Effects of different dosage of Myelin Oligodendrocyte Glycoprotein on visual function in the Dark Agouti rat model of Experimental Autoimmune Encephalomyelitis

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Introduction

Experimental autoimmune encephalomyelitis (EAE) is the most common preclinical model for multiple sclerosis (MS), characterized by inflammation, demyelination, axonal loss and gliosis[1]. EAE can be actively induced by immunization with CNS-derived tissues, as well as myelin peptides, like Myelin Oligodendrocyte Glycoprotein (MOG)[2]. The Dark Agouti (DA) rat strain immunized with MOG develops a protracted and relapsing EAE[3]. Spinal cord pathology had already been studied in MOG-EAE DA rats[4], which also presents visual function abnormalities[5], a typical feature of MS patients[6]. In particular, we explored the involvement of visual pathways in MOG-EAE using Visual Evoked Potentials (VEPs), which are reliable biomarkers of neurophysiological dysfunctions affecting optic nerves (ONs)[7]. The injection of different quantities of MOG can give different outcomes in terms of ON pathology. Interestingly, increasing MOG dosage in C57BL/6 mouse resulted in different profiles of optic neuritis, with an earlier onset with different peaks of inflammation and demyelination using higher doses of MOG[8]. In the present study, we will analyze VEPs in DA rats immunized with increasing doses of MOG (40, 50 and 60 µg), together with the monitoring of motor symptoms. For VEP recording, we will take advantage of a non-invasive technique already tested by our group[9] at two different time points (0 and 35 days post immunization, dpi). At the end of the study (35 dpi), ON histology will detect inflammation, demyelination and axonal loss in order to validate VEP outcomes.

Methods

Animals

Twenty four (n = 24) female DA rats, 8 weeks aged, with a body weight of 110-130 g were used in this experiment.

Experimental protocol

At day 0 (baseline), 18 rats were injected with different doses of MOG. In particular, 6 rats were immunized with 40 µg (MOG 40), 7 with 50 µg (MOG 50) and 5 with 60 µg (MOG 60) of MOG, whereas 6 rats were left untouched and considered as controls (MOG 0). VEPs from left and right eyes were recorded at two time points: the first recording was performed before immunization (baseline), then at 35 days post immunization (dpi). Clinical score of immunized rats was monitored daily from 0 to 35 dpi. After the last VEP session (35 dpi), the rats were sacrificed for histological analysis of left and right ONs.

Clinical assessment of EAE rats

Clinical signs of EAE in immunized rats were scored daily from 0 to 35 dpi. Clinical score ranged from 0 to 5. (0: no signs; 0.5: tail weakness; 1: complete tail paralysis; 1.5: complete tail paralysis and weakness of the hind limbs; 2: complete tail paralysis and one hind limb paralyzed; 2.5: hind limbs do not support the body weight, but without complete paralysis; 3: complete paralysis of the hind limbs; 3.5: complete paralysis of the hind limbs and paresis of front paws; 4: complete paralysis of front and hind limbs; 4.5: moribund; 5: death due to the severity of EAE).

VEP recording

Flash VEPs from left and right eyes were recorded under ketamine (40 mg/kg) and xylazine (5 mg/kg) using 6 mm Ø Ag/Cl epidermal cup electrodes over the contralateral primary visual cortex, with a reference needle electrode inserted in the nose. For each recording session, 4 averages of 20-30 trains each were used for measuring N1 latency and N1-P2 amplitude (expressed in % of change from latency/amplitude measured at the baseline) from the complex P1-N1-P2 of flash-VEPs. When VEP response was not elicited, we assigned a value of 0 µV for N1-P2 amplitude and an arbitrarily value of 99.99 ms for N1 latency, as similarly reported in literature[10].

Histology

Rats were sacrificed with cervical dislocation under ketamine/xylazine anaesthesia. ONs were removed and fixed in 4% paraformaldehyde at 4°C for 48 hours, then embedded in paraffin and cut at the microtome. For each group, 8 ONs were randomly selected for histological quantifications. Three 8 µm longitudinal sections of ON were collected and stained with luxol fast blue (LFB), SMI312 antibody and Iba1 antibody to detect myelin, axons and activated microglia/macrophages, respectively. The percentage of demyelination/axonal loss and the number of activated microglia/macrophages were quantified averaging the three ON sections.

Statistical analysis

Clinical scores of immunized rats were analysed through Friedman test with "time" as "within subjects" main factor (36 levels: from 0 to 35 dpi), followed by Dunn post-hoc test. N1 latency and N1-P2 amplitude were compared using two-way ANOVA for repeated measures with "time" (2 levels: baseline and 35 dpi) as "within subjects" main factor and "MOG dose" as "between subjects" main factor (5 levels: 0, 30, 40, 50, 60 µg), followed by LSD post-hoc test for "MOG dose" factor and protected paired t-tests for "time" factor. For histological measurements, "MOG dose" groups were compared through one-way ANOVA followed by LSD post-hoc test. Data were considered significant at $p < 0.05$.

Results

Clinical assessment of EAE

In MOG 40 rats, EAE onset ranged from 9 to 11 dpi (**Fig 1A**). At 35 dpi, complete remissions were observed in 2 out of 6 rats (MOG 40_3 and 6; 33.3%) and occurred at 31 and 35 dpi. Relapses were seen in all the 6 rats. At 35 dpi, left and right eyes of MOG 40_4 did not respond to visual stimuli, resulting in absent VEPs. All the immunized rats showed clinical symptoms from 11 to 13 dpi , whereas the minimal percentage of diseased animals (16.7%) was observed at 9 dpi (**Fig 1B**). The EAE clinical course was characterized by three significant remissions (at 17 and 18 dpi, 21 and 22 dpi, 31 and 32 dpi; **Fig 1C**). In MOG 50 rats, EAE onset ranged from 9 to 14 dpi (**Fig 2A**). At 35 dpi, complete remissions were observed in 3 out of 7 rats (MOG 50_1, 4 and 7; 42.9%) and occurred between 33 and 35 dpi. Relapses were seen in 6 out of 7 rats (MOG 50_1, 3, 4, 5, 6 and 7; 85.7%). At 35 dpi, both eyes of MOG 50_5 and the right eye of MOG 50_7 did not respond to visual stimuli, resulting in absent VEPs. The totality of immunized rats showed clinical symptoms from 27 to 32 dpi, whereas the minimal percentage of diseased animals (14.3%) was observed at 9 dpi (**Fig 2B**). The EAE score revealed two significant remissions (from 17 to 23 dpi and at 35 dpi; **Fig 2C**). In MOG 60 rats, EAE onset ranged from 8 to 12 dpi (**Fig 3A**). At 35 dpi, complete remissions were not observed. Relapses were seen in 2 out of 5 rats (MOG 60_3 and 5; 40%). At 35 dpi, left and right eyes of MOG 60_1, MOG 60_2, MOG 60_4 and MOG 60_5 did not respond to visual stimuli, resulting in absent VEPs. All the immunized rats showed clinical symptoms at 12, 13 and 16 dpi, from 20 to 22 dpi and from 24 to 35 dpi, whereas the minimal percentage of diseased animals (40%) was observed at 8 dpi (**Fig 3B**). The EAE motor disability showed a significant remission from 14 to 18 dpi (**Fig 3C**).

VEP latency and amplitude in MOG rats

During each VEP recording session, we succeeded in obtaining a good signal-to-noise ratio, with P1-N1-P2 complex that was clearly distinguishable and measurable in terms of latency and amplitude in all the experimental groups (**Fig 4A**). For N1 latency analysis, two-way ANOVA for repeated measures detected significant main effects of "time" ($F_{1,44} = 58.231$, $p < 0.0001$), "MOG dose" ($F_{3,44} = 14.687$, $p < 0.0001$) and a significant interaction between "time" and "MOG dose" ($F_{3,44} = 14.687$, $p < 0.0001$; **Fig 4B**). Post-hoc analysis revealed that, compared to MOG 0, mean N1 latencies at 35 dpi were significantly increased in MOG 40 ($p = 0.032$), MOG 50 ($p = 0.007$) and MOG 60 ($p < 0.0001$). N1 latency of MOG 60 was significantly increased with respect to MOG 0 ($p < 0.0001$), MOG 40 ($p < 0.0001$) and MOG 50 ($p = 0.00013$). Regarding N1 latency changes over time, protected paired t-tests highlighted significant increases in MOG 40 ($t_{11} = 2.263$, $p = 0.045$), MOG 50 ($t_{13} = 3.145$, $p = 0.008$) and MOG 60 ($t_9 = 9.837$, $p < 0.0001$). On the other hand, N1 latencies did not significantly change over time in MOG 0 ($t_{11} = 0.934$, $p = 0.37$). For N1-P2 amplitude analysis, two-way ANOVA for repeated measures detected significant main effects of "time" ($F_{1,44} = 33.213$, $p < 0.0001$), "MOG dose" ($F_{3,44} = 4.482$, $p = 0.008$) and a significant interaction between "time" and "MOG dose" ($F_{3,44} = 4.482$, $p = 0.008$; **Fig 4C**). Post-hoc analysis revealed that, compared to MOG 0, mean N1-P2 amplitudes at 35 dpi were significantly decreased in MOG 40 ($p = 0.014$), MOG 50 ($p = 0.036$) and MOG 60 ($p = 0.001$). Regarding N1-P2 amplitude changes over time, protected paired t-tests highlighted significant decreases in MOG 40 ($t_{11} = -5.364$, $p = 0.0002$), MOG 50 ($t_{13} = -3.326$, $p = 0.005$) and MOG 60 ($t_9 = -3.280$, $p = 0.009$). On the other hand, N1-P2 amplitudes did not significantly change over time in MOG 0 ($t_{11} = 0.055$, $p = 0.957$).

Histological analysis of ONs

At 35 dpi, rats were sacrificed and longitudinal ON sections from MOG 0, MOG 40, MOG 50 and MOG 60 were analysed. MOG 0 ONs showed a regular morphology, whereas ONs from MOG 40, 50 and 60 groups were characterized by strong positive Iba1 and weak positive LFB and SMI312 stainings (**Fig 5A**). Regarding microglia/macrophages ($Iba1^+$) quantification (**Fig 5B**), one-way ANOVA showed significant differences between groups ($F_{3,31} = 9.886$, $p = 0.0001$). Post-hoc analysis highlighted that, compared to MOG 0, microglia/macrophages were significantly increased in MOG 40 ($p = 0.003$), MOG 50 ($p = 0.0006$) and MOG 60 ($p < 0.0001$) ONs. Concerning myelin (LFB^+) quantification (**Fig 5C**), one-way ANOVA detected significant differences between groups ($F_{3,31} = 21.225$, $p < 0.0001$). Post-hoc analysis revealed that, compared to MOG 0, myelin was significantly decreased in MOG 40 ($p = 0.0002$), MOG 50 ($p < 0.0001$) and MOG 60 ($p < 0.0001$) ONs. Moreover, there was a significant decrease of myelin in MOG 60 compared to both MOG 40 ($p = 0.001$) and MOG 50 ($p = 0.009$) ONs. As for neurofilaments ($SMI312^+$) quantification, (Figure 5D), one-way ANOVA reported significant differences between groups (F3,31 = 11.092, $p < 0.0001$). Post-hoc analysis indicated that, compared to MOG 0, neurofilaments were significantly decreased in MOG 40 ($p = 0.017$), MOG 50 ($p = 0.0005$) and MOG 60 ($p < 0.0001$) ONs. In addition, there was a significant decrease of neurofilaments in MOG 60 with respect to MOG 40 ($p = 0.005$) ONs.

Correlations between VEP and ON histology

A negative correlation was found between N1 latency at 35 dpi and the level of ON myelination (Pearson's correlation: $r = -0.772$, $p < 0.0001$; **Fig 6A**). Moreover, there was a positive correlation between N1-P2 amplitude measured at 35 dpi and the amount of neurofilaments (Pearson's correlation: $r = 0.623$, $p = 0.0001$; **Fig 6B**).

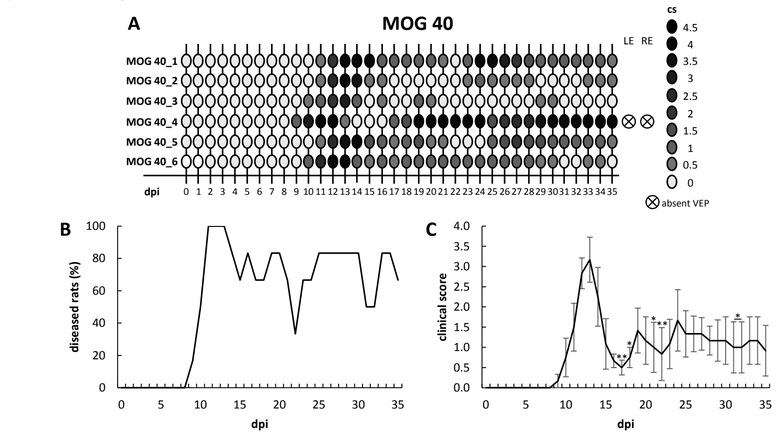

Figure 1. A: *Graphic representation of individual clinical profile in MOG 40 rats from 0 to 35 dpi.* ***B:*** *Kaplan-Meier curve representing MOG 40 rats (n = 6) with clinical symptoms.* ***C:*** *Clinical score of MOG 40 rats (n = 6) from 0 to 35 dpi. Asterisks indicate significant changes compared with the first peak of the disease (13 dpi). *: $p < 0.05$; **: $p < 0.01$. Data are expressed as mean ± SEM.*

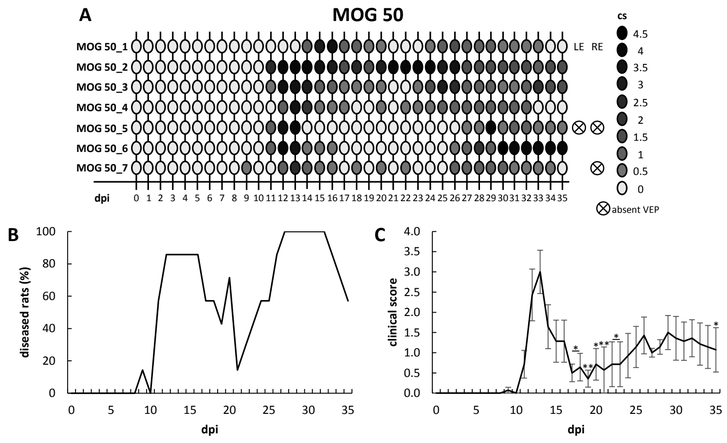

Figure 2. A: *Graphic representation of individual clinical profile in MOG 50 rats from 0 to 35 dpi.* ***B:*** *Kaplan-Meier curve representing MOG 50 rats (n = 7) with clinical symptoms.* ***C:*** *Clinical score of MOG 50 rats (n = 7) from 0 to 35 dpi. Asterisks indicate significant changes compared with the first peak of the disease (13 dpi). *: $p < 0.05$; **: $p < 0.01$. Data are expressed as mean ± SEM.*

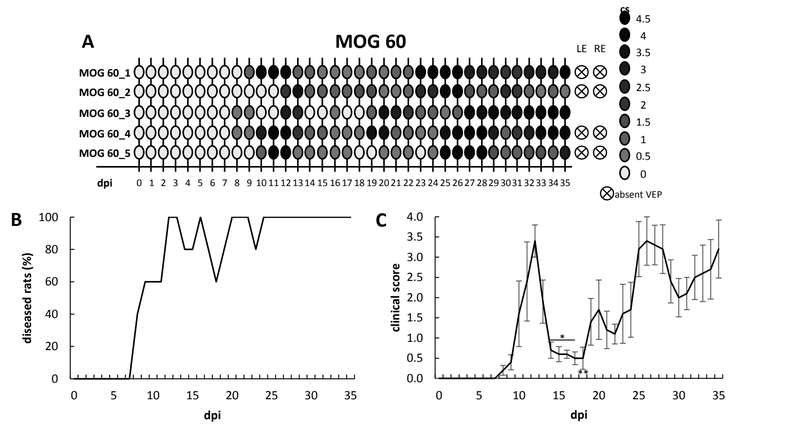

Figure 3. A: *Graphic representation of individual clinical profile in MOG 60 rats from 0 to 35 dpi.* ***B:*** *Kaplan-Meier curve representing MOG 60 rats (n = 5) with clinical symptoms.* ***C:*** *Clinical score of MOG 60 rats (n = 5) from 0 to 35 dpi. Asterisks indicate significant changes compared with the first peak of the disease (12 dpi). *: $p < 0.05$. Data are expressed as mean ± SEM.*

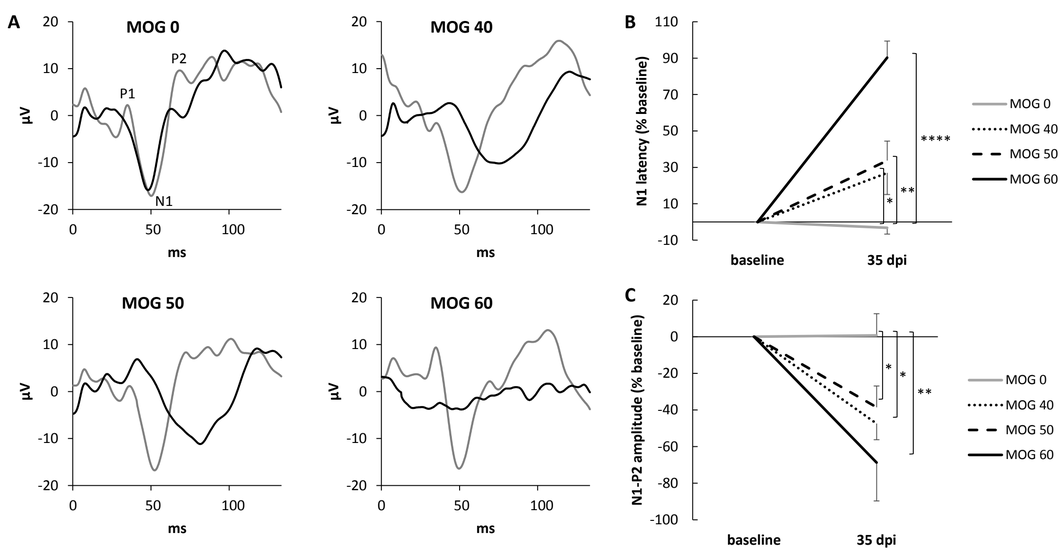

Figure 4. *Representative VEP traces (**A**) recorded from MOG rats at baseline (dashed lines) and 35 dpi (black line), in which the P1-N1-P2 complex is highlighted. N1 latency (**B**) and N1-P2 amplitude (**C**) measured at baseline and 35 dpi in MOG 0 (n = 12 eyes), MOG 40 (n = 12 eyes), MOG 50 (n = 14 eyes) and MOG 60 (n = 10 eyes). Data are normalized as % of baseline and expressed as mean ± SEM (*: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$).*

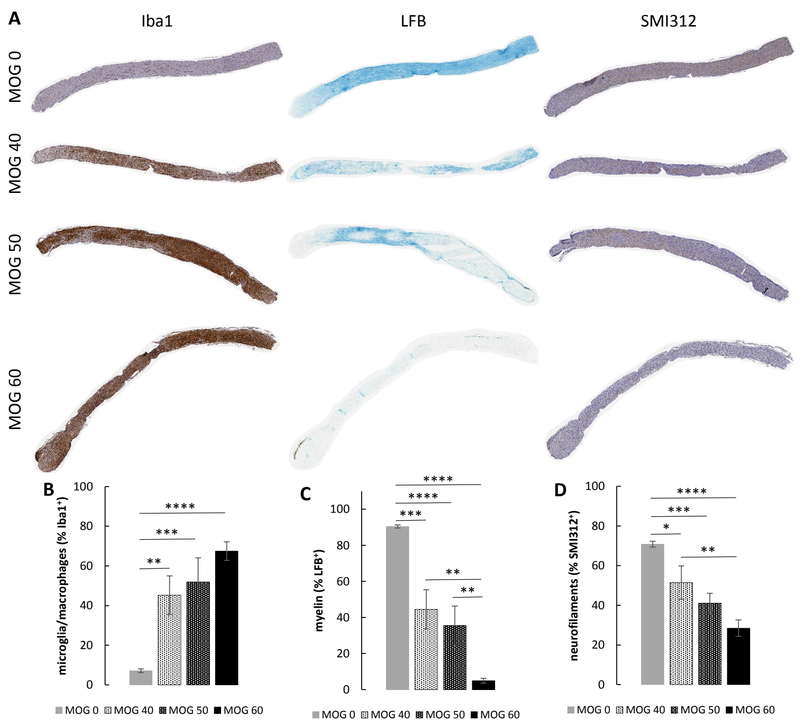

Figure 5. A: *Representative ON sections stained to detect microglia/macrophages (Iba1), myelin (LFB), and neurofilaments (SMI312) in MOG 0, MOG 40, MOG 50 and MOG 60 at 35 dpi (magnification: 10×). Histograms show the quantifications of microglia/macrophages (**B**), myelin (**C**) and neurofilaments (**D**) detected in ONs from MOG 0 (n = 8), MOG 40 (n = 8), MOG 50 (n = 8) and MOG 60 (n = 8). Data are expressed as mean ± SEM (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$).*

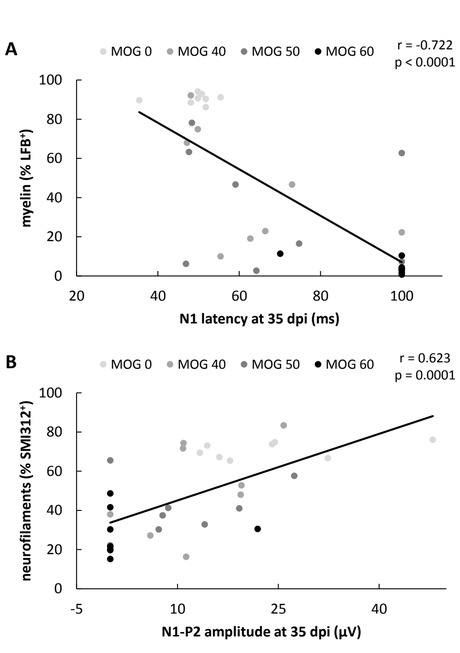

Figure 6. *Pearson's correlations between VEP and ON histology at 35 dpi, in which Pearson's r coefficient and p value are indicated.* ***A:*** *significant negative correlation between N1 latency and percentage of myelin detected with LFB staining;* ***B:*** *significant positive correlation between N1-P2 amplitude and percentage of neurofilaments detected with SMI312 staining.*

Conclusions

Our findings confirmed the relapsing-remitting clinical course of the MOG-EAE DA rat model. We showed clear ON dysfunctions in DA rats immunized with 40, 50 and 60 µg of MOG, as VEP latencies were delayed and VEP amplitudes were decreased at 35 dpi. It is noteworthy that VEP abnormalities were detected in a dose-dependent gradient. In particular, MOG doses of 40 and 50 µg could be used to study VEP delay, which might be caused by demyelination. On the other hand, with a MOG dose of 60 µg, VEP responses were mostly suppressed, probably due to axonal loss along ONs. Consequently, 60 µg of MOG could be administered to DA rats in order to investigate axonal degeneration occurring in EAE. Accordingly, ON histology validated the VEP outcomes obtained, together with significant correlations between VEP and ON histology, which suggested that VEP latency delay and amplitude decrease were actually caused by demyelination and axonal loss, respectively. In experimental procedures involving EAE DA rats, adopting the more suitable dose of MOG might be crucial when testing treatments that could counteract demyelination and/or axonal loss of the ON: in the former case, 40 or 50 µg of MOG should be used, whereas in the latter case 60 µg of MOG should be adopted.

Bibliography & Acknowledgements

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