The final blink: intact cerebellar associative learning in isolated dystonia

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Abstract

Impaired eyeblink conditioning is often cited as evidence for cerebellar dysfunction in isolated dystonia. However, the results from individual studies are conflicting and underpowered. This collaborative project collated all published data and systematically re-examined the contribution of the predictors dystonia and its subtypes within a statistical model which controlled for the co-variates age and sex.

Original neurophysiological data were shared and a sex and age matched control group were collected (dystonia n=52, controls n=50). Two raters blinded to participant identity rescored all recordings (6732 trials). After high inter-rater agreement was confirmed, mean conditioning per block was entered into a mixed repetitive measures model to evaluate the influence of sex, age, dystonia subtype (focal hand dystonia, cervical dystonia, DYT-TOR1A and DYT-THAP1) and clinical features such as tremor.

There was a wide range of conditioning behavior across individuals in both groups. Young age (p=0.031) was associated with higher conditioning. All dystonia versus controls showed no difference in conditioning (p=0.517). Analysis of dystonia subgroup, with age and sex as co-variates, showed that conditioning in cervical dystonia, focal hand dystonia and DYT-TOR1A was comparable to controls. DYT-THAP1 was characterized by high levels of conditioning. Clinical features such as tremor did not significantly influence conditioning. Sample size estimates for future work are provided based on the variance observed.

Eyeblink conditioning, a proxy for cerebellar function, appears intact in the subtypes of isolated dystonia examined. Precise mechanisms for how the cerebellum interplays mechanistically with other key neuroanatomical nodes within the dystonic network remains elusive.
Introduction

Dystonia is a hyperkinetic movement disorder characterized by involuntary sustained muscle contractions which lead to twisting and repetitive movements or abnormal postures of the affected body part. Classically dystonia was considered a disorder of basal ganglia function. However, more recent research has pointed to abnormalities of multiple brain regions and within this wider sensorimotor network the cerebellum is thought to be a key node.

Eyeblink conditioning is a cerebellar dependent experimental paradigm that can been used to study cerebellar function. Pavlovian in its design, a biologically potent stimulus (the unconditioned stimulus) is paired with a previously neutral stimulus (the conditioning stimulus). Experimentally in humans the unconditioned stimulus is typically supraorbital nerve stimulation causing a blink (the unconditioned response). The conditioning stimulus (usually an auditory tone) always occurs before the unconditioned stimulus and with time the tone alone yields a conditioned blink (Fig 1). Informatively, eyeblink conditioning can be adapted and tested across species and elements of the paradigm can be specifically mapped to the function of individual cells within the cerebellar circuitry. For example, the magnitude of the conditioned eyeblink responses correlates on a trial-by-trial manner to the level of firing of Purkinje cells (magnitude of simple spike suppression). Studying eyeblink conditioning is therefore an attractive experimental method with the chance to offer real insights into cerebellar dysfunction in disease.

Impaired eyeblink conditioning is widely cited as evidence of functional cerebellar disturbance in the dystonia literature, yet, collectively findings across studies are conflicting. A preliminary study examined a mixed group of cervical dystonia and focal hand dystonia and found that both had lower rates of conditioning. Subsequently, genetic subtypes were evaluated and normal conditioning was recorded in DYT-TOR1A (DYT1) and higher levels of conditioning in DYT-THAP1 (DYT6). However, the young age of the DYT-THAP1 group was noted and when compared to an age-matched control group levels of conditioning were similar. A later study examined a larger group of patients with cervical dystonia. Conflicting with the earlier study, levels of conditioning in cervical dystonia were normal and only low with co-existing head tremor. In summary, high, normal and low levels of conditioning have been observed across the isolated dystonia subtypes.

There are many potential reasons for the differences observed across studies. Firstly, levels of eyeblink conditioning in the healthy population are highly variable. Clinical studies are therefore vulnerable to inconsistency if underpowered and with small numbers of participants uncontrolled covariates such as sex and age can confound. Much recent research also points to the subtypes of...
isolated dystonia having unique etiologies and/or neuroanatomical substrates and thus uniform patterns of conditioning behavior across the subtypes are not necessarily anticipated\(^\text{13}\).

This current study capitalized on an unusual opportunity in isolated dystonia by recognizing that it was possible to combine data from individual studies into a ‘mega’ dataset. Our motivation was to better answer three questions: (i) Is isolated dystonia as a group associated with reduced conditioning? (ii) Does eyeblink conditioning distinguish between subtypes of isolated dystonia? (iii) Does tremor or other clinical features associated with dystonia influence conditioning? Importantly, all of these questions were probed within a statistical model which included age and sex as co-variates as young age and female sex are associated with higher levels of conditioning.

**Figure 1. Eyeblink conditioning in humans.** The unconditioned stimulus consists of electrical stimulation to the supraorbital nerve at 800ms which causes a blink (unconditioned response). The conditioning stimulus is an auditory tone that starts at 400ms with a duration of 400ms. With repeated pairings of the US/UR and the CS a conditioned blink response emerges prior to the delivery of the US. The lower panel shows rectified electromyographic traces from a single trial early with no conditioned response and a later trial when conditioning has developed.
Method

PubMed and MEDLINE were searched with the terms dystonia AND ((eyeblink conditioning) OR (associative learning)) and identified three studies for inclusion (Fig 2). Demographic and neurophysiological data files were collated and shared between investigators. All followed the same experimental paradigm and had been tested at University College London between the period 2009-2019.

For each participant demographic (age, sex) and, if dystonia was present, clinical details were noted (subtype of dystonia, duration of symptoms, severity of dystonia, presence of tremor, medication and botulinum toxin use). Severity of dystonia was described as a percentage relative to the total of the scoring scale used for each subtype of dystonia. As many of these studies had been compared to a historical numerically small control group we collected further control data. This study had been approved by the local ethics committee and written informed consent was obtained.

The eyeblink conditioning paradigm was identical across studies (Fig 1). Eye blinks were captured by surface electromyographic (EMG) electrodes over right and left orbicularis oculi muscles. Signal was amplified (gain 2000), bandpass filtered (20Hz–3000Hz) and digitized (5kHz). The unconditioned stimulus (US) was an electrical stimulus (200µsec, 5 times sensory threshold) to the supraorbital nerve at the termination of the conditioning stimulus which elicited a blink reflex (UR). Supraorbital nerve was applied with chloride disc surface electrodes (cathode: right supraorbital foramen, anode: 2 cm above). The conditioning stimulus (CS) was a loud (~70dB), 2000Hz, 400ms tone played via binaural headphones. Repeated pairs of CS and US yielded conditioned blink responses (CRs) occurring before the US (figure 1b). Experimental structure was as follows: conditioning blocks consisted of six blocks of eleven trials (9 x CS-US, 1 x US and 1 x CS). US-only detects rates of spontaneous blinks and CS-only confirms that CRs are acquired independent of US. EMG bursts were regarded as CRs if latency was >200ms after onset of CS but before the US. The seventh block measured extinction with eleven CS-only trials in which EMG bursts occurring 200–600ms after the CS were considered CRs.

Data were analyzed offline analysis using Cambridge Electronic Design (CED) 1401 hardware and Signal software (CED). Every trial (11 trials x 6 blocks x 102 participants = 6732 trials) was assessed for the presence of a conditioned response by two assessors blinded to the participants identity (AS and LR). One participant with dystonia was excluded due to irregularities with the experimental paradigm. In order to assess the agreement across assessors the concordance correlation coefficient (CCC) was calculated for each block of the experiment (Fig 3):
The concordance correlation coefficient is given by:

\[
\text{concordance correlation coefficient} = \frac{2 \sigma_{12}}{\sigma_1^2 + \sigma_2^2 + (\mu_1 + \mu_2)^2}
\]

where \(\sigma_{12}\) is the covariance between the two distributions and \(\sigma_1^2, \sigma_2^2, \mu_1, \mu_2\) are the variance and mean of distribution \(x\) respectively. This quantifies the similarity between distributions based on their covariance and average values; CCC values >0.7 are considered reasonable\(^{14}\).

We used a mixed-effects model for repeated measures (MMRM) to analyze conditioning outcomes\(^{15}\). The model used variables to estimate the effects associated with age, sex, patient group, experimental block, and the intersections of age, sex and group with block. Treating the first assessment as a baseline, exploratory data analysis revealed that post-baseline outcomes had approximately constant variance and serial correlation, irrespective of the lag. This suggested that a compound symmetric covariance structure was appropriate and so patient-level intercepts were used in the MMRM. Model checks revealed that the residuals were approximately independent, homoskedastic, and normally distributed, and that patient-level intercepts were approximately normal. Tests of difference between subgroups were made using likelihood ratio tests of nested models. Further fixed effects were added to this model to test the explanatory power of clinical variables such as tremor.

Sample size estimates for the minimum size of each arm were made by extrapolating from the variability of conditioning levels observed in the final block 6.

\[
\text{Sample size} = \frac{2 \sigma^2 (Z_a + Z_\beta)^2}{d^2} = \frac{2 \sigma^2 (1.96 + 0.84)^2}{d^2} = \frac{16 \sigma^2}{d^2}
\]

where \(\sigma\) is the standard deviation, \(d\) is the effect size and \(Z\) constants are calculated on the basis of desired significance level (type 1 error, alpha) and power (probability of rejecting null hypothesis when false). Constants were defined for a standard two sided comparison with a significance level of 5\% and power of 80\%.\(^{16}\) All results are given to three significant figures.
Figure 2. Study and participant identification  

A: Eight studies that have examined eyeblink conditioning and dystonia were identified. Studies were excluded if they did not examine isolated dystonia (n=3): myoclonus dystonia or fixed/functional dystonia.12-14 Another paper did not contain novel data in isolated dystonia (n=1).15 Neurophysiological data for a final study were not available and this would have contributed eight further patients with cervical dystonia, same historical control group.16  

B: the number of patients and controls for each study are noted. Further new control data were collected for this study.

| Study   | Dystonia group                     | Control group  |
|---------|------------------------------------|----------------|
| Teo     | focal hand (n=5)                  | novel (n=8)    |
|         | cervical (n=7)                    |                |
| Antelmi | cervical (n=24)                   | historical (n=8)|
| Sadnicka| DYT-TOR1A (n=11)                  | historical (n=8)|
|         | DYT-THAP1 (n=5)                   | novel (n=8)    |
| Current study | no novel                        | novel (n=36) |
| Total   | all dystonia (n=52)               | all control (n=50) |
Figure 3 Rater reliability A: Two assessors rated every trial of every participant whilst blinded to the participants’ identity. The concordance correlation coefficient was excellent across all blocks (values >0.7 marked with solid line are considered reasonable) B: A comparison of score for the final block revealed an average difference of <1. C: In some participants, assessors had different thresholds for labelling conditioned responses as the ‘difference between scorers’ had a unilateral tail (negative on this plot).

Results

In total data for 52 individuals with dystonia (28 females, mean age 56.0 years (SD 15.1 years)) and 50 controls (27 females, mean age 55.2 years (SD 15.5 years)) were available for analysis. High inter-rater agreement of scores across all blocks of conditioning assessed was found suggesting that levels of conditioning were reliably identified from data (Fig 3). The mean % conditioning value for each block across the two assessors was subsequently used for data visualization and statistical analysis.

A wide range of conditioning behavior was observed in both controls (Fig 4A) and patients with dystonia (Fig 4B). Some subjects did not exhibit any conditioned responses (0% conditioning across all blocks) whereas others had conditioned responses counted in early blocks. A plot of mean rates of conditioning by group (control, all dystonia, Fig 4C) demonstrated that the level of conditioning were similar across groups (p=0.517) and the two groups had very similar demographic features in terms of age (Fig 4D, t (99)=0.0901, p=0.928) and sex (Fig 4E, equivalent proportions in each group).

Despite the variability in conditioning across individuals, age significantly influenced the levels of conditioning and younger age was associated with higher conditioning (Fig 5A, p=0.031). On average, women also had higher rates of conditioning; a finding that was not significant in this study but has been confirmed in previous studies (Fig 5B, p=0.143).
We then analyzed the different subtypes of dystonia: focal hand dystonia, cervical dystonia, DYT-TOR1A and DYT-THAP1 dystonia and compared conditioning behavior to controls. Here the influence of age and sex were treated as covariates and in Fig 6A we show behavior across subgroup adjusted for these variables. There was some segregation by subgroup. Patients with DYT-THAP1 dystonia showed conditioning scores 26 units higher than controls, on average (p=0.049). Conditioning levels in focal hand, cervical and DYT-TOR1A dystonia subtypes were similar to controls, with mean differences < 10 units (all p-values > 0.5).

Finally, we reviewed key clinical parameters to assess whether they influenced levels of conditioning. We examined disease features (severity, duration, tremor) and active treatments at the time of study (botulinum toxin injections and/or the medications trihexyphenidyl and clonazepam). In the dystonia group, the presence of tremor did not affect conditioning (p=0.943, Fig 6B), nor did any of the other disease features or active treatments at the time of study (all p-values > 0.5).

Based on the variance we observed in the final conditioning block we performed sample size calculations as a guide for future studies. The standard deviation in controls was 35.3%, standard deviation in dystonia 32.7%, with a mean standard deviation of 34.0%. Therefore in order to detect a difference in conditioning of 5% a sample size of 739 per group is required. A difference of conditioning of 10% requires 185 per group, 20% requires 46 per group and 30% requires 21 per group.
**Figure 4. Dystonia and controls have similar patterns of conditioning.** Range of conditioning across individuals is demonstrated in three dimensional plots for controls (A) \((n=50)\) and dystonia (B) \((n=51)\). % conditioning for each block is marked by a filled circle and connected by a thin line of the same color. Block number 1-6 is shown on the x-axis, participant number on the y-axis and the % conditioning plotted on the z-axis. For each group the profile of responses is sorted according to the % conditioning achieved by the final block (participant one lowest level of conditioning). C: Group mean and shaded standard error are shown (control=green, dystonia=orange). All individuals \((n=101)\) are plotted in grey in the background. The groups were matched for age (D) and sex (E).
**Figure 5. Young age and female sex is associated with higher levels of conditioning**  
A: For the purpose of illustration data are split into three age categories to show that younger age is associated with higher levels of conditioning (<=40 years yellow, 41-60 orange, >60 pink).  
B: Increased conditioning in females has been shown in previous studies but was not statistically significant in this study (female green, male blue). In both plots, all participants are plotted in grey in the background to illustrate degree of variability across individual participants.
Figure 6 Evaluation of dystonia subtype and tremor with covariates age and sex. On the y-axis model counterfactuals are plotted, reflecting the model's best estimate for conditioning behavior across block for each age/sex category. A: Focal hand, cervical and DYT-TOR1A dystonia show similar conditioning behavior to controls. DYT-THAP1 has high levels of conditioning across categories. B: Tremor did not influence the levels of conditioning.
Discussion

This current study capitalized on an unusual opportunity in isolated dystonia to combine data from individual studies into a ‘mega’ dataset. The inter-rater agreement for scoring the dataset was very good suggesting that levels of conditioning were reliably identified. We evaluated three questions within a statistical model which included age and sex as co-variates: (i) Is isolated dystonia as a group associated with reduced levels of conditioning? (ii) Do levels of conditioning distinguish between subtypes of isolated dystonia? (iii) Does tremor or other clinical features associated with dystonia influence conditioning?

Firstly, grouping all subtypes of dystonia into a single dystonia group did not reveal any differences in the level of conditioning observed (Fig 4). This is in concordance with observations from previous studies that used subsets of this data, and found eyeblink conditioning to be either low, normal or high in comparison to controls. Thus, in response to our first question, eyeblink conditioning seems not to be a marker for isolated dystonia as a group. The full range of conditioning responses in both healthy controls and patients with dystonia was also better appreciated. Despite such variability we confirmed that age influences rates of conditioning (Fig 5). Age-related effects on behavioral performance and neural activity have been consistently demonstrated in human and animal studies across a number of different conditioning paradigms and age-associated functional and morphological change within the conditioning network (such as the cerebellum and hippocampus) are thought to underpin these effects. Potential reasons for increased conditioning in females are also multiple such as sexual dimorphism of brain development due to sex hormones.

As different subtypes of isolated dystonia may have distinct etiologies and/or neuroanatomical substrates our second aim was to analyze subgroups of dystonia within a model that controlled for age and sex as co-variates. The data suggested that focal hand dystonia, cervical dystonia and DYT1 dystonia had normal levels of conditioning (Fig 6). The finding that the DYT-THAP1 group had relatively high levels of conditioning is preliminary, as there were only five participants. Overall, our results suggest that eyeblink conditioning, a proxy for cerebellar function is intact across the subtypes of isolated dystonia (+/- ‘hyper’ functioning in DYT-THAP1) which revises the conclusion of earlier reports with a subset of this data which may have erroneously concluded that lower rates of conditioning are a feature of focal dystonia.

What does intact associative conditioning reveal about the pathophysiology of dystonia? Eyeblink conditioning examines associative learning and different elements of the paradigm have been mapped to the firing of individual cells within the cerebellar circuitry. The homogenous nature of the cerebellar micro-circuitry has given rise to the idea that the cerebellum performs the same function at
the algorithmic level across diverse domains (‘universal transform’). In such a scenario the broad functional heterogeneity of different cerebellar regions across motor control, perception, language, and cognition is primarily determined by connectivity patterns to cortical and subcortical targets rather than the micro-circuitry\textsuperscript{23}. If we interpret our results in line with the ‘universal cerebellar transform’ theory, most simply, confirmation of the integrity of the general micro-circuitry and plasticity of the cerebellum via eye-blink conditioning, could be seen as a sample of global cerebellar health across all functional domains. However, an alternate viewpoint is that the complete range of cerebellar function entails multiple specialized algorithms across different cerebellar regions\textsuperscript{23}. In this scenario the same underlying circuit implements functionally distinct algorithms subserving different functional modules and tasks (‘multiple functionality’)\textsuperscript{23,24}. Correspondingly, it may be that eyeblink conditioning is not tapping into the specific functionality at play in dystonia pathophysiology, a specific algorithm which underwrites the dystonic phenotype.

In general terms, the finding of normal eyeblink conditioning in people with dystonia does reveal that there must be a relative subtlety to any cerebellar involvement in dystonia. Neurodegenerative disorders such as spinocerebellar ataxia (type 6) affect the cerebellum relatively uniformly and are associated with broad functional impairments ranging from delayed eyeblink conditioning and force adaptation in reaching\textsuperscript{24-28} and to abnormalities in predictive timing in sequence control\textsuperscript{29} and cognition\textsuperscript{30,31}. More circumscribed cerebellar deficits, e.g. caused by cerebellar stroke, can produce highly task-specific impairments, such as abnormal adaptation to force but not to visuo-motor adaptation and vice versa\textsuperscript{32,33}. Overall, normal eye blink conditioning does not support a widespread cerebellar deficit. This is in keeping with the clinical syndrome of dystonia as a dynamic and selective abnormality of posture/movement and an absence of overt cerebellar signs.

How do these results relate to the network theory for dystonia etiology in which the cerebellum is a key neuroanatomical node? Deep cerebellar nuclei and cerebellar output tracts are increasingly targeted therapeutically with techniques such as deep brain stimulation and therefore our data are important as we strive to develop accurate mechanistic models in parallel. While the cerebellum is a component part of the “dystonic network”, it is still not really known how the cerebellum interplays with other implicated neuroanatomical nodes in the genesis of the genetic/idiopathic isolated dystonia in humans. In the future, we are likely to lean on techniques which evaluate multiple nodes within the network simultaneously in order to gain broader, less one-dimensional insight. Indeed, attempting to identify roles for individual nodes with paradigms that aim to examine a single node may not be fruitful. Synergistic and/or compensatory interactions between nodes may define novel mechanisms in dystonia.
In response to our final question, tremor and other clinical features such as severity or medications did not appear to influence conditioning (Fig 6). Statistically, when controlling for the covariates age and sex we did not replicate findings in a previous study in which low eyeblink conditioning was found in the dystonic tremor group\(^\text{10}\). Similar to dystonia, a network model of the pathophysiology of tremor is proposed in which the olivo-cerebellar system is thought to be critical. Patients with essential tremor (some with cerebellar signs) have been studied in a two-day paradigm in which ten blocks of ten CS-US pairings were delivered using an air puff rather supraorbital nerve stimulation as the US\(^\text{34}\). In this study, a mean total difference of 20% in conditioning was noted on the two consecutive days across groups. Yet, a study by the same authors has revealed that another type of paradigm, long trace eyeblink conditioning (CS and US separated by an interval) conditioning was preserved in essential tremor\(^\text{35}\). Long trace version of associative conditioning is thought to be more reliant on the prefrontal cortex and hippocampus (stimulus interval is likely to require a memory trace to encode the association). However cerebellar cells show similar responses across paradigms and thus findings in essential tremor require further studies to establish the fidelity of the original findings\(^\text{35}\). Of note, low levels of cerebellar conditioning do not appear to be a marker of any tremor syndrome, as tremor associated with neuropathy is thought to be associated with normal levels of eyeblink conditioning\(^\text{36}\).

In the wider movement disorders literature, other patient populations have also had eyeblink conditioning tested. For example, data support the idea that eyeblink conditioning is intact in Parkinson’s disease\(^\text{37}\). In myoclonus-dystonia the first multicenter study demonstrated normal associated learning (n=11, conditioning 10-20% higher in myoclonus-dystonia, extinction/forgetting of response reduced)\(^\text{17}\). By contrast, a later study examining this same disorder found reduced eyeblink conditioning (n=17, conditioning 20% lower in myoclonus-dystonia)\(^\text{18}\). Thus results across studies in many patient groups are mixed and such differences may reflect low number of subjects in a paradigm that is highly variable in health. Indeed, our sample size calculations reveal large studies are required to confidently assess for changes in eyeblink conditioning behavior as most individual studies in the literature are under-powered. For example to detect a 20% difference in the level of conditioning across groups a minimum of 46 participants per group is required.

Limitations of our study are that the numbers of the subgroups of dystonia remained small for focal hand dystonia and DYT-THAP1. Nonetheless we extended previous conclusions by modelling the co-variates age and sex and making comparisons to the large control group. Also, although identical methods, software and equipment are documented and reported by authors some of the reasons for variability of conditioning response is still likely to reflect non-biological influences such as differences in experimental technique across investigators.
In summary, eyeblink conditioning in isolated dystonia appears intact and clinical features of dystonia such as tremor did not significantly modulate the level of conditioning observed. Collaborative efforts such as this article allow larger number of patients to be evaluated in rarer diseases to provide more balanced estimates of disease effects over experimental paradigms. Normal eyeblink conditioning is against a global cerebellar learning deficit in isolated dystonia. Future studies are required to elucidate exactly how cerebellar involvement interplays with other key neuroanatomical nodes implicated in the dystonic network.
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