2. Donepezil treatment

DPZ (Sigma Aldrich, St-Louis, MO, USA) was dissolved in a sterile 0.9% NaCl solution. The drug was administered i.p. from a stock solution, daily for two weeks starting on the first day of visual training. The two doses tested, 0.5 mg/kg or 1 mg/kg, are commonly used for behavioral and physiological experiments and were chosen based on previous studies (Cutuli et al., 2008; Soma et al., 2013b) and on pilot experiments. DPZ was injected 30 min before the beginning of the exposure to the visual stimulus to reach the maximum effect of the drug (Soma et al., 2013a) during the stimulation. Control animals received the same treatment with saline injections.

2.3. Visual evoked potential recording procedures

Visual evoked potential (VEP) were recorded as described (Cooke and Bear, 2010; Kang et al., 2015) before and one week after the last visual training session in the same location (although the electrode was not chronically implanted, it was inserted at the same coordinates). Briefly, animals were anesthetized with isoflurane (induction 5%, maintenance 1.5%) and placed in a stereotaxic apparatus. Core body temperature was maintained at 37 °C using a thermostatic controlled heating pad (FHC, Bowdoinham, ME, USA). A hole adjusted to the diameter of the electrode was made in the skull with a dental drill to access V1 and a recording tungsten-electrode (FHC, <1 MOhm) was acutely inserted into the left hemisphere (mm from Bregma AP −7.5, ML +4.0, DV −0.5) (Paxinos et al., 1980). Rats were then maintained in the dark for the rest of the procedure. Eight different spatial frequencies (0.08, 0.12, 0.3, 0.5, 0.7, 0.8, 0.9, 1.0 CPD) at two different orientations, 30° (the visual training orientation) and 120° (the orthogonal orientation) were presented in the right hemifield. Evoked responses were amplified (5000×), filtered at 3 Hz ∼ 1 kHz (Grass Inc., West Warwick, RI, USA) and collected with the MP100 data acquisition system and Acqknowledge software (v 3.8; Biopac system Inc., Goleta, CA, USA). Signal amplitude was calculated by measuring peak-to-peak differences between 0 and 500 ms after the stimulus onset. The baseline amplitude was measured during grey screen display. VEPs were expressed as the change from baseline (%) using the following equation

$$\textit{change from baseline}\ (\%) = \frac{\text{signal amplitude} - \text{baseline amplitude}}{\text{baseline amplitude}} \times 100$$

VEP amplitudes were calculated by averaging change from baseline (%) of 40 repetitions of each orientation (30° and 120°) and the eight spatial frequencies. Cortical activation after 2 weeks of visual training was measured by comparing pre-training and post-training VEPs.

2.4. Visual training procedure

The same visual training as described previously (Kang et al., 2014a, 2015) was used in this study for comparison purposes. Briefly, awake rats were restrained and surrounded by three monitors at a distance of 21 cm: one frontal and two lateral (LG, luminance 37 cd/m^2). The visual stimulus was chosen to examine the improvement of response to an orientation of poor saliency. Consequently, the optimal spatial frequency of this stimulus was voluntarily chosen to spare attentional resources for orientation detection. The stimulus consisted of a sine-wave grating of 0.12 cycle/degree, orientation 30°, phase converting at 1 Hz (Vpixx software, v 2.79, Vpixx technologies Inc., Saint-Bruno, QC, Canada). Rats were trained daily for 10 min for 14 consecutive days (Table 1). Each training session was performed at the same time of day for each rat.

Table 1
Experimental procedures.

Experimental steps	Description	Days (timeline)
1. VEP pre-training	Assessment of the VEPs in naive animals: baseline measurements	Day 1
2. Training sessions	Visual training paired or not with donepezil injection	Days 2-15
3. Resting week	Donepezil washout period (no handling)	Days 16-23
4. VEP post-training	Assessment of the VEPs after training: long-term influence of the training	Day 24
5. PCR	The visual cortex is processed	Day 25

2.5. Tissue sampling

One week after the last training session, which coincided with the day following the post-training VEP recording, rats were deeply anesthetized with isoflurane and sacrificed by decapitation. The brain was rapidly collected on a cold plate and a 2 mm^2 piece of the visual or somatosensory cortex (approximately Bregma AP −7 and −3 mm, respectively, and ML ± 2 mm) were dissected within 60 s with the help of a millimeter graduated ruler and put in RNA*later* stabilization reagent (QIAGEN, Valencia, CA, USA) for 24–48 h. The somatosensory cortex was chosen as a control region of cortex since it is a sensory area with a similar cytoarchitecture, cholinergic innervation and identical cholinergic receptors (Aubert et al., 1996) but should not be affected by the visual training. Subsequently, supernatant was removed and samples were stored at −80 °C until assayed.

2.6. Primer designing

The following reference genes representing different functional classes were selected: mAChR (M1-M5) and nAChR (α4, α7 and β2). Primer design was performed with PRIMER3 (www. genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and VectorNTI software based on the NCBI (National Centre for Biotechnology Information) BLAST database (Table 2). Forward and reverse primers were positioned in different exons to reduce the chance of amplifying genomic DNA.

2.7. Measurement of cholinergic receptor expression by quantitative RT-PCR

Total RNA was extracted from the RNA later fixed visual cortex samples using Qiazol reagent and RNeasy®Lipid Tissue Mini Kit

Table 2
Primer list.

		Sequence	
18S	Forward	5' TCA ACT TTC GAT GGT AGT CGC CGT	3'
	Reverse	5' TCC TTG GAT GTG GTA GCC GTT TCT	3'
M1	Forward	5' AGC TCA GAG AGG TCA CAG CCA	3'
	Reverse	5' GGG CCT CTT GAC TGT ATT TGG GGA	3'
M2	Forward	5' CAA GAC CCA GTA TCT CCG AGT CTG	3'
	Reverse	5' CGA CGA CCC AAC TAG TTC TAC AGT	3'
M3	Forward	5' ACA GAA GCG GAG GCA GAA AAC TTT	3'
	Reverse	5' CTT GAA GGA CAG TAG AGT AGC	3'
M4	Forward	5' AAG GAG AAG AAG GCC AAG ACT CTG	3'
	Reverse	5' GCG AGC AAT GCT GGC AAA CTT TCG	3'
M5	Forward	5' TGT AGC AGC TAC CCC TCT TCA GAG	3'
	Reverse	5' AGC AGC AGC TGG AGA CAG AAA GTA	3'
α4	Forward	5' GAC CAC CTC AAG GCA GAA GA	3'
	Reverse	5' CCC AGA AGG CAG ACA ATG AT	3'
α7	Forward	5' TAT CAC CAC CAT GAC CCT GA	3'
	Reverse	5' CAG AAA CCATGC ACA CCA GT	3'
β2	Forward	5' TGC GAA GTG AGG ATG ATG AC	3'
	Reverse	5' ACG GTC CCA AAG ACA CAG AC	3'

(QIAGEN, Valencia, CA, USA) according to manufacturer's protocol. RNA consistency was determined using a Nanodrop (ND-1000) measuring 260/280 and 260/230 ratios, respectively. A single-strand cDNA was synthesized with 250 ng of total RNA in a total reaction volume of 20 µl using the QuantiTect Rev Transcription Kit (Qiagen Toronto, On, Canada). After cDNA synthesis, a tenfold-diluted cDNA was used for the SYBR Green based real-time quantitative PCR reaction. The reaction contained 12.5 µl of 2 × IQ Biotool SYBR Green (Biotool, Cedarlane, Montreal, QC, Canada), 200 nM of each primer (Table 2), 1 µl cDNA template and ultrapure water to a reaction volume of 25 µl. The qPCR reaction was performed on a Mx3000P Q-PCR System (Stratagene, La Jolla, CA, USA) with cycling conditions of 3 min at 95 °C, followed by 45 cycles with denaturing template for 30 s at 95 °C, followed by 1 min at melting temperature (Tm), and elongation at 72 °C for 30 s. Both targeted and referenced genes were amplified in duplicate in the same run. The relative quantification of target genes was determined using the MxProTM Q-PCR software version 3.00 (Stratagene, La Jolla, CA, USA) where mRNA levels were normalized to 18S housekeeping gene expression levels. Briefly, the cycle threshold (Ct) average of each duplicate was calculated for each gene and 18S and the ΔCt (Ctgene-Ct18S) was

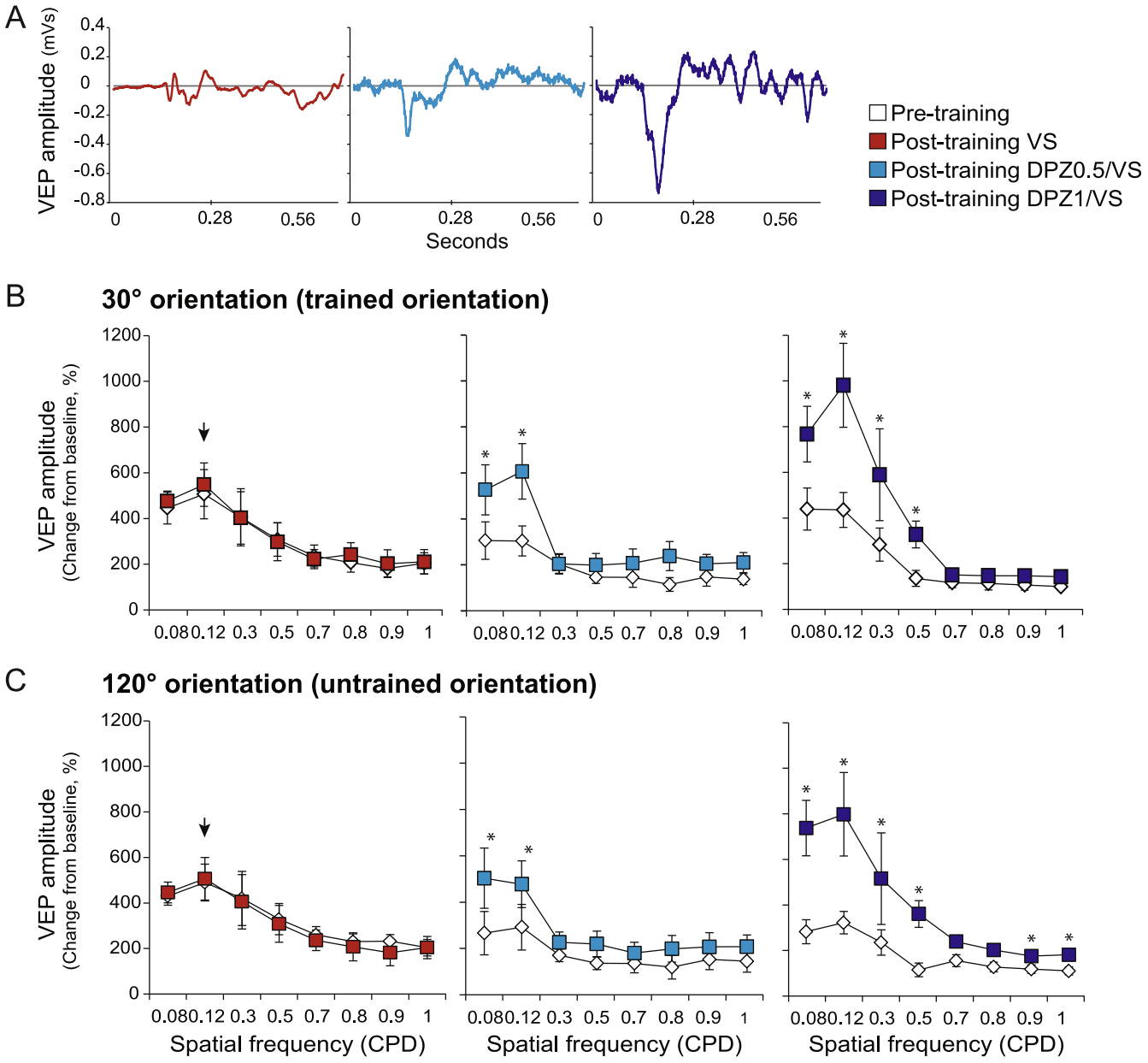

Fig. 1. VEP amplitude before and 2 weeks after daily exposure to a 30° and 0.12 CPD sinusoidal grating with donepezil administration. (A) VEP examples from the VS (red), DPZ0.5/VS (turquoise) and the DPZ1/VS group (blue) after visual training. Peak-to-Peak measures were extracted between 0 and 500 ms after the stimulus onset. (B) VEP amplitude, change from baseline (%) were recorded in V1 in response to a 30° (trained orientation) sinusoidal grating of different spatial frequencies. There was no significant difference between the pre- and post-values of the VEP amplitude after VS alone (red). However, there was an increase in VEPs in response to the trained spatial frequency (0.12 CPD, arrow head)) and the one lower (0.08 CPD) after VS paired to 0.5 mg/kg DPZ (DPZ0.5/VS, turquoise) or 1 mg/kg DPZ (DPZ1/VS, blue) and for higher spatial frequencies (0.3 CPD and 0.5 CPD) for the latest condition. (C) VEP amplitude, change to baseline (%) were recorded in response to a 120° (untrained orientation) sinusoidal grating of different spatial frequencies. The results were similar to the trained orientation. In addition, there was an increase in VEPs in response to the 0.9 CPD and 1 CPD spatial frequency after VS paired to 1 mg/kg DPZ.

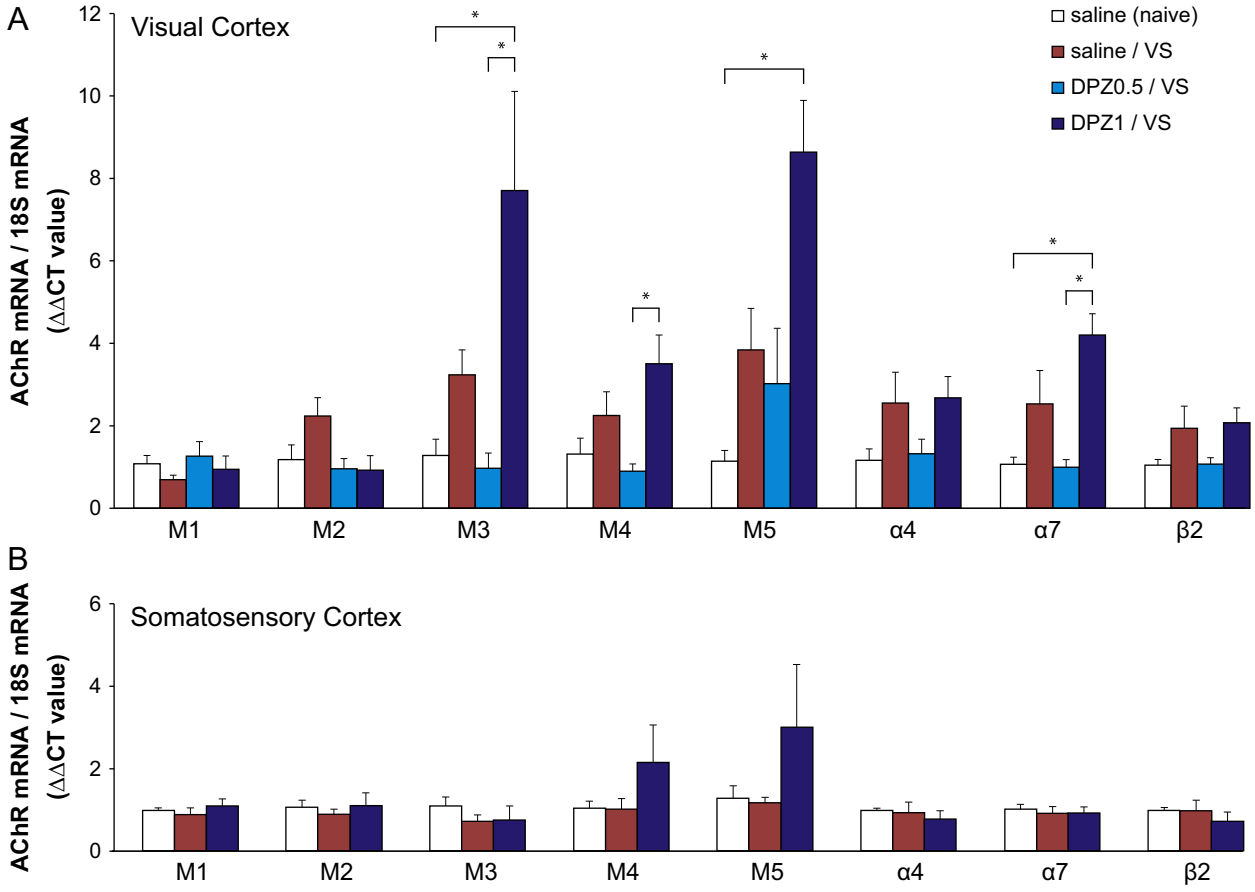

Fig. 2. Effect of visual training and donepezil treatment on cholinergic receptors mRNA expression. (A) Two weeks of visual exposure alone (VS, red) or paired to 0.5 mg/kg DPZ (DPZ0.5/VS, turquoise) does not alter the cholinergic mRNA receptor expression (M1-M5 mAChR receptors subtypes and α4, α7 and β2 nAChR receptors subunits) in the visual cortex. A 1 mg/kg of donepezil combined with visual exposure (DPZ1/VS, blue) increases mRNA expression for M3, M5 and α7 compared to naive and M4 and α7 compared to the DPZ05/VS group. (B) Two weeks of visual exposure paired or not (red) to 1 mg/kg DPZ (DPZ1/VS, blue) does not alter the mRNA expression in a non-visual area for either the mAChRs or the nAChRs. As the results were not significant in V1 for the 0.5 mg/kg DPZ, the somatosensory cortex mRNA were not analyzed for this dose. All the test values were normalized with respect to 18S values.

Table 3
Significance table.

Receptor	Kruskal-Wallis	Pairwise Comparaison					
		Naïve-VS	Naïve-DPZ05VS	Naïve-DPZ1VS	VS-DPZ05VS	VS-DPZ1VS	DPZ05VS-DPZ1VS
M1	0.317						
M2	0.088						
M3	**0.002***	0.498	1.000	**0.032***	0.189	1.000	0.060
M4	**0.012***	1.000	1.000	0.111	0.503	1.000	**0.016***
M5	**0.003***	0.545	1.000	**0.002***	1.000	0.785	0.057
α4	0.090						
α7	**0.002***	0.702	1.000	**0.007***	0.752	1.000	**0.009***
β2	0.062						

p values for Kruskal-Wallis and Pairwise Comparaison tests

determined. The relative quantification of gene expression was analyzed by the $2-\Delta\Delta Ct$ method and normalized by respective 18S values (Livak and Schmittgen, 2001; Pouliot et al., 2012).

2.8. Statistical analyses

Non-parametric statistical analyses were calculated using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The intragroup differences of pre-training and post-training visual cortical activity were determined by using the Wilcoxon Signed-Rank test. VEP amplitude (Post-Pre) comparisons between groups—VS, DPZ0.5/VS and DPZ1/VS—were performed using Kruskal-Wallis tests and post-hoc pairwise comparisons. For the PCR parameters, the comparison of the gene expression of the mAChR and nAChR (fold change) between the basal level (naive group) and VS, DPZ0.5/VS or DPZ1/VS were performed individually using the Kruskal-Wallis

test and pairwise comparison was applied to compensate for multiple testing conditions. Although non-parametric statistics were used, bars graph representing mean ± S.E.M. were used for clarity.

3. Results

3.1. Visual exposure without cholinergic enhancement does not alter the cortical responsiveness or the expression of cholinergic receptors

The averaged VEP amplitude was not altered by two weeks of visual exposure without pharmacological treatment (VS group) compared to pre-training data for any spatial frequency at 30° or 120° orientation (Fig. 1A). The expression of mAChR subtypes (M1-M5) or any nAChR subunits measured (α4, α7 and β2) mRNA was not significantly changed in the VS group compared to naive animals (Kruskal-Wallis, Fig. 2, see Table 3 for the significance *p* values) in both the visual and the somatosensory cortices. These results suggest that 2 weeks of repeated visual exposure (and animal handling) is not associated with a significant change in the cholinergic receptor expression and cortical reactivity.

3.2. Combined visual exposure and 0.5 mg/kg dose of donepezil increases the cortical visual response without any cholinergic receptor expression change

Two weeks of visual exposure combined with daily injection of 0.5 mg/kg DPZ, significantly increased VEP amplitude compared to pre-training for the trained orientation at 0.08 and 0.12 CPD spatial frequencies (Fig. 1B, Wilcoxon, $p = 0.028$). This effect was also observed for the 120° orientation for both 0.08 and 0.12 CPD (Fig. 1B, Wilcoxon, $p = 0.028$). The VEP amplitude at other spatial frequencies was not affected by the training. The VEP increase was not associated with a change in the expression of mAChR nor the nAChR mRNAs compared to the naive group or to the VS group (Fig. 2 and Table 3).

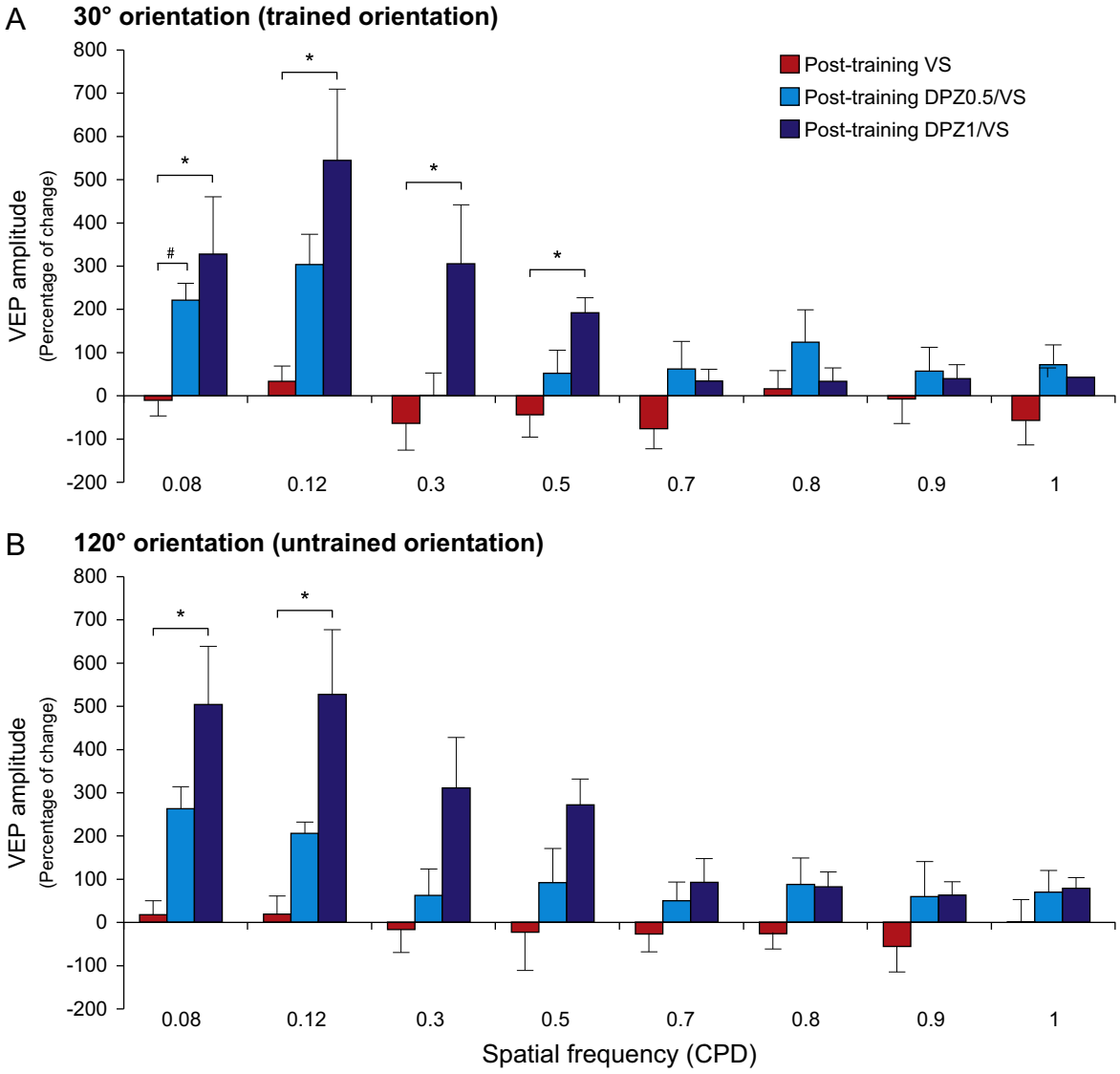

Fig. 3. Effect of visual training and donepezil treatment on VEP amplitude (percentage of change) for the different treatments. Post-training/Pre-training ratio of VEP amplitude was determined for each tested group for the different spatial frequencies (0.08–1 CPD) 30° orientation (A) and 120° orientation (B). VEP amplitude was significantly enhanced for the 0.08 CPD between VS-DPZ0.5/VS (#) and VS- DPZ1/VS (∗) for both orientations. The VEP amplitudes were also significantly enhanced for 0.12 CPD, 0.3 CPD and 0.5 CPD for the 30° orientation and for 0.12 CPD for the 120° orientation in the DPZ1/VS group.

3.3. Combined visual exposure and 1 mg/kg dose of donepezil induces broader VEP effect and alters both muscarinic and nicotinic receptor expression

Two weeks of visual exposure combined with a daily injection of 1 mg/kg DPZ, significantly increased VEP amplitude compared to pre-training at 30° orientation not only for the trained spatial frequency 0.08 CPD and 0.12 CPD (Fig. 1C, Wilcoxon, $p = 0.028$ but also for higher spatial frequencies (0.3, 0.5 CPD) (Wilcoxon, $p = 0.018$, $p = 0.018$). In addition, at 120° orientation the DPZ1/VS group showed an increase in cortical activity for the trained frequency (Fig. 1C, Wilcoxon, $p = 0.018$), as well as higher frequencies (0.3, 0.5, 0.9 and 1 CPD), compared to pre-training recordings (Wilcoxon, $p = 0.018$, $p = 0.018$, $p = 0.043$, $p = 0.018$, respectively). Moreover, two weeks of visual training paired with 1 mg/kg of DPZ produced a significant increase in the expression of M3, M5 and α7 compared to naive animals (Fig. 2, Table 3) but not M1, M2, M4, α4 and β2. Additionally, the expression of mRNA for all the cholinergic receptors at 1 mg/kg of DPZ was not different from the VS only group. The change in cholinergic receptor expression upon visual training coupled to DPZ (1 mg/kg) was also evaluated in a non-visual area (somatosensory cortex) to assess the specificity of the changes observed. The expression of mAChR or nAChR subtypes was not significantly altered in this structure: M1 ($p = 0.872$); M2 ($p = 0.430$); M3 ($p = 0.570$); M4 ($p = 0.308$); M5 ($p = 0.061$); α4 ($p = 0.705$); α7 ($p = 0.702$) and β2 ($p = 0.501$) (Fig. 2).

Intergroup comparisons demonstrated that the only significant change induced by 0.5 mg/kg DPZ in post-pre variation of VEP amplitude compared to VS group was seen for the trained orientation (30°) and the lowest spatial frequency of the stimulus (0.08 CPD) (Fig. 3, Kruskal-Wallis, $p = 0.020$). However, 1 mg/kg DPZ induced a significant increase of the VEP amplitude for 0.08, 0.12, 0.3 and 0.5 CPD, 30° orientation (Kruskal-Wallis: $p = 0.025$, $p = 0.015$, $p = 0.019$, $p = 0.015$, respectively) and for 0.08 and 0.12 CPD, 120° orientation (Kruskal-Wallis: $p = 0.003$, $p = 0.004$, respectively) compared to VS counterparts. This suggests that a higher dose of DPZ induces a spreading of the enhancement of cortical activity. In addition, the comparison of the treatments indicated a significant increase in the mRNA expression of mAChRs M3 and M4 in DPZ1/VS compared to DPZ0.5/VS as well as the α7 nAChR subunits (Fig. 2, Table 3). No significant differences between DPZ0.5/VS and DPZ1/VS were observed for M1, M2, M5, α4, β2 mRNA expression. This suggests that the two doses of DPZ modulate the cholinergic receptor expression differently.

4. Discussion

In this study, we used DPZ administration combined with 2 weeks of daily visual training to improve long-term V1 reactivity. The profile of the mAChRs and nAChRs expression in V1 was also examined by measuring mRNA by RT-PCR at the end of the experiment. Doses of 0.5 or 1 mg/kg of DPZ induced a long-term increase of cortical VEPs compared to pre-training values which were not observed when the visual stimulation was performed without cholinergic enhancement. This increase was spread to neighboring spatial frequencies for the 1 mg/kg DPZ group. Additionally, an upregulation of M3, M4, M5 and α7 expression was observed in the DPZ1/VS group selectively in V1 (not in the somatosensory cortex, taken as a non-visual control sensory area). Together these results indicate that the higher extracellular concentration of ACh induces long-term cortical hyper-reactivity and cholinergic receptors overexpression in V1 whereas the lower ACh concentration induces a more limited increase in the cortical responsiveness without any subsequent cholinergic receptor mRNA variation.

4.1. Dose-dependent effect of repetitive visual exposure combined with DPZ on functional activity

Two weeks of visual exposure alone was not sufficient to induce an increase in cortical reactivity to any of the tested spatial frequencies or orientations. This is consistent with previous studies showing no change of VEPs in the VS group in a similar visual training paradigm (Kang et al., 2014b, 2015) but not with other studies showing gradual increases in VEP amplitudes to a 5-day trained visual stimulus (Cooke and Bear, 2010). These discrepancies could be due to the regimen of the visual stimulation used by Cooke and collaborators which consisted of a wide array of spatial frequencies and orientation which stimulated a larger amount of visual cells compared to our training focussed on one spatial frequency and one orientation. These discrepancies might also be due to recording procedures, since Cooke and Bear recorded VEP in awake rats resulting in increased amplitude of VEPs and increased levels of attention. In our study, the repeated visual exposure using one spatial frequency and one orientation was not sufficient to change the expression of the mAChR or nAChR receptors. This suggests that the natural release of ACh occurring during visual stimulation (Collier and Mitchell, 1966a; Laplante et al., 2005) or the feedforward visual input itself is not sufficient to increase persistent cortical activity and regulation of the AChR expression.

A dose of 0.5 mg/kg of DPZ induced an enhancement of the trained stimulus that was transferred to the orthogonal orientation (120°) for 0.08 and 0.12 CPD but no spreading of the enhancement effect was observed to the higher spatial frequencies. When a higher dose of DPZ administration (1 mg/kg) was combined with visual training, a significant increase in cortical response that spread beyond the trained spatial frequency for both tested orientations was observed. Orientation tuning depends mostly on thalamocortical inputs and horizontal local connections whereas spatial frequency changes results from thalamocortical but also cortico-cortical connections including long-range feedback connections (Angelucci et al., 2002). The spreading of VEP enhancement in spatial frequency seen in the rat due the training-induced strengthening of the cortical connections could be due to the salt and pepper organization of V1 in rodents and the small distance of the lateral and feedback connections compared to other species. It can also be due to an effect of the DPZ on higher cognitive areas, facilitating feedback top-down mechanisms. The transfer of increased sensitivity for the orthogonal orientation observed in this study is consistent with a previous study (Cooke and Bear, 2010; Kang et al., 2015). However, a 2-week cholinergic/visual stimulation selectively improved the visual acuity for a 30° pattern but no other orientations, as measured behaviorally (Kang et al., 2014a). The discrepancy between behavioral or electrophysiological studies related to orientation selectivity of the effect may depict (1) an increased number of cells changing or enlarging their orientation selectivity or (1) an enhanced response for cells selective for 30° and 120° patterns induced by ACh transmission (see Kang and Vaucher, 2009; Kang et al., 2014a for further discussion). Moreover, tetanic burst stimulation of the lateral dorsal geniculate nucleus also induces enhancement of VEPs to generalize to other orientation or spatial frequencies (Cooke and Bear, 2010). As cholinergic potentiation induces long-term potentiation-like mechanisms in V1 (Brocher et al., 1992; Kirkwood et al., 1995; Kang and Vaucher, 2009), these broad effects of cholinergic potentiation of the visual training effects could indicate plasticity or reinforcement mechanisms in V1 induced by increased levels of ACh. The enhancement of VEP amplitude on neighboring spatial frequencies is also consistent with previous studies using electrical stimulation to potentiate cholinergic transmission (Kang et al., 2015). Thus, even if the cholinergic transmission is more diffusely potentiated by DPZ treatment that is not restricted to periods of pattern visual

stimulation compared to timely coupled electrical stimulation, the effect of the two treatments on the potentiation of the cortical responses is similar and of equivalent strength.

The DPZ dose-dependence of the evoked V1 responses obtained are consistent with previous studies in a mouse model that showed a dose-dependent DPZ effect (0.3 mg/kg and 1.0 mg/kg) on the relief of cognitive rigidity. In this study, 1 mg/kg DPZ showed greater cognitive enhancement compared to the 0.3 mg/kg dose (Karvat and Kimchi, 2014). Additionally, a higher dose of DPZ (3 mg/kg) was proven to antagonize the scopolamine-induced performance deficit in mice, which was not the case with a lower dose of DPZ (0.75 mg/kg) (Spowart-Manning and van der Staay, 2004). In our study, the dose-dependent effect could arise from increased levels of extracellular ACh in the cortex, as these levels seem to double with a 1 mg/kg compared to 0.5 mg/kg dose measured in the hippocampus and prefrontal cortex (Hatip-Al-Khatib et al., 2004; Naik et al., 2009). The dose-dependent effect seems to affect the sensitivity of the cortex to detect high spatial frequency visual stimuli and elaborate a stronger response which could, in turn, induce a stronger feedforward processing, as already suggested in other cortical areas (Hasselmo, 2006; Giocomo and Hasselmo, 2007).

4.2. Repetitive visual exposure combined with 1 mg/kg DPZ induces long-term changes in cholinergic receptor expression

The lower dose of DPZ combined with visual stimulation did not induce changes in the synthesis of new mRNA, i.e., either the intrinsic quantity of receptors was sufficient to support this strong cholinergic activity or the receptors mRNA expression was changed at other time points of the stimulation (or in other brain regions).

The 1 mg/kg dose of DPZ induced an increase in V1 cholinergic receptors mRNA expression compared to basal levels in naive animals. The regulation of mRNA expression was selective for the stimulated cortical area (V1) compared to the non-visual area. The receptors overexpressed were α7, M3 and M5. In addition, the M4 receptor was overexpressed in DPZ1/VS compared to DPZ0.5/VS.

Because the mRNA expression was measured one week after the last training session to allow a partial drug washout, we considered that the change in mRNA expression at this time point reflects an involvement of these receptors in long-term effects of visual/-cholinergic training rather than in an acute effect such as a change in ionic channel conductance. It can also reflect the return of ACh concentrations to pre-DPZ levels because of DPZ washout, but the stability of the mRNAs expression in the control cortex suggests that DPZ has no effect on cholinergic receptor expression without sensory challenge. It has to be noted that the mRNA located in the cell bodies that showed increased expression detected in the present study is related to local neurons in the sampled areas, i.e. cortical cells. Thus possible changes at the presynaptic level (thalamocortical, corticocortical or cholinergic basalocortical fibers) were not assessed, although they might play a role in the process. The results are consequently discussed in terms of involvement of the V1 cholinergic receptors in V1 functioning. VEP changes were compared with mRNA level changes although not measured in the same animals, considering that the VEP changes could also result from DPZ-induced changes at the level of the lateral geniculate nucleus or retina.

The α7 receptor, present on pyramidal cells (Aramakis and Metherate, 1998; Aztiria et al., 2004), is usually associated with long-term cortical plasticity and attention (Young et al., 2004). Moreover, it has been demonstrated that this receptor is present on GABAergic neurons from Layer I and could induce disinhibition of pyramidal cells (Christophe et al., 2002). The lack of the α7 nAChR subunit in rodents alters the visual cortex synaptic plasticity (Criscuolo et al., 2015) and reduces visual acuity (Origlia et al., 2012). Knowing that the α7 subunit is involved in visual cortical synaptic plasticity (Criscuolo et al., 2015), the increase in its expression observed in the 1 mg/kg dose of DPZ group may be involved in long-term changes of plasticity in V1. Moreover, this expression was not related to visual experience (Origlia et al., 2012).

The excitatory M3 mAChR subtype role in visual processing is not clear. M3 is present on GABAergic interneurons (Amar et al., 2010), even if a previous report suggests a scarce presence of the M3 subtype in the rodent's visual cortex (Levey et al., 1994). Since the activation of M3 by ACh on GABA cells expands the inhibitory conductance, an increase in its mRNA could indicate an intensification of the release of GABA, whose receptor is involved in cortical plasticity (Yazaki-Sugiyama et al., 2009). M3 has also an influence on cortical properties such as contrast sensitivity or spatial frequency (Groleau et al., 2014) and long-term depression (Origlia et al., 2006) in V1. Moreover, in adult mice, the absence of M1 and M3 produces an increase in the size of the visual cortical receptive field population (Groleau et al., 2014). Therefore, the changes observed in M3 mAChR mRNA expression could also be associated with the broader cortical responsiveness at 1 mg/kg.

An increase in M5 expression was observed in the 1 mg/kg dose of DPZ group. This subtype is mainly found on endothelial cells resulting in vasodilation of the vessels (Elhusseiny and Hamel, 2000). The M5 overexpression may thus be involved in regulation of cerebral perfusion and oxygenation upon repetitive transient visual activity. However, some studies showed a neuronal rather than vascular effect of acetylcholinesterase inhibitors (Silver et al., 2008; Ricciardi et al., 2013).

The increase in M4 mRNA expression observed at the higher dose of DPZ could indicate an inhibitory effect of ACh in the layer IV, as observed in the somatosensory cortex (Eggermann and Feldmeyer, 2009). This would lead to filter weak sensory inputs in this layer. Consistently, an increased cortical ACh level by DPZ has been shown to decrease the propagation of the excitatory response following a visual stimulation in rats (Kimura et al., 1999) and in humans (Silver et al., 2008), inducing a reduction in excitatory activity by an increased intracortical inhibition. The increase in M4 obtained with a higher concentration of ACh could thus favor geniculocortical inputs. This shift from cortico-cortical to thalamo-cortical inputs matches previous studies suggesting that low cortical ACh concentration is related to cortical circuits dominated by local cortical recurrent activity whereas high ACh is related to cortical circuits dependent on thalamic inputs (Oldford and Castro-Alamancos, 2003; Hasselmo and Giocomo, 2006; Giocomo and Hasselmo, 2007; Wester and Contreras, 2013; Shah et al., 2015). Therefore, the upregulation of M3 and M4 mAChR mRNA receptors in addition to an overexpression of nAChR subunit α7 obtained suggests a greater effect of a high dose of DPZ on cortical activity.

Unexpectedly, the mRNA expression of M1, M2 and α4β2, which are the main AChRs present in the cerebral cortex, was not regulated by visual stimulation combined with cholinergic enhancement. Either these receptors do not contribute to long-term changes following our type of stimulation or their basal expression in V1 is so strong (Krejci and Tucek, 2002) that there is no need to synthesize new receptors upon intensive use. This absence of regulation of M1 receptor upon cholinergic stimulation is, however, also observed *in vitro* (Cabadak et al., 2011). In addition, the M1 mAChR do not appear to be involved in the visually increased VEP amplitude since M1 mAChR blockade during the cholinergic/visual training did not abolish the potentiation of VEP (Groleau et al., 2015). The M1 subtype is found on postsynaptic pyramidal cells (Mrzljak et al., 1993; Gu, 2003; Gulledge et al., 2009). Moreover, the absence of regulation of the M2 mRNA

expression after 14 days of training could be linked to the stability of the inhibitory system as the inhibitory M2 subtype is largely present on GABAergic neurons (reviewed by Groleau et al. (2015)). The $\alpha4\beta2$ is also found on GABAergic neurons (Lucas-Meunier et al., 2009) and on thalamocortical terminals. Therefore, it is possible that these receptors could modulate the cortical activity even if their expression is not altered in the visual cortex.

5. Conclusions

A 1 mg/kg dose of DPZ induced a greater cortical enhancement, diffusing to other spatial frequencies and orientations, than did a lower dose. The DPZ cholinergic potentiation of the visual training induced a change in the mRNA expression of the M3, M4, M5 mAChRs and the $\alpha7$ nAChR subunit. Two weeks of visual exposure alone did not result in long-term functional or structural changes. Therefore, these results suggest that coupling of both visual training and a sufficient dose of cholinergic enhancer would be beneficial for visual processing efficiency.

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