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ORIGINAL PAPER

Long Trace Eyeblink Conditioning Is Largely Preserved in Essential Tremor

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Abstract

The cerebellum and the prefrontal cortex are assumed to play a role in the pathophysiology of essential tremor (ET). Trace eyeblink conditioning with a long interstimulus interval relies on an intact function of the hippocampus, prefrontal cortex (PFC), and, although marginally, of the cerebellum. The aim of the present study was to evaluate whether long trace eyeblink conditioning is impaired in patients with ET. In 18 patients with ET and 18 controls, a long trace conditioning paradigm was applied. Following 100 paired conditioned response-unconditioned response trials, 30 conditioned response alone trials were given as extinction trials. The degree of tremor and the presence of accompanying cerebellar signs were determined based on clinical scales. The acquisition of conditioned eyeblink responses was not impaired in the group of all patients compared to controls (mean total incidences of conditioned responses in patients 23.3 ± 14.5%, in controls 24.1 ± 13.9%; $P = 0.88$). In the subgroup of six patients with cerebellar signs, incidences of conditioned responses were numerically but not significantly lower (16.4 ± 9.9%) compared to patients without cerebellar signs (26.8 ± 15.5%; $P = 0.16$). Trace eyeblink conditioning with a long interstimulus interval was not impaired in subjects with ET. Patients with clinical cerebellar signs presented slightly reduced conditioning. Areas of the PFC contributing to trace eyeblink conditioning appear less affected in ET. Future studies also using a shorter trace interval should include a larger group of subjects in all stages of ET.

Keywords Essential tremor · Trace eyeblink conditioning · Cerebellum · Associative learning

Introduction

Essential tremor (ET) is one of the most prevalent movement disorders [1]. The pathophysiology remains incompletely understood. There is ongoing debate whether ET is a more complex degenerative disorder or a reversible functional disturbance of neuronal oscillation [2]. Clinical, neuroimaging, and post-mortem studies suggest a contribution of the olivo-cerebellar system in the pathogenesis of ET [3–6]. There is evidence from clinical, animal, and imaging studies that, besides the cerebellum, the prefrontal cortex (PFC) is involved in the pathophysiology of ET [7–11].

The aim of the current study was to assess prefrontal and cerebellar involvement in ET using trace eyeblink conditioning. The acquisition of trace conditioned eyeblink responses strongly depends on the forebrain, i.e., the hippocampal formation [12–15] and the PFC [16–21], given that the trace interval is long enough. Animal studies suggest a role of the cerebellum, i.e., of the deep nuclei as well as the cerebellar cortex [22–25]. However, as revealed by studies in humans, the cerebellar involvement using a long hippocampus-dependent trace interval appears substantially less compared to a shorter trace interval or delay eyeblink conditioning [26, 27]. The PFC might be less involved in longer CS-US intervals as has been shown for delay conditioning [28]. We were interested if long trace eyeblink conditioning is affected in ET subjects and whether this might be prominent in ET subjects with accompanying clinical signs.

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Methods

Subjects

A total of 18 ET subjects (12 male, 6 female; mean age 56.9 ± 19.2 years, range 23 to 83 years) diagnosed according to the criteria established by Deuschl et al. [29] and 18 age- and sex-matched control subjects (10 male, 6 female; mean age 56.7 ± 10.9 years, range 27 to 74 years) were included in the study. Mean duration of tremor in the ET group from symptom onset until examination was 20.1 ± 11.7 years, with a range of 2 to 40 years. The family history for ET was positive in eight subjects, i.e., in 44.4% with at least one affected first-degree relative. Nine subjects were medicated with propranolol for treatment of tremor in ET, but none had a dose of more than 1 mg per kg body weight which could impede eye blink conditioning [30]. Subjects using other centrally acting drugs, e.g., gabapentin or primidone were excluded. None of the control subjects had a history of neurological diseases. All ET and control subjects were naïve for eyeblink conditioning. The study was approved by the local ethics committee and written informed consent was obtained from all subjects.

An experienced neurologist (KS) performed a complete neurological examination. The degree of tremor was evaluated using the "Clinical Rating Scale for Tremor," part 1 (CRST, max. score 80) [31] and an "Intention tremor score" as described by Helmchen et al. [5]. To evaluate possible accompanying ataxia symptoms, the "International Cooperative Ataxia Rating Scale" (ICARS, max. score 100) [32] was used.

Six subjects presented with cerebellar signs. Three of them (ID 2, 6, and 7 in Table 1) showed intention tremor of both upper limbs based on scores of at least two points in an intention tremor score [5, 33]. Further three subjects had postural instability based on at least two points on the posture and gait sub-score of the ICARS [33, 34] (ID 8, 11 and 16 in Table 1). The CRST and ICARS were correlated in the patient group ($R = .486$, $P = 0.041$). Controls did not present with abnormalities in CRST and ICARS. None of the subjects had clinical signs of brainstem dysfunction, dystonia, or other abnormalities on clinical examination Clinical characteristics of the ET subjects are summarized in Table 1.

Trace Eyeblink Conditioning

Details of the trace eyeblink conditioning procedure were reported previously and are repeated in brief [27]. A long trace interval was used. A conditioned stimulus (CS), such as a tone, is followed by an unconditioned stimulus (US), such as an air-puff delivered close to the eye. In delay conditioning, the CS and US partly overlap and terminate at the same time. Different from that, trace eyeblink conditioning is a more complex form of associative learning. Here, CS and US are separated by a stimulus-free time interval requiring a "memory trace." It has to be noted that delay conditioning might also require a "memory trace", because stimuli that are not modified decrease exponentially in their activation of the corresponding sensory receptors and pathways. First, ten CS alone and ten US alone trials were presented in an unpaired and random order, followed by 100 paired CS-US trials and 30 CS alone trials as extinction trials at the end of the experiment. A tone as CS was presented to the right ear, followed by the stimulus-free trace interval of 1000 ms. An air-puff was used as US. In the acquisition phase, 9–10 US alone trials were interspersed. Surface EMG recordings were taken from orbicularis oculi muscles ipsilaterally to the US with electrodes fixed to the lower eyelid and to the nasion. Trace conditioned eyeblink responses were automatically identified within the CS-US interval in paired and extinction trials using custom-made software. Responses occurring within the 150 ms interval after CS-onset were considered as reflexive responses to the tone (i.e., alpha-responses) and not conditioned responses [35]. Trials with spontaneous blinks (SB) occurring prior to CS onset were excluded from the analysis [36]. The investigator (S.J.O) was blinded regarding the participants (ET subjects vs. controls).

The total number of paired trials was subdivided into blocks of ten trials each. The number of conditioned responses was expressed as the percentage of trials containing responses with respect to each block of ten trials (percentage CR-incidence) and the total number of trials (total percentage CR-incidence).

Three extinction blocks of 10 trials each were administered at the end of the experiment. To evaluate extinction effects, CR-incidence within the last block of paired trials (block 10) was compared with the last block of extinction trials (extinction block 3). Moreover, the decline of CR-incidence across the three extinction blocks was analyzed. Analysis of extinction was performed only in those subjects who exhibited at least one CR in the first extinction block to ensure a remaining ability of learning in the paired trials [26, 33].

Onset and peaktime of CRs in paired trials und URs in unpaired trials were automatically quantified as outlined above (Fig. 1). US onset was set as 0 ms. CR onset and peaktime were expressed as negative values (prior US onset). Individual values for CR and UR onset and time to peak were averaged. EMG peak amplitudes were measured ipsilaterally. It has to be noted that due to methodological conditions sizes of peak amplitudes in surface EMG recordings might vary and have to be considered with care [37].

In each session, the frequency of SB was measured within 1 min both at the beginning and the end of the experiment. The rate of alpha-blinks was analyzed within the 150 ms interval after CS onset of 100 paired trials.

Table 1 Patient characteristics

ID	Sex	Age (years)	Disease duration (years)	Family history of ET	CRST	Intention tremor score		ICARS	
					total of part 1	right arm	left arm	total	Posture and Gait
01	m	23	8	–	6	0	1	4	0
02	*m*	*24*	*24*	–	*30*	*4*	*4*	*12*	*0*
03	m	32	31	–	2	0	0	3	0
04	f	39	19	+	1	0	0	2	1
05	m	61	16	–	7	0	0	4	1
06	*f*	*69*	*29*	+	*12*	*3*	*3*	*9*	*0*
07	*m*	*63*	*33*	+	*15*	*2*	*3*	*9*	*0*
08	*f*	*74*	*4*	–	*2*	*0*	*0*	*11*	*3*
09	m	55	39	–	4	0	0	6	1
10	m	71	11	–	2	0	0	6	1
11	*m*	*66*	*16*	–	*2*	*0*	*0*	*5*	*3*
12	m	69	2	–	11	0	1	6	1
13	m	69	30	–	4	0	0	3	1
14	f	25	11	–	8	0	0	3	0
15	f	70	5	–	4	0	0	2	1
16	*m*	*83*	*15*	+	*6*	*1*	*1*	*11*	*3*
17	f	69	40	–	4	1	0	9	1
18	m	63	28	–	5	0	0	5	1

Assessment of tremor: "Clinical Rating Scale for Tremor" (CRST, part 1); "Intention tremor score". Assessment of ataxia: "International Cooperative Ataxia Rating Scale" (ICARS)

ID identity, *m* male, *f* female, *ET* essential tremor. Subjects with cerebellar signs are shown in Italics

Awareness

To assess awareness, subjects were asked a 15-item true or false questionnaire following the conditioning session about details of the stimuli within the main, i.e., the middle part of the conditioning trial (presentation of paired stimuli). Subjects who gave more than three wrong answers were defined as "unaware" [38, 39]. Mean CR-incidences were compared between subjects with and without awareness using unpaired *t* tests in controls and ET subjects.

Data Analysis

In paired trials, analysis of variance (ANOVA) with repeated measures was calculated with percentage CR-incidence as dependent variable, block (1–10), extinction block (1–3) or block 10 and extinction block 3 as within subject factor and group (controls vs. ET subjects) as between subject factor. Group differences (controls vs. ET subjects) of total CR-incidence, timing parameters or conditioned and unconditioned eyeblink responses, effects of awareness and the

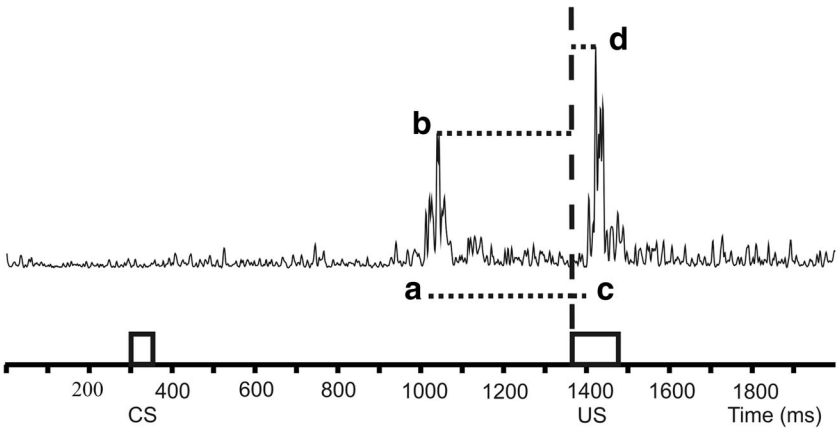

Fig. 1 CRs were visually identified in a time window from 150 ms after CS-onset to the onset of the US where EMG activity deviated at least twice from the EMG baseline level and lasted at least 50 ms. CR latencies were calculated as onset (a) and peak time (b) of CRs before onset of US, with time of the US set as 0 ms. Onset of responses was defined at 10% of peak amplitude. Peak amplitude was measured for the peak with the largest amplitude in case of multiple peaks. UR onset (c) and peak time (d) latencies were assessed as time following US-onset

spontaneous blink rate were calculated using unpaired *t* tests. Level of significance was set at $P < 0.05$. The degrees of freedom were adjusted, if appropriate, according to Greenhouse and Geisser (ANOVA).

Results

CR-Incidences

Mean percentage CR-incidences ± standard error (SE) in ET subjects and controls are shown in Fig. 2a (ET subjects 23.3 ± 14.5%, controls 24.1 ± 13.9%). Control subjects increased percentage CR-incidences across the ten learning blocks (Block 1 12.7 ± 15.9%, Block 10 24.7 ± 24.8%). Also in ET subjects, there was an increase across blocks (Block 1 18.8 ± 21.2%, Block 10 27.0 ± 24.1%). Analysis of variance (ANOVA) with percentage of CR-incidence as dependent variable, block (1–10) as the within subject factor and group (ET vs. controls) as the between subject factor did not reveal a significant main effect of group [$F(1, 34) = 0.023$; $P = 0.88$]. The block effect was significant [$F(1, 34) = 4.3$; $P < 0.001$], the block by group interaction effect was not significant [$F(1, 34) = 1.1$; $P = 0.39$].

ET Subjects with Cerebellar Signs

To evaluate possible effects of accompanying clinical signs of cerebellar dysfunction on eyeblink conditioning mean percentage of CR-incidences ± standard error (SE) in paired trials were compared between ET subjects with clinical cerebellar signs ($n = 6$), ET subjects without cerebellar signs ($n = 12$), and all control subjects ($n = 18$). Compared to controls (24.1 ± 13.9%), mean percentage CR-incidence was numerically lower in ET subjects with clinical cerebellar signs (16.4 ± 9.9%) but not in ET subjects without cerebellar signs (26.8 ± 15.5%; Fig. 2b). The difference was not significant. Comparison of controls vs. both ET subgroups did not reveal a significant effect of group [$F(2, 33) = 1.1$; $P = 0.34$]. The block effect was significant [$F(1, 33) = 2.8$; $P = 0.01$], the block by group effect was not significant [$F(2, 33) = 1.7$; $P = 0.30$]. Similarly, comparison of ET subjects with clinical cerebellar signs with ET subjects without cerebellar signs did not reveal a significant group effect [$F(1, 16) = 2.2$; $P = 0.16$], also the block-effect [$F(1, 16) = 1.9$; $P = 0.08$] and the block by group effect [$F(1, 16) = 1.5$; $P = 0.18$] were not significant. ET subjects with cerebellar signs presented numerically smaller mean peak amplitudes compared to ET subjects without cerebellar signs (0.22 and 0.28 mV; $P = 0.36$). However, findings were not significant and analysis might present only an approximation due to methodological considerations. ET subjects with cerebellar signs were not significantly older than ET subjects without cerebellar signs (63.2 ± 20.4 vs. 53.8 ± 18.7 yrs.; $T(16) = -0.94$; $P = 0.37$).

ET Subjects with Beta-Blockers

To analyze possible effects of low-dose beta-blocker therapy on mean percentage of CR-incidences, ET subjects with medication of beta-blockers (24.7 ± 12.6%; $n = 9$) were compared to ET subjects without any medical treatment (21.9 ± 16.8%; n = 9) and all controls. ANOVA revealed a significant block effect [$F(1, 33) = 3.5$; $P = 0.002$]. The block by group effect [$F(2, 33) = 9.0$; $P = 0.56$] and the group effect [$F(2, 33) = 0.1$; $P = 0.91$] were not significant. Similarly, comparison of ET subjects with medication of beta-blockers and ET subjects without any medical treatment was not significant ($P = 0.70$).

Awareness

All subjects described both stimuli correctly. The majority of controls (16 out of 18) and ET subjects (11 out of 18) identified the correct order of CS and US. Four of the six ET subjects with accompanying clinical cerebellar signs showed awareness. Differences in the number of aware subjects between the group of all controls and all ET-subjects did not reach significance (chi-square test, $P = 0.054$). Mean CR-incidences were slightly decreased in subjects without

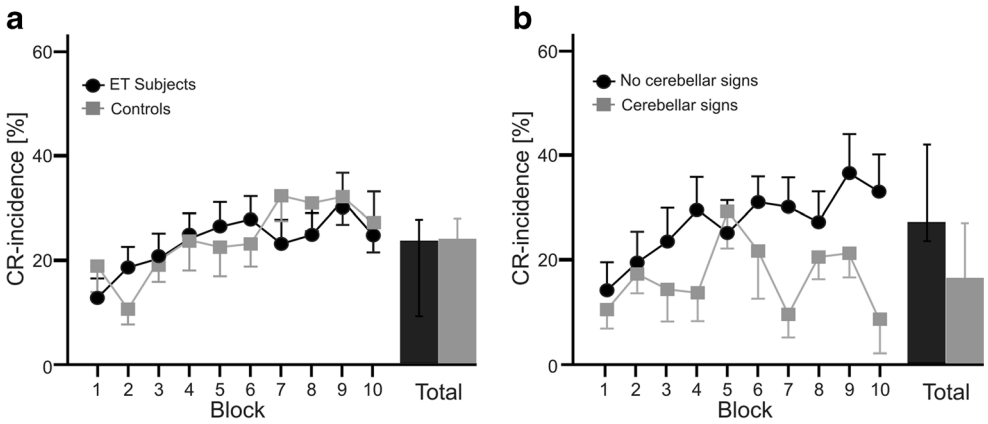

Fig. 2 Mean percentage CR-incidence ± standard error (SE) in paired trials shown per block of ten trials and mean total percentage CR-incidence (Total) in **a** ET and control subjects and in **b** ET subjects without and with accompanying cerebellar signs

awareness but did not differ significantly neither between ET subjects nor controls with and without awareness [ET subjects with (25.6 ± 16.7%) and without (19.7 ± 10.0%) awareness, $T(1, 16) = 0.84$, $P = 0.41$; controls with (24.8 ± 14.5%), and without (18.2 ± 8.9%) awareness, $T(1, 16) = 0.62$, $P = 0.55$]. Significant differences of mean CR-incidences between groups, calculated separately between aware and unaware subjects, were not observed [ET subjects and controls with awareness, $T(1, 25) = 0.14$, $P = 0.89$); ET subjects and controls without awareness, $T(1, 7) = 0.19$, $P = 0.86$].

Timing of Conditioned and Unconditioned Eyeblink Responses

The comparison of mean CR onset and peaktime latencies between ET subjects (− 438.5 ± 130.3 and − 407.0 ± 129.6 ms, respectively) and controls (− 485.3 ± 92.5 and − 452.5 ± 92.0 ms, respectively) did not reveal significant group differences ($P = 0.22$, $P = 0.23$; unpaired t test; Fig. 3a). Timing parameters are illustrated by individual EMG recordings in a control subject and ET patient (Fig. 4).

There were no significant group differences ($P = 0.46$, $P = 0.17$; unpaired t test) comparing UR onset and peaktime latencies between ET subjects (64.6 ± 23.0 and 110.0 ± 32.2 ms, respectively) and controls (60.3 ± 8.0 and 97.7 ± 19.0 ms, respectively; Fig. 3b, see also Fig. 4).

Extinction of Conditioned Eyeblink Responses

In each group, 16 subjects exhibited at least one CR in the first extinction block and were included in the analysis [26, 33]. Neither in ET patients nor in controls CRs showed a significant decline comparing the last block of paired trials (block 10) and the last block of extinction trials (extinction block 3; ET subjects: 24.7 ± 24.8 to 34.5 ± 24.0%; controls 27.0 ± 24.0 to 29.3 ± 24.8%; Fig. 5a). ANOVA with percentage CR-incidence as dependent variable, block 10 and extinction block 3 as the within subject factor and group as the between subject factor revealed no significant effects of block [$F(1, 30) = 1.4$; $P = 0.24$]. Likewise, the group [$F(1, 30) = 0.011$; $P = 0.92$] and extinction by group effects were not significant [$F(1, 30) = 0.64$; $P = 0.43$]. Only controls showed a slight decline of CRs comparing extinction blocks 1–3 (34.0 ± 24.8 to 29.3 ± 24.8%), while CRs tended to increase across extinction blocks 1–3 in ET patients (22.4 ± 16.5 to 34.5 ± 24.0%). ANOVA did not reveal significant effects of block [$F(2, 60) = 0.19$; $P = 0.83$], block by group [$F(2, 60) = 3.1$; $P = 0.054$] and group [$F(1, 30) = 0.18$; $P = 0.67$] (Fig. 5b).

Spontaneous Blink-Rate and Alpha-Blinks

The mean number of SB did not differ significantly comparing ET subjects and controls (SB/min, at the beginning: 15 ± 11 vs. 13 ± 7, $P = 0.64$; SB/min. at the end: 19 ± 13 vs. 18 ± 12, $P = 0.85$; unpaired t-test). ET subjects presented numerically more alpha-blinks than controls (4.8 ± 3.4 vs. 2.7 ± 2.4, $P = 0.039$; unpaired t test).

Discussion

Trace eyeblink conditioning was investigated in subjects with ET using a long trace interval. Acquisition of conditioned eyeblink responses was not impaired in the group of all ET subjects compared to controls. Although the number of conditioned responses was numerically reduced in ET subjects with accompanying cerebellar signs, this difference was not statistically significant.

The knowledge of etiologies and pathogenesis of ET is still at a low level [40]. Evidence has accumulated for a cerebellar and prefrontal contribution in this condition [7–11].

A subset of ET subjects exhibits cerebellar signs including intention tremor, disturbances of gait [4, 41, 42], eye movements [5], balance, and speech [34]. Functional imaging studies indicate a hyperactivity of the cerebellar cortex as the most consistent finding [43]. Furthermore, post-mortem studies revealed neurodegenerative pathologies in cerebellar structures of ET subjects [44–46]. Finally, ET patients, even in the very

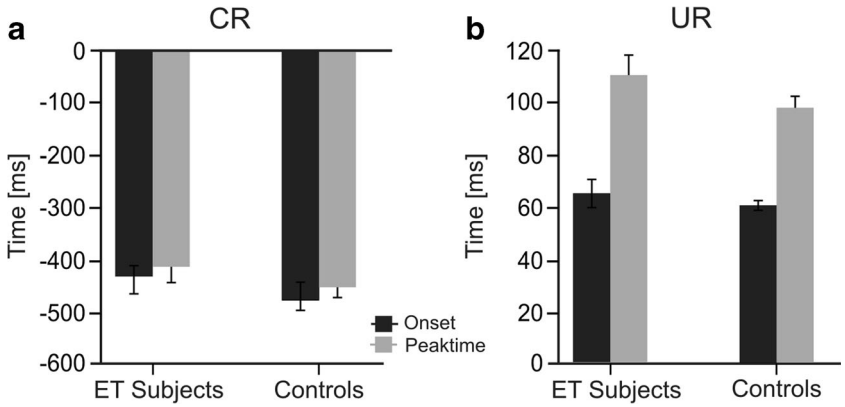

Fig. 3 Timing of **a** conditioned (CR) and **b** unconditioned eyeblink responses (UR) in ET subjects and controls. Group mean values ± SE are shown for response onset and time to peak latencies. CR timing parameters are expressed in time prior US (air-puff)-onset

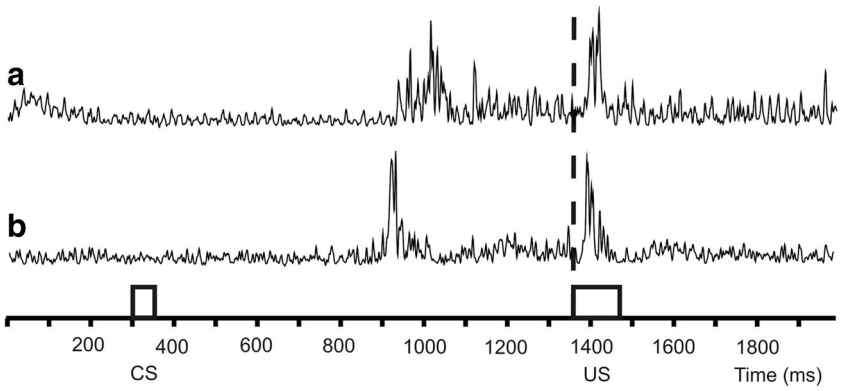

Fig. 4 Individual EMG recordings illustrating onset and peaktime latencies of CR and UR in an exemplary control (a) and ET subject (b, ID 12 in Table 1). CS and US onset and period are indicated as rectangle at the time scale. Dotted vertical lines indicate onset of the US (air puff)

early stages of ET, have been shown impaired in cerebellar dependent delay eyeblink conditioning [33, 47]. Cerebellar contribution appears to be substantially weaker in trace in comparison to delay eyeblink conditioning although cerebellar nuclear cells present similar responses to both delay and trace conditioning paradigms [22–26, 48]. A parallel pontine projection from prefrontal areas to the cerebellar cortex as well as nuclei presenting the CS directly to the interposed nucleus is supported by animal data [49]. However, the role of the posterior interposed nucleus might include enhancement of the blinkreflex and conditioned eyelid responses, but does not involve the learning process itself [50]. It has been suggested that forebrain information processing with projection to the pontine nuclei and to the interposed nucleus may make the cerebellar cortex in trace conditioning not necessary [51]. Using a long trace interval acquisition was not impaired in patients with cortical cerebellar degeneration [27]. Considering a cerebellar involvement in trace eyeblink conditioning, the numerically reduced CR-incidences in the subgroup of ET subjects with clinical cerebellar signs may be conclusive. It has to be debated that a cerebellar contribution to a long trace eyeblink conditioning paradigm may be too slight to give rise to distinct changes in a group of ET subjects. It might be of interest to investigate in future studies whether ET subjects are impaired using a shorter trace interval, which is more likely to be processed by cerebellar circuits.

Subjects with ET exhibit deficits in cognitive functions that require an intact PFC [52]. Imaging studies revealed an altered connectivity [10, 11] and atrophy of the gray and white matter [7, 8] of the PFC in ET. Especially in trace, but also in delay eyeblink conditioning the hippocampus and PFC are essentially involved [21, 28, 53]. Animal studies have shown that the activity of forebrain regions is critical [16–20]. While the hippocampus is, although transiently, essential for the acquisition of trace CRs [12–15, 54, 55], the PFC and the sensory cortex are most likely involved in the timing of eyeblink conditioning [56, 57]. More specific, while the caudal medial PFC bridges the temporal gap between the end of the CS and the beginning of the US [58], dorsolateral PFC neurons are involved in the generation of timed CRs [59]. The rostral MFC is probably involved in the determination of CS-US intervals of an intermediate range. It has been shown that rostral medial prefrontal neurons in rabbits modify their oscillation as a function of CS-US intervals and discharge no significant neural responses at very short or long CS-US delay conditioning intervals [28]. The PFC projects to cerebellar circuits where

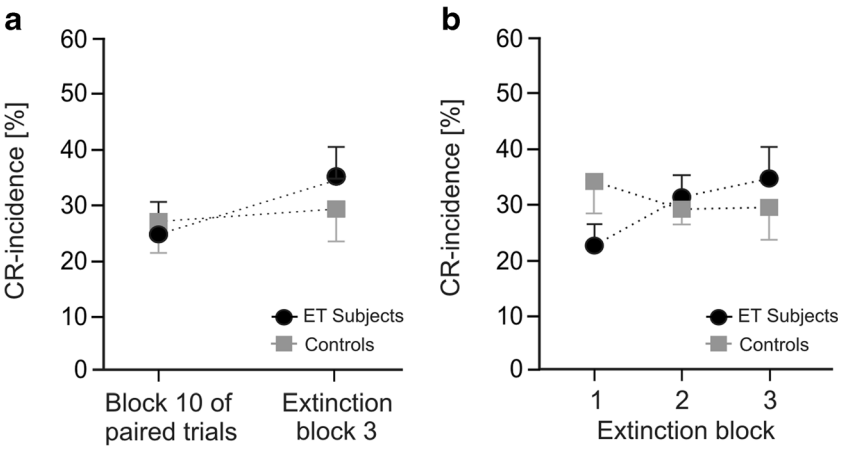

Fig. 5 Mean percentage CR-incidences ± SE in the last block (block 10) of paired trials compared with extinction block 3 (**a**) and across the three extinction blocks (**b**) in ET and control subjects

the CS and US are thought to be associated to generate a trace conditioned response [59]. Based on findings of a prefrontal dysfunction in ET, impaired trace eyeblink conditioning might be expected. However, neuroimaging studies in ET show an involvement of the dorsolateral but not of the medial PFC [11]. The latter is of relevance in trace eyeblink conditioning. The involvement of different anatomical locations (although within the PFC) may explain why the present ET subjects did not exhibit significant deficits in long trace eyeblink conditioning. Moreover, involvement of the PFC in the ET subject population in the advanced stage may have been not severe enough to cause deficits in trace conditioning.

Acquisition of trace eyeblink conditioning with long trace intervals is dependent on the awareness of the stimulus contingencies [39]. In the present study, most of the controls (16 of 18) and more than half of the ET subjects (11 out of 18) showed awareness. CR-incidences tended to be higher in aware compared to unaware subjects of both groups. Differences were not significant, corresponding to findings of a previous study in cerebellar patients using the same trace paradigm [27]. In the present study, more out of the ET subjects than controls remained unaware, only two of the controls were classified as unaware. Four of the ET subjects with accompanying cerebellar signs were aware and two were unaware, respectively. Therefore, awareness seems not a critical factor to explain the tendency of lower conditioning rates in this subgroup. It has to be noted that groups were small and no clear conclusion on effects of awareness could be drawn.

Extinction is an active process which involves the cerebellum as well as the hippocampus and PFC [60]. The cerebellum has been shown to play a role in the extinction of delay conditioned eyeblink responses in rabbits [61, 62], though there may be differences concerning delay and trace eyeblink conditioning. In cerebellar patients and controls, extinction of CRs was previously observed using a long as well as a short trace interval. Similar to the present findings the variability across extinction blocks was high [27].

This study had strengths and limitations. A well-established trace eyeblink conditioning paradigm was used in a sizeable group of participants. ET was diagnosed following recognized criteria [36] and participants did not take centrally acting medication, which could impede eyeblink conditioning. Analysis of eyeblink conditioning was performed blinded in respect to the participant's group and subgroup. There was, however, no correlation with imaging data and no neuropsychological assessment was done.

Conclusion

Long trace eyeblink conditioning was not impaired in the whole group of ET subjects we studied compared to healthy controls. The patients with clinical cerebellar signs showed lower, but not statistically significant impaired long trace eyeblink conditioning. Areas of the PFC which are involved in trace eyeblink conditioning appear less affected in ET. To contribute to a better understanding of brain structures involved in ET, future studies should include a larger group of patients in all stages of ET using different eyeblink conditioning paradigms and shorter trace intervals.

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Author Contributions KS: Research project; Statistical Analysis; Manuscript Preparation.
SJO: Research project; Statistical Analysis; Manuscript Preparation.
MK: Research project; Manuscript Preparation.
DT: Research project; Statistical Analysis; Manuscript Preparation.
MG: Research project; Statistical Analysis; Manuscript Preparation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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