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Impairment of brainstem implicit learning paradigms differentiates multiple system atrophy (MSA) from idiopathic Parkinson syndrome

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ABSTRACT

Objectives: Learning as measured by eyeblink classical conditioning is preserved in patients with idiopathic Parkinson's disease, but severely affected in patients with progressive supranuclear palsy. We here sought to clarify whether procedural learning is impaired in multiple system atrophy, and whether it may be helpful for the differentiation of Parkinsonian syndromes.

Design: We investigated learning using (1) eyeblink classical conditioning with a delay (interstimulus interval 0 ms) and a trace (600 ms) paradigm and (2) a serial reaction time task.

Setting: Participants were recruited from academic research centers.

Participants: 11 patients with multiple system atrophy and 11 healthy controls.

Results: Implicit learning in eyeblink classical conditioning (acquisition of conditioned responses) as well as the serial reaction time task measures of implicit learning (reaction time change) are impaired in multiple system atrophy patients as compared to controls, whereas explicit learning as measured by the sequence recall of the serial reaction time task is relatively preserved.

Analysis: We hypothesize that the MSA patients' learning deficits are due to lesions of cerebellar and connected brainstem areas.

Conclusions: A retrospective synopsis of these novel data on multiple system atrophy patients and groups of idiopathic Parkinson's disease patients and progressive supranuclear palsy patients studied earlier suggests that eyeblink classical conditioning may contribute to the early differentiation of atypical Parkinson syndromes from idiopathic Parkinson's disease. This hypothesis should be tested in a prospective trial.

ARTICLE SUMMARY:

Article focus:

- We tested if the non-motor feature of procedural learning is impaired in patients afflicted with multiple system atrophy.
- We studied the eyeblink classical conditioning and a serial reaction time task in 11 MSA patients and matched control subjects.

Key messages:

- Blink reflex pathways were intact, but eyeblink conditioning was significantly impaired in MSA patients. By contrast, the serial reaction time task was difficult and inconclusive in these patients due to motor constraints impairing finger tapping.
- A retrospective comparison with previously studied groups patients with idiopathic Parkinson's disease or Progressive Supranuclear Palsy points to a putative role of eyeblink conditioning in distinguishing typical from atypical Parkinsonian disorders.

Strength and limitations:

- The study differentiates feasible and non-feasible assessments of procedural learning in multiple system atrophy.
- The comparison to other patient groups is clearly retrospective and needs to be validated by a prospective trial.

INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurodegenerative disorder characterized by an absent or poorly levodopa responsive Parkinsonism, cerebellar dysfunction and autonomic failure.[1] A predominantly Parkinsonian (MSA-P) and a cerebellar subtype (MSA-C) are recognized. Despite the development of consensus criteria,[2] the differential diagnosis between MSA and other hypokinetic rigid syndromes, such as idiopathic Parkinson's disease (IPD) or progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome, PSP), remains a clinical challenge.[3 4]

Recent years have seen an increasing focus on non-motor symptoms such as cognitive deficits in Parkinsonian disorders. Different patterns of cognitive impairment including deficits in executive function and learning abilities have been described.[5-9]

A well established task to study associative, procedural learning[10] is eyeblink classical conditioning (EBCC), which some regard as a model of implicit learning.[11] Previous studies have shown that learning as measured by EBCC is normal in patients with IPD, but, severely affected in patients with PSP.[12-14] The serial reaction time task (SRTT) is another established task for which the implicit measures of motor skill (reaction time and errors) were close to normal in IPD patients, but impaired in PSP patients, whereas sequence recall as measure of explicit learning were largely preserved in both groups.[12 14] We sought to investigate whether implicit learning deficits are specific for PSP or present in MSA as well, thereby comparing the clinical feasibility of SRTT and of EBCC in this patient group.

METHODS

Subjects

11 MSA patients were recruited from the outpatient clinics in Munich and Göttingen between 1999 and 2008 (**table 1**). The clinical diagnosis of "probable MSA" was established following consensus criteria.[2] 7 MSA patients were taking L-Dopa, two dopamine agonists (ropinirole, pramipexole), one amantadine, one budipine, one biperiden, another metixen, and two were taking antidepressants.

L-Dopa equivalent doses (LEDs) were calculated according to Tomlinson and colleagues[15] except for budipine, biperiden and metixen, where no conversion factor was given.

To rule out an immediate impact of medication on the patients' memory performance, the anti-parkinsonian medication was discontinued on the morning of the day of the study. MSA patients were compared with 11 age matched healthy control subjects (mean age 59.5±10.0 years, 6 male, 5 female), of which a subgroup was already involved in our earlier published study (numbers 2,3,5,6,8,9,11,12,14 according to Table 2 in[12]). All participants gave written informed consent; the research protocol was approved by the local ethics committee. Neither the patients nor the control subjects had any sign of cranial nerve impairment or auditory deficits in routine neurological examination.

Table 1

Pat Nr.	MSA Type	Age [year]	Sex	Duration [year]	L-Dopa response	LED [mg]	UPDRS Max=108	Cerebellar Max=4	Autonomic Max=5[f], 6[m]	Pyramidal Max=2	Hamilton Max=69	MMS Max=30
1	P	66	F	9	Poor	0+	50	0	1	0	11	27
2	P	69	M	4.5	Poor	125	20	0	2	0	20	30
3	P	73	M	8	Absent	255	16	0	3	0	15	28
4	P	59	F	1.5	Poor	125#	30	0	2	1	16	26
5	P	71	M	4	Absent	150	35	0	4	0	6	29
6	P	75	M	5	Modest	524	38	0	3	0	6	29
7	P	75	F	3	Poor	375	40	0	3	0	10	28
8	P	58	M	3	Poor	105#	18	0	1	0	2	30
9	C	64	M	2	Poor	900	69	2	2	0	22	27
10	C	56	M	2.5	*	0	5	3	1	0	16	28
11	C	60	F	8	*	0	30	3	3	1	14	26
Mean		66.0		4.6			31.9	0.7	2.3	0.2	12.5	28.0
S.D.		7.1		2.6			17.7	1.3	1.0	0.4	6.2	1.4

Clinical testing procedures

The Hamilton rating scale for depression[16] and the Mini-Mental state examination[17] were used to quantify the affective and general cognitive status, respectively, with pragmatic and established tests. Motor features in patients were evaluated with the Unified Parkinson Disease Rating Scale (UPDRS, part III).[18] Further clinical assessments are listed in table 1.

To investigate eyeblink function we used the blink reflex (BR) and its R2 recovery cycle detailed elsewhere.[12 19 20] In brief, a single electrical stimulation of the supraorbital nerve (duration: 0.2 ms) elicits an early unilateral (R1) and a late bilateral (R2) blink response. Paired-pulse supraorbital stimulation probes brainstem interneuronal excitability and yields an inhibition of the R2 of the second stimulus. We used interstimulus intervals (ISIs) of 100 ms, 300 ms and 600 ms on both sides. For EBCC we stimulated the side in which amplitudes and latencies were within or close to the normal range or arbitrarily the right supraorbital nerve if no side-differences were found.

EBCC-implicit learning

The procedures were virtually identical to and detailed in the earlier studies from our group.[12 14] In brief, an unconditioned stimulus, i.e. en electric pulse over the supraorbital nerve, invariably induces an eyeblink reflex. It is paired with a preceding conditioned stimulus, i.e. a tone, which by itself may elicit early startle responses (so called alpha blinks, within 200 ms after the tone). With repeated presentation of the tone and the electric pulse, a conditioned eyeblink response is expected to occur before the onset of the electrical stimulus. In the current study, the tone (conditioned stimulus, CS, 400 ms duration) was generated by a custom built sound generator (Department of Electronic Engineering, University of Goettingen) and presented via earphones (Cherry Inc., Japan) at an intensity perceived as very loud by each subject, usually 80 dB. A brief electrical stimulus to the supraorbital nerve eliciting a blink reflex served as unconditioned stimulus (UCS). We tested the EBCC with a two different interstimulus intervals between the end of the tone and the beginning of the electric pulse, either ISI 0 ms (delay paradigm) or 600 ms (trace paradigm), in randomized order. For each paradigm we administered six learning blocks with CS and UCS in trials 1-9, UCS only in trial 10 (to control for random blinks) and CS only in trial 11 (to test for an independent learning effect). To control for extinction of learned responses a seventh block consisted of 11 trials with CS only.[21] The intertrial interval was randomized between 10 and 30 seconds.

Electromyographic (EMG) activity was recorded using silver-silverchloride cup electrodes fixed with adhesive tape over the lower eyelid and over the ipsilateral temple.[12 14] EMG signals were fed into a recording device and filtered at 100 Hz and 5 kHz (SynAmps, Neuroscan Inc, Virginia, USA). To detect any ongoing muscular activity we recorded 400 ms before and 1600 ms after CS onset.

Serial reaction time task (SRTT)

The SRTT is established as a test of implicit learning.[12 23] Subjects were sitting in front of a computer screen, and were told that single asterisks would appear in one out of four positions on a computer screen. They were instructed to press a marked key on a computer keyboard that was underneath the position of the asterisk on the screen. The asterisks were presented in three random blocks (blocks 1, 2, 7) and four identical sequence blocks (blocks 3-6), in each of which a sequence of 10 elements (CBDABCDCBA) was presented 10 times. After each block subjects were asked to repeat the last 10 asterisk positions manually on the computer keyboard. We analyzed reaction time, errors and number of correctly repeated parts of the sequence. This test was difficult for many patients: Only 6 patients completed the test as required, one patient discontinued after block 1 and was excluded from the analysis. Two others discontinued after block 4, one after block 3. To enable some kind of statistical analysis, the result that these patients reached in their last sequence block was carried forward to the following sequence blocks, and the result of the second random block was assumed for block 7. One patient apparently responded with random typing to the letters presented and was therefore excluded from the analysis.

Comparison of MSA patients with PSP and IPD patients studied earlier

While clearly retrospective, we have taken the liberty to compare the MSA patients results obtained here with the group of PSP patients that we studied in 2001[14] and a subgroup of IPD patients studied in 1999 with identical methods (numbers 1-4 and 6-11 according to Table 1 in[12], selected to

match the current MSA group with regard to the disease severity according to UPDRS part III). Demographical data are cited in table 2. In figure 1, 2 and 4, data from these IPD and PSP patients are given in dashed lines.

Table 2

Nr.	group	Age [year]	Sex	Duration [year]	UPDRS Max=108	BDI Max=63	MMS Max=30	MDRS Max=144
1	C	57	m	-	-	2		144
2	C	60	f	-	-	9		142
3	C	50	m	-	-	0		141
4	C	64	f	-	-	0		142
5	C	58	m	-	-	1		138
6	C	73	m	-	-	6		134
7	C	49	f	-	-	0		143
8	C	45	m	-	-	1		144
9	C	53	m	-	-	1		142
10	C	73	f	-	-	11	30	
11	C	72	f	-	-	0	30	
Mean		59.5				2.6		
S.D.		10.0				3.6		
1	IPD	69	f	2	45	11		138
2	IPD	64	f	6	39	15		143
3	IPD	62	m	5	21	9		132
4	IPD	45	m	6	28	5		141
5	IPD	47	m	7	31	6		139
6	IPD	49	m	7	25	6		140
7	IPD	64	m	9	47	11		135
8	IPD	63	m	8	16	11		141
9	IPD	61	m	5	33	8		143
10	IPD	50	m	3	44	11		143
Mean		57.4		5.8	32.9	9.3		139.5
S.D.		8.7		2.1	10.7	3.1		3.7
1	PSP	54	m	2	22	6	30	127
2	PSP	69	m	9	34	0	28	110
3	PSP	65	f	2	44	13	28	107
4	PSP	57	f	3	30	35	30	135
5	PSP	66	m	2	30	13	28	135
6	PSP	59	m	6	50	4	22	100
7	PSP	68	f	4	43	50	18	116
8	PSP	63	m	5	54	*	23	112
Mean		62.6		4.1	38.4	17.3	25.9	117.8
S.D.		5.4		2.5	11.1	18.4	4.4	13.1

Data analysis

R2 latencies were measured off-line. For analysis of the blink reflex recovery cycle, we entered the R2 amplitudes of the second pulse normalized to the R2 amplitudes of the first pulse into a repeated measures analysis of variance (ANOVA) with “interstimulus interval” (100; 300, 600 ms) as within

subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) as between subject factor.[12 19 20] In the EBCC, EMG bursts were regarded as alpha-blinks, i.e. startle responses, or conditioned responses (CRs) if they occurred within the appropriate time window (alpha blinks: within 200ms after onset of tone (CS); CRs: within 200 ms before electrical stimulus (UCS)) and if their amplitude exceeded the baseline noise by at least 1.5 fold and reached at least 50 μV. For the tone-alone-trials we extended the time window until 300 ms after the end of the UCS to detect delayed CRs.[22] We analyzed the percentage of conditioned eyeblink responses and of alpha blinks with separate repeated measures ANOVAs with "block" (blocks 1-6, CS only block) as within subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) and "paradigm" (delay versus trace) as between subject factors. In addition, we repeated the ANOVAs for conditioned eyeblink responses with the individual average alpha blink rate across all seven blocks as covariate.

For SRTT, we analyzed reaction time, accuracy errors and retrieval using separate repeated measures ANOVAs with "block" (blocks 1-7) as within subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) as between subject factor. In all analyses, Mauchly's sphericity test was performed and Greenhouse–Geisser correction was applied when necessary. The level of significance was set at $p<0.05$. Post-hoc t-tests were Bonferroni-corrected. A correlation between two parameters was determined by calculating Pearson's correlation coefficient and was reported if it was higher than 0.75 or lower than -0.75. The results are given as mean values ± one standard deviation.

RESULTS

Rating scales

Details of age, sex, disease duration, L-Dopa response and rating scales of MSA patients are displayed in **table 1**. UPDRS scores for motor impairment placed the patients in an intermediately impaired range. The Hamilton depression score revealed mild depressive symptoms (mean 12.5±6.2 out of 69 points) and the Mini-Mental State scored between 26 and 30 points (mean 28.0±1.4)

indicating mild cognitive impairment in more than half of the patients. These results are comparable to the IPD and PSP groups reported earlier.[12 14]

Blink reflex pathways

Blink reflex responses in MSA patients showed normal latencies (ipsilateral R1: 11.6±1.0 ms, ipsilateral R2: 31.5±4.9 ms contralateral R2: 34.4±4.1 ms). An R2 recovery cycle could be obtained in all patients (**figure 1**) with no significant side difference between the ipsi- and contralatral R2 recovery. MSA patients showed significantly less R2 inhibition compared to the control group (repeated-measures ANOVA MSA-controls, effect of group, $F(1, 20)= 15.0$. $p=0.001$).

-- Please insert fig. 1 about here --

Conditioned eyeblink responses

All MSA patients showed few random blinks as assessed by the UCS only trials (3.0±6.7 % across both paradigms). The conditioned responses (CRs) were first analyzed for block 1-6 excluding the tone alone trials (see below). In either paradigm MSA patients showed significantly fewer CRs than the control group (**figure 2;** repeated-measures ANOVA MSA-control**,** effect of group, $F(1, 39)= 37.1$, $p<0.0001$; effect of block, $F(3.4, 39)= 7.0$, $p<0.0001$; interaction of group by block, $F(3.4, 266)= 3.325$, $p=0.017$, no main effect of paradigm). Adding the rate of alpha blinks as covariate to the ANOVA did not abolish the effect of group ($F(1, 38)= 31.5$, $p<0.0001$). These results were supported by a separate analysis of the tone alone trials (trial 11, block 1-6), in which the MSA group yielded an average number of CRs of 14±17 % in the delay and 12±17 % in the trace paradigm, which was significantly less than the control group with 73±23 % and 55±27 % of CRs respectively (ANOVA MSA-control, effect of group, $F(1,37)=59.1$, $p<0.0001$). There was again no main effect of paradigm, i.e. trace and delay paradigm did not differ from each other.

Considering the MSA patients only, there was no difference in the occurrence of CRs between MSA-P and MSA-C patients (ANOVA, effect of MSA subtype, F(1,17)=2.5, p=0.13).

-- Please insert fig. 2 about here -

Alpha Blinks

In either paradigm MSA patients tended to show less alpha blinks than controls (repeated measures ANOVA, effect of group F(1,38)=4.0, p=0.054; **figure 3**.). The mean percentage of alpha blinks across all blocks in MSA patients was 17.6±4.6 % in the delay and 14.4±4.1 % in the trace paradigm, for control subjects 31.5±11.1 % and 35.2±11.3 % respectively. There were significantly more alpha blinks in earlier blocks than in late blocks for both paradigms (effect of block, F(4.3, 163.7)=8.5, p<0.0001). Considering the MSA patients only, there was no statistically significant difference in the occurrence of alpha blinks between MSA-P and MSA-C patients ~~(repeated measures ANOVA, effect of MSA subtype F(1,17)=1.5, p=0.23)~~.

-- Please insert fig. 3 about here --

Serial reaction time task (SRTT)

Reaction time

MSA patients showed longer reaction times compared with controls (repeated measures ANOVA MSA-control, effect of group, F(1,18)=20.2, p<0.0001; and a trend for an interaction of group by block, F(1.52, 27.34)=2.77, p=0.10, **figure 4A**). In both groups reaction times decreased from block 1 to the sequence blocks 3, 4, 5 and 6 (effect of block**,** F(1.52, 27.34)=5.5, p=0.016). The reaction time increase from sequence block 6 to random block 7, which is considered ~~being~~ a measure of implicit learning, was significant in the control group only (t-test p<0.01; MSA p=0.1).

-- Please insert fig. 4 about here --

Accuracy errors

The average error rate of MSA patients across blocks was 19.7±4.2 %, which is significantly higher compared to controls with a rate of 2.6±0.8 % (repeated measures ANOVA MSA-control, effect of group, $F(1,18)=10.1$, $p=0.005$). In both groups error rates decreased from the first random to the sequence blocks (effect of block, $F(3.37, 42.66)=3.9$, $p=0.022$) and tended to increase between the last sequence block and the random block 7 without being significant.

Retrieval of sequence

There was no significant difference between MSA patients and controls in the measures of sequence detection (manual sequence retrieval, ANOVA MSA-control, effect of group, $F(1,18)=0.7$, $p=0.42$). Both groups detected an increasing amount of the sequence during the course of the experiment (ANOVA, effect of block, $F(3.58, 64.48)=31.0$, $p<0.001$). A small percentage of repetition was seen even before the sequence was presented, which indicates the baseline guessing rate (**figure 4B)**.

Correlation analyses for MSA patients

We did not find a significant correlation between the average number of CRs across block 3-6 (steady state) and the duration of disease, the Hamilton scale score, the MMST, the motor examination of the UPDRS, the cerebellar or autonomic impairment as assessed by the scores (see table 1) for either paradigm. The percentage of manual retrieval in the SRTT did not correlate with any of these parameters either.

Retrospective comparison of all patient groups (MSA, PSP, IPD) and the control group

In the blink reflex recovery cycle, the R2 inhibition was not different between the patient groups (figure 1). For the EBCC paradigms, the lack of eyeblink conditioning in MSA patients was similar to the PSP group and differed significantly from the increasing occurrence of CRs in IPD patients and controls (ANOVA, effect of group, $F(3,63)=23.2$, $p<0.0001$; interaction of group by block, $F(11.1, 233.0)=3.6$, $p<0.0001$, post-hoc t-test with Bonferroni correction; figure 2). Adding the rate of alpha blinks as covariate did not abolish the effect of group ($F(1, 32)= 16.7$, $p<0.0001$). Also in the tone alone trials, MSA and PSP patients showed fewer CRs than the IPD and control groups (ANOVA, effect of group, $F(3,64)=19.0$, $p<0.0001$; interaction of group by block, $F(15,320)=1.8$, $p=0.04$). MSA and PSP groups both showed fewer alpha blinks than IPD patients and controls (ANOVA, effect of group, $F(3,61)=3.5$, $p=0.02$; interaction of group by block ($F(12.73, 259.0)=2.0$, $p=0.025$; see figure 3). However, the post-hoc t-test analysis indicated these differences to be non-significant.

For the differentiation of MSA and PSP from IPD we considered the mean percentage of CRs in the steady state part of the EBCC learning curve (blocks 3-6). The mean percentage of CRs allows a non-overlapping separation of the MSA and PSP groups from IPD in the trace paradigm with a cutoff at 26% (**figure 5).** In the delay paradigm, the separation between groups was less complete. Whereas all PSP patients exhibited less than 26% of CRs, the fraction of MSA patients with values equal or below 26% was 0.91. This corresponds to sensitivity values for the separation of PSP and MSA from IPD of 100% and 91%, respectively. If the specificity is defined as the fraction of IPD patients with CRs values above 26%, the specificity in the delay paradigm is 75%. As reported above, the sensitivity and specificity values for the trace paradigm are 100%. The slightly better performance of IPD patients[13] as compared to controls was not significant.

-- Please insert fig. 5 about here --

In the SRTT, MSA patients showed significantly longer reaction times than the IPD patients, but no significant difference compared to the PSP group (ANOVA: effect of group F(3,30)=7.4, p=0.001; see figure 4). With regard to the error rate, MSA patients performed again very similar to the PSP patients, who showed 19.5±1.8 % accuracy errors, but significantly worse than the IPD patients (error rate 4.8±1.7 %; ANOVA, effect of group, F(3,32)=6.1, p=0.002). The sequence recall measurements revealed no statistically significant differences between groups.

DISCUSSION

The differentiation of IPD and atypical Parkinsonian syndromes (MSA, PSP) constitutes a challenge for neurologists, as the motor symptoms often present very similarly, in particular in the early stages. Additional markers such as imaging have been evaluated,[24 25] but these provide ~~still~~ insufficient sensitivity values or are technically challenging. In addition, macroscopically discernible structural changes as detectable by MRI are likely to occur some time after functional loss has begun. Therefore functional tests might be better suited because they reveal deficits before discernible structural changes occur. In this study we focus on the differential leaning abilities tested by eyeblink conditioning (EBCC) and a serial reaction time task (SRTT). First, the results of the MSA patients will be discussed, followed by a comparison with PSP and the putative impact for differentiation from IPD.

The MSA patients showed severely impaired implicit learning in the trace as well as in the delay eyeblink conditioning paradigm, with standard deviations in the range of other studies,[10 26] whereas non-learning associated blink reflex latencies as indicators of oculomotor pathways were normal.

Histopathological alterations in both subtypes of MSA include the substantia nigra, putamen, descending and ascending fiber tracts of the motor system, olivary and pontine nuclei, brainstem-cerebellar circuits as well as cerebellar structures (hemispheres and vermis).[27-29] This has been confirmed *in vivo* by diffusion tensor imaging of white matter microstructure.[30] We suggest that

damage of cerebellar structures and associated brainstem-cerebellar circuits are responsible for the failure of EBCC learning in MSA patients. This assumption is supported by the findings of impaired EBCC in patients with cerebellar damage,[22 31-33] positron-emission tomography (PET) measurements in healthy humans showing changes in glucose metabolism in the cerebellum and pons during EBCC[21 34] as well as in experiments studying the influence of selective pharmacological blockade of cerebellar input on EBCC in rabbits.[35] Most patients in our study were clinically characterized as MSA-P and not MSA-C. However, the failure in blink reflex acquisition in both groups points to a subclinical cerebellar involvement in MSA-P, which is in accordance with the histopathological studies.[28 29] EBCC therefore seems to detect cerebellar involvement at a subclinical stage.

In addition to the cerebellum, several studies indicate that acquisition of CR in the trace paradigm depends on forebrain areas such as the hippocampus or prefrontal cortex, in particular for longer interstimulus intervals.[36 37] In our study, the failure of CR acquisition in MSA patients was slightly more pronounced in the trace compared to the delay paradigm. Therefore, lesions of the frontal lobe, which have been suggested by neuropsychological testing[6 38] and confirmed histopathologically in a variety of MSA cases,[39 40] may have contributed to impaired EBCC acquisition in the trace paradigm.

An alternative explanation that was brought up by an anonymous reviewer is that the tone may be a less salient CS to the MSA patients than to the control group. The reduced number of alpha blinks would support this assumption. Following that very elegant line of thought, the EBCC group difference between MSA patients and control subjects would have to do less with implicit learning and more with responsiveness and associative processes related to external stimuli. While this may have some relevance, adding the number of alpha blinks to the ANOVAs on conditioned eyeblink responses did not abolish the between-group differences.

In the SRTT the MSA patients showed severe impairment in terms of prolonged reaction time, high error rate and, in some patients, early discontinuation due to fatigue and exhaustion. In contrast

to the control group they showed no significant reaction time increase between block 6 (random) and 7 (sequence) and this is indicative of implicit learning deficits. On the other hand they showed good performance on the parameters of sequence recall (explicit learning). This preservation of SRTT explicit learning parts may be explained by the relative preservation of posterior association (temporal and parietal) cortex and hippocampus in MSA. However, the validity of the SRTT learning results is limited by the discontinuation of patients and our "last observation carried forward approach" (see Methods). In addition, the patients' motor impairment, which may interfere with the motor part of the task, and the fact that sequence learning and movement preparation seem to share similar attentional and working memory resources[41] have to be considered. Therefore the SRTT seems to be inappropriate to assess learning abilities in MSA patients. This is in contrast to the EBCC, which is independent of the motor performance of patients. Furthermore, EBCC circuits are located anatomically closer to the affected brainstem regions.

With all the limitations of such a retrospective comparison of data acquired in different patient groups by the same authors, the implicit learning impairment in MSA patients as clearly revealed by the EBCC and with some limitation by the SRTT parallels the previously reported findings in PSP patients.[14] Interestingly, MSA and PSP are characterized by different histopathological alterations, α-synuclein positive inclusions versus tau-positive aggregations, which lead to presume different pathophysiological mechanisms. However, the common involvement of cerebellar structures in both diseases[27 42] seems to be responsible for the clinical phenomenology independent of the cellular mechanism.

In contrast to MSA and PSP, IPD patients show normal[12] or even enhanced[13] acquisition of conditioned responses in EBCC. EBCC may therefore contribute to distinguish IPD from these atypical Parkinsonian syndromes. As the development of cerebellar and related neuropathology in MSA or PSP often occurs prior to or even without clinical manifestation,[29 42] we propose impaired EBCC particularly in the trace paradigm as an early indicator of neurodegeneration of areas beyond those typically affected in IPD. However, as the retrospective synopsis is clearly limited, the pivotal

questions of whether EBCC can serve as predictor for the development of typical or atypical disease and whether EBCC is a useful addition to imaging techniques in establishing an early differential diagnosis are unanswered yet and require further prospective investigation.

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Contributors

All authors listed above fulfill all three International Committee of Medical Journal Editors (ICMJE) guidelines for authorship which are (1) substantial contributions to conception and design, acquisition of data or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be published. MSo, CT and WP conceived the study. MSch, FvL and MSo were responsible for obtaining the data. FvL and MSo conducted the data analysis. All authors contributed to decisions on the interpretation of results. FvL and MSo contributed to the drafting of the manuscript. CT and WP were responsible for editing and providing guidance on the paper. All authors were responsible for critically revising the paper. All authors approved the final version of the manuscript prior to submission.

Competing interests None

Ethics approval Ethics committee of the Medical Faculty of the University of Goettingen.

Data sharing statement There are no additional data available.

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Legends to tables and figures

Table 1: Characteristics of the MSA patients. Type: Parkinsonian (MSA-P) or cerebellar (MSA-C) predominance; LED = L-Dopa equivalent dose, + indicates additional budipine medication, # additional anticholinergic medication; UPDRS = Unified Parkinson's disease rating scale, motor examination only (high number of points indicates high disability); MMS= Mini Mental State (30 points are normal, ≤26 is usually considered as cognitive impairment). Cerebellar impairment was evaluated for ataxia (arm, leg), saccades and intention tremor (finger-nose test), autonomic impairment for postural faintness, syncopes, urinary incontinence or retention, faecal incontinence, and impotence (males only). Pyramidal tract impairment was scored for hyperreflexia and Babinski sign. Each item was scored if present with 1, otherwise 0. In the Hamilton scale a low score indicates few depressive symptoms. *not investigated.

Table 2: Characteristics of controls, IPD and PSP patients in part taken from earlier publications.[12] [14] Dementia had been ruled out using the Mattis Dementia Rating scale (MDRS)[43 44], where higher scores out of a maximum of 144 indicate better performance, with a cut-off ≤123 considered as cognitive impairment[45]. Depression had been assessed using the Beck Depression Inventory (BDI), where higher scores out of a maximum of 63 points indicate a more severe depressed state, and a score of 15 is regarded as cut off for a self report of mild depression.[46 47] *not investigated. The MDRS was not available at the German study sites.

Figure 1: Blink reflex recovery cycle with interstimulus intervals of 100, 300 and 600 ms. In MSA patients, the inhibition of the ipsilateral R2 response to the second stimulus is weaker than in the

control group. Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 2: Conditioned eyeblink responses in two different paradigms: left, delay (interstimulus interval 0 ms), right, trace paradigm (interstimulus interval 600 ms). In both paradigms, the number of conditioned responses was significantly lower in MSA and PSP patients than in the control and IPD groups indicating impaired implicit learning. CS, conditioned stimulus (tone), UCS, unconditioned stimulus (electrical stimulation to the supraorbital nerve). Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 3: Occurrence of 'alpha-blinks'. These bursts are a startle reaction to the tone (CS) and are less frequent in MSA and PSP patients than in the control and IPD groups. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Data are indicated as average value and single standard deviation and were pooled for both paradigms.

Figure 4: A Reaction time in a serial reaction time task (SRTT). An implicit learning effect is indicated by the reaction time increase between the last sequence block (6) and the following random block (7). **B** Explicit learning in the SRTT was tested after each block by manual retrieval of the sequence (repetition of the last 10 key presses) and revealed no significant difference between groups. Data are indicated as average value and single standard deviation. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Asterisks indicate a significant difference for the comparison of blocks 6 and 7 ($p<0.05$).

Figure 5: Overview of the mean percentage of conditioned eyeblink responses (CRs) across blocks 3-6 in the EBCC paradigms in MSA patients and control subjects from this study and in IPD and PSP patients from earlier studies.[12 14] With the trace paradigm a complete separation between IPD and the atypical Parkinsonian syndromes MSA and PSP is achieved (cutoff 26%), whereas in the delay paradigm there is a small overlap between these groups. Overall, IPD patients perform slightly better than control subjects,[13] further enhancing the group distinction between IPD and atypical syndromes.

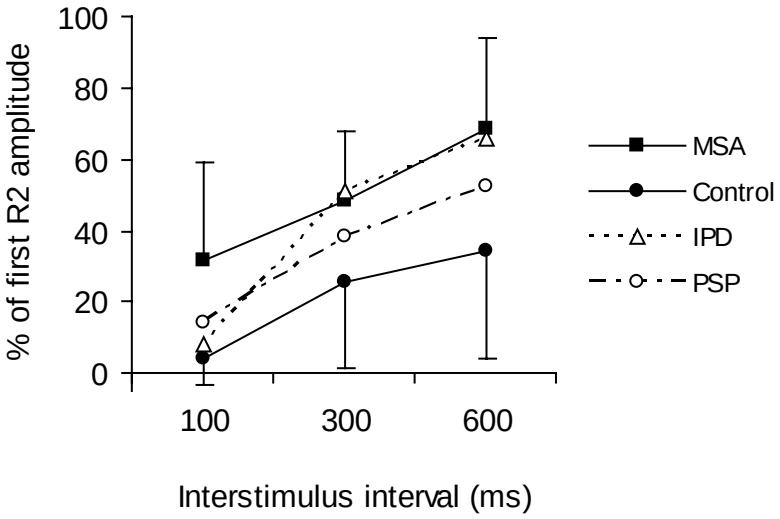

Figure 1, von Lewinski et al.

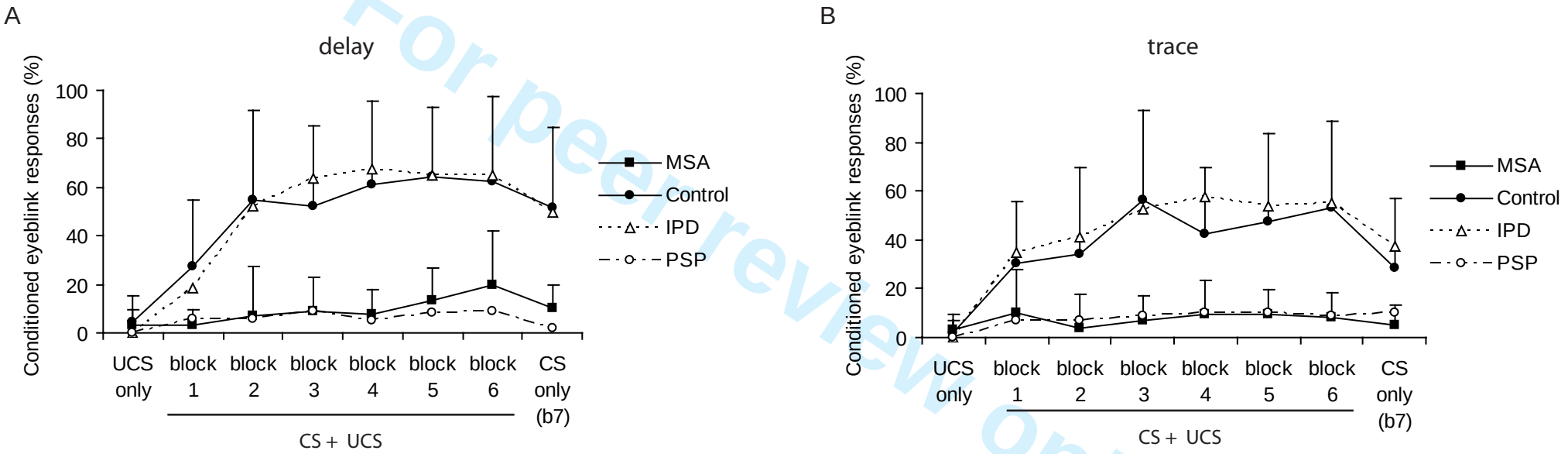

Figure 2, von Lewinski et al.

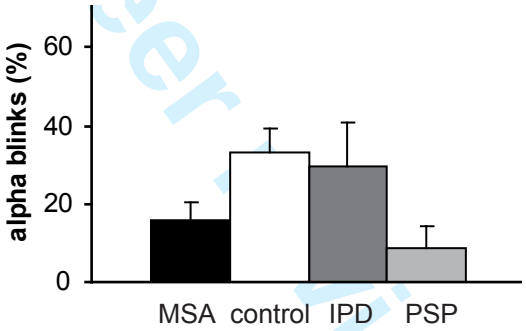

Figure 3, von Lewinski et al.

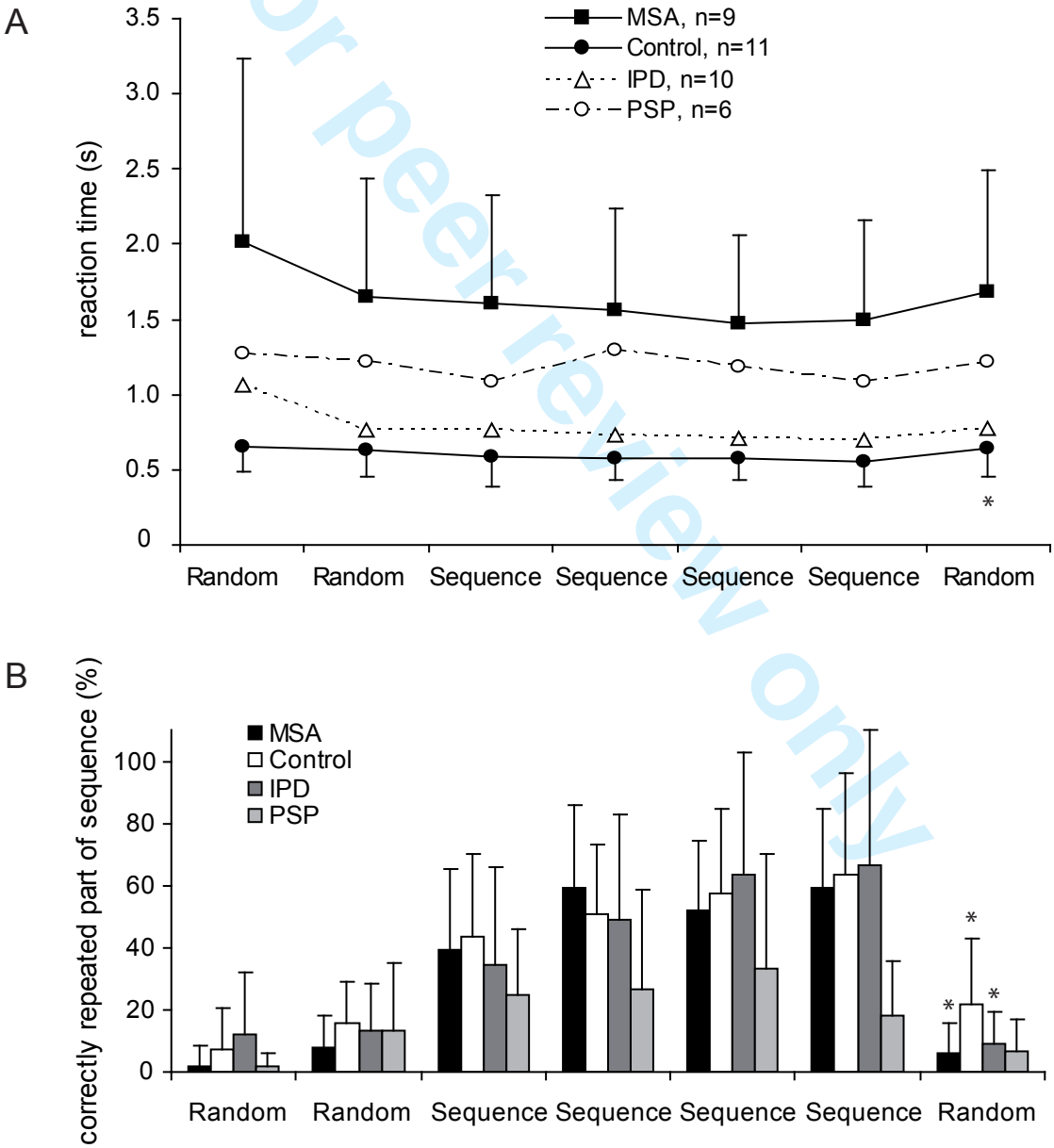

Figure 4, von Lewinski et al.

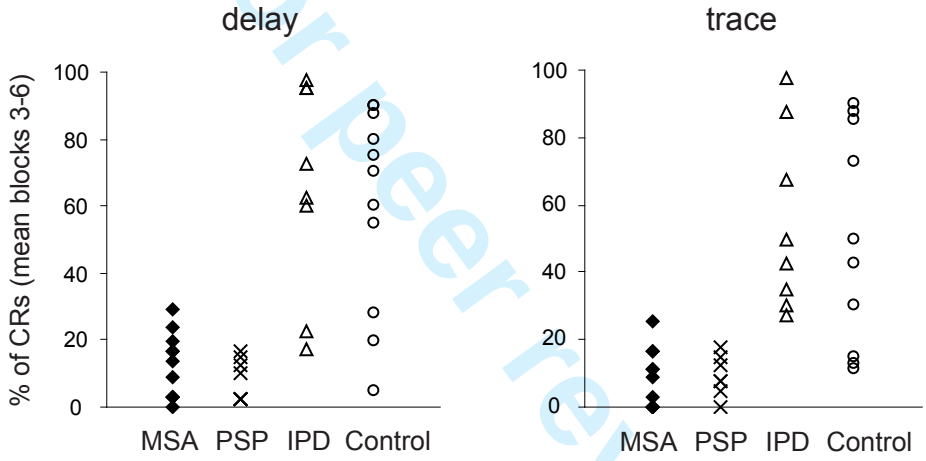

Figure 5, von Lewinski et al.

Impairment of brainstem implicit learning paradigms differentiates multiple system atrophy (MSA) from idiopathic Parkinson syndrome

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ABSTRACT

Objectives: Learning as measured by eyeblink classical conditioning is preserved in patients with idiopathic Parkinson's disease, but severely affected in patients with progressive supranuclear palsy. We here sought to clarify whether procedural learning is impaired in multiple system atrophy, and whether it may be helpful for the differentiation of Parkinsonian syndromes.

Design: We investigated learning using (1) eyeblink classical conditioning with a delay (interstimulus interval 0 ms) and a trace (600 ms) paradigm and (2) a serial reaction time task.

Setting: Participants were recruited from academic research centers.

Participants: 11 patients with multiple system atrophy and 11 healthy controls.

Results: Implicit learning in eyeblink classical conditioning (acquisition of conditioned responses) as well as the serial reaction time task measures of implicit learning (reaction time change) are impaired in multiple system atrophy patients as compared to controls, whereas explicit learning as measured by the sequence recall of the serial reaction time task is relatively preserved.

Analysis: We hypothesize that the MSA patients' learning deficits are due to lesions of cerebellar and connected brainstem areas.

Conclusions: A retrospective synopsis of these novel data on multiple system atrophy patients and groups of idiopathic Parkinson's disease patients and progressive supranuclear palsy patients studied earlier suggests that eyeblink classical conditioning may contribute to the early differentiation of atypical Parkinson syndromes from idiopathic Parkinson's disease. This hypothesis should be tested in a prospective trial.

ARTICLE SUMMARY:

Article focus:

- We tested if the non-motor feature of procedural learning is impaired in patients afflicted with multiple system atrophy.
- We studied the eyeblink classical conditioning and a serial reaction time task in 11 MSA patients and matched control subjects.

Key messages:

- Blink reflex pathways were intact, but eyeblink conditioning was significantly impaired in MSA patients. By contrast, the serial reaction time task was difficult and inconclusive in these patients due to motor constraints impairing finger tapping.
- A retrospective comparison with previously studied groups patients with idiopathic Parkinson's disease or Progressive Supranuclear Palsy points to a putative role of eyeblink conditioning in distinguishing typical from atypical Parkinsonian disorders.

Strength and limitations:

- The study differentiates feasible and non-feasible assessments of procedural learning in multiple system atrophy.
- The comparison to other patient groups is clearly retrospective and needs to be validated by a prospective trial.

INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurodegenerative disorder characterized by an absent or poorly levodopa responsive Parkinsonism, cerebellar dysfunction and autonomic failure.[1] A predominantly Parkinsonian (MSA-P) and a cerebellar subtype (MSA-C) are recognized. Despite the development of consensus criteria,[2] the differential diagnosis between MSA and other hypokinetic rigid syndromes, such as idiopathic Parkinson's disease (IPD) or progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome, PSP), remains a clinical challenge.[3 4]

Recent years have seen an increasing focus on non-motor symptoms such as cognitive deficits in Parkinsonian disorders. Different patterns of cognitive impairment including deficits in executive function and learning abilities have been described.[5-9]

A well established task to study associative, procedural learning[10] is eyeblink classical conditioning (EBCC), which some regard as a model of implicit learning.[11] Previous studies have shown that learning as measured by EBCC is normal in patients with IPD, but, severely affected in patients with PSP.[12-14] The serial reaction time task (SRTT) is another established task for which the implicit measures of motor skill (reaction time and errors) were close to normal in IPD patients, but impaired in PSP patients, whereas sequence recall as measure of explicit learning were largely preserved in both groups.[12 14] We sought to investigate whether implicit learning deficits are specific for PSP or present in MSA as well, thereby comparing the clinical feasibility of SRTT and of EBCC in this patient group.

METHODS

Subjects

11 MSA patients were recruited from the outpatient clinics in Munich and Göttingen between 1999 and 2008 (**table 1**). The clinical diagnosis of "probable MSA" was established following consensus criteria.[2] 7 MSA patients were taking L-Dopa, two dopamine agonists (ropinirole, pramipexole), one amantadine, one budipine, one biperiden, another metixen, and two were taking antidepressants.

L-Dopa equivalent doses (LEDs) were calculated according to Tomlinson and colleagues[15] except for budipine, biperiden and metixen, where no conversion factor was given.

To rule out an immediate impact of medication on the patients' memory performance, the anti-parkinsonian medication was discontinued on the morning of the day of the study. MSA patients were compared with 11 age matched healthy control subjects (mean age 59.5±10.0 years, 6 male, 5 female), of which a subgroup was already involved in our earlier published study (numbers 2,3,5,6,8,9,11,12,14 according to Table 2 in[12]). All participants gave written informed consent; the research protocol was approved by the local ethics committee. Neither the patients nor the control subjects had any sign of cranial nerve impairment or auditory deficits in routine neurological examination.

Table 1

Pat Nr.	MSA Type	Age [year]	Sex	Duration [year]	L-Dopa response	LED [mg]	UPDRS Max=108	Cerebellar Max=4	Autonomic Max=5[f], 6[m]	Pyramidal Max=2	Hamilton Max=69	MMS Max=30
1	P	66	F	9	Poor	0+	50	0	1	0	11	27
2	P	69	M	4.5	Poor	125	20	0	2	0	20	30
3	P	73	M	8	Absent	255	16	0	3	0	15	28
4	P	59	F	1.5	Poor	125#	30	0	2	1	16	26
5	P	71	M	4	Absent	150	35	0	4	0	6	29
6	P	75	M	5	Modest	524	38	0	3	0	6	29
7	P	75	F	3	Poor	375	40	0	3	0	10	28
8	P	58	M	3	Poor	105#	18	0	1	0	2	30
9	C	64	M	2	Poor	900	69	2	2	0	22	27
10	C	56	M	2.5	*	0	5	3	1	0	16	28
11	C	60	F	8	*	0	30	3	3	1	14	26
Mean		66.0		4.6			31.9	0.7	2.3	0.2	12.5	28.0
S.D.		7.1		2.6			17.7	1.3	1.0	0.4	6.2	1.4

Clinical testing procedures

The Hamilton rating scale for depression[16] and the Mini-Mental state examination[17] were used to quantify the affective and general cognitive status, respectively, with pragmatic and established tests. Motor features in patients were evaluated with the Unified Parkinson Disease Rating Scale (UPDRS, part III).[18] Further clinical assessments are listed in table 1.

To investigate eyeblink function we used the blink reflex (BR) and its R2 recovery cycle detailed elsewhere.[12 19 20] In brief, a single electrical stimulation of the supraorbital nerve (duration: 0.2 ms) elicits an early unilateral (R1) and a late bilateral (R2) blink response. Paired-pulse supraorbital stimulation probes brainstem interneuronal excitability and yields an inhibition of the R2 of the second stimulus. We used interstimulus intervals (ISIs) of 100 ms, 300 ms and 600 ms on both sides. For EBCC we stimulated the side in which amplitudes and latencies were within or close to the normal range or arbitrarily the right supraorbital nerve if no side-differences were found.

EBCC-implicit learning

The procedures were virtually identical to and detailed in the earlier studies from our group.[12 14] In brief, an unconditioned stimulus, i.e. en electric pulse over the supraorbital nerve, invariably induces an eyeblink reflex. It is paired with a preceding conditioned stimulus, i.e. a tone, which by itself may elicit early startle responses (so called alpha blinks, within 200 ms after the tone). With repeated presentation of the tone and the electric pulse, a conditioned eyeblink response is expected to occur before the onset of the electrical stimulus. In the current study, the tone (conditioned stimulus, CS, 400 ms duration) was generated by a custom built sound generator (Department of Electronic Engineering, University of Goettingen) and presented via earphones (Cherry Inc., Japan) at an intensity perceived as very loud by each subject, usually 80 dB. A brief electrical stimulus to the supraorbital nerve eliciting a blink reflex served as unconditioned stimulus (UCS). We tested the EBCC with a two different interstimulus intervals between the end of the tone and the beginning of the electric pulse, either ISI 0 ms (delay paradigm) or 600 ms (trace paradigm), in randomized order. For each paradigm we administered six learning blocks with CS and UCS in trials 1-9, UCS only in trial 10 (to control for random blinks) and CS only in trial 11 (to test for an independent learning effect). To control for extinction of learned responses a seventh block consisted of 11 trials with CS only.[21] The intertrial interval was randomized between 10 and 30 seconds.

Electromyographic (EMG) activity was recorded using silver-silverchloride cup electrodes fixed with adhesive tape over the lower eyelid and over the ipsilateral temple.[12 14] EMG signals were fed into a recording device and filtered at 100 Hz and 5 kHz (SynAmps, Neuroscan Inc, Virginia, USA). To detect any ongoing muscular activity we recorded 400 ms before and 1600 ms after CS onset.

Serial reaction time task (SRTT)

The SRTT is established as a test of implicit learning.[12 23] Subjects were sitting in front of a computer screen, and were told that single asterisks would appear in one out of four positions on a computer screen. They were instructed to press a marked key on a computer keyboard that was underneath the position of the asterisk on the screen. The asterisks were presented in three random blocks (blocks 1, 2, 7) and four identical sequence blocks (blocks 3-6), in each of which a sequence of 10 elements (CBDABCDCBA) was presented 10 times. After each block subjects were asked to repeat the last 10 asterisk positions manually on the computer keyboard. We analyzed reaction time, errors and number of correctly repeated parts of the sequence. This test was difficult for many patients: Only 6 patients completed the test as required, one patient discontinued after block 1 and was excluded from the analysis. Two others discontinued after block 4, one after block 3. To enable some kind of statistical analysis, the result that these patients reached in their last sequence block was carried forward to the following sequence blocks, and the result of the second random block was assumed for block 7. One patient apparently responded with random typing to the letters presented and was therefore excluded from the analysis.

Comparison of MSA patients with PSP and IPD patients studied earlier

While clearly retrospective, we have taken the liberty to compare the MSA patients results obtained here with the group of PSP patients that we studied in 2001[14] and a subgroup of IPD patients studied in 1999 with identical methods (numbers 1-4 and 6-11 according to Table 1 in[12], selected to

match the current MSA group with regard to the disease severity according to UPDRS part III). Demographical data are cited in table 2. In figure 1, 2 and 4, data from these IPD and PSP patients are given in dashed lines.

Table 2

Nr.	group	Age [year]	Sex	Duration [year]	UPDRS Max=108	BDI Max=63	MMS Max=30	MDRS Max=144
1	C	57	m	-	-	2		144
2	C	60	f	-	-	9		142
3	C	50	m	-	-	0		141
4	C	64	f	-	-	0		142
5	C	58	m	-	-	1		138
6	C	73	m	-	-	6		134
7	C	49	f	-	-	0		143
8	C	45	m	-	-	1		144
9	C	53	m	-	-	1		142
10	C	73	f	-	-	11	30	
11	C	72	f	-	-	0	30	
Mean		59.5				2.6		
S.D.		10.0				3.6		
1	IPD	69	f	2	45	11		138
2	IPD	64	f	6	39	15		143
3	IPD	62	m	5	21	9		132
4	IPD	45	m	6	28	5		141
5	IPD	47	m	7	31	6		139
6	IPD	49	m	7	25	6		140
7	IPD	64	m	9	47	11		135
8	IPD	63	m	8	16	11		141
9	IPD	61	m	5	33	8		143
10	IPD	50	m	3	44	11		143
Mean		57.4		5.8	32.9	9.3		139.5
S.D.		8.7		2.1	10.7	3.1		3.7
1	PSP	54	m	2	22	6	30	127
2	PSP	69	m	9	34	0	28	110
3	PSP	65	f	2	44	13	28	107
4	PSP	57	f	3	30	35	30	135
5	PSP	66	m	2	30	13	28	135
6	PSP	59	m	6	50	4	22	100
7	PSP	68	f	4	43	50	18	116
8	PSP	63	m	5	54	*	23	112
Mean		62.6		4.1	38.4	17.3	25.9	117.8
S.D.		5.4		2.5	11.1	18.4	4.4	13.1

Data analysis

R2 latencies were measured off-line. For analysis of the blink reflex recovery cycle, we entered the R2 amplitudes of the second pulse normalized to the R2 amplitudes of the first pulse into a repeated measures analysis of variance (ANOVA) with "interstimulus interval" (100; 300, 600 ms) as within

subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) as between subject factor.[12 19 20] In the EBCC, EMG bursts were regarded as alpha-blinks, i.e. startle responses, or conditioned responses (CRs) if they occurred within the appropriate time window (alpha blinks: within 200ms after onset of tone (CS); CRs: within 200 ms before electrical stimulus (UCS)) and if their amplitude exceeded the baseline noise by at least 1.5 fold and reached at least 50 μV. For the tone-alone-trials we extended the time window until 300 ms after the end of the UCS to detect delayed CRs.[22] We analyzed the percentage of conditioned eyeblink responses and of alpha blinks with separate repeated measures ANOVAs with "block" (blocks 1-6, CS only block) as within subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) and "paradigm" (delay versus trace) as between subject factors. In addition, we repeated the ANOVAs for conditioned eyeblink responses with the individual average alpha blink rate across all seven blocks as covariate.

For SRTT, we analyzed reaction time, accuracy errors and retrieval using separate repeated measures ANOVAs with "block" (blocks 1-7) as within subject factor and "group" (control and MSA; or control, MSA, IPD, PSP) as between subject factor. In all analyses, Mauchly's sphericity test was performed and Greenhouse–Geisser correction was applied when necessary. The level of significance was set at $p<0.05$. Post-hoc t-tests were Bonferroni-corrected. A correlation between two parameters was determined by calculating Pearson's correlation coefficient and was reported if it was higher than 0.75 or lower than -0.75. The results are given as mean values ± one standard deviation.

RESULTS

Rating scales

Details of age, sex, disease duration, L-Dopa response and rating scales of MSA patients are displayed in **table 1**. UPDRS scores for motor impairment placed the patients in an intermediately impaired range. The Hamilton depression score revealed mild depressive symptoms (mean 12.5±6.2 out of 69 points) and the Mini-Mental State scored between 26 and 30 points (mean 28.0±1.4)

indicating mild cognitive impairment in more than half of the patients. These results are comparable to the IPD and PSP groups reported earlier.[12 14]

Blink reflex pathways

Blink reflex responses in MSA patients showed normal latencies (ipsilateral R1: 11.6±1.0 ms, ipsilateral R2: 31.5±4.9 ms contralateral R2: 34.4±4.1 ms). An R2 recovery cycle could be obtained in all patients (**figure 1**) with no significant side difference between the ipsi- and contralatral R2 recovery. MSA patients showed significantly less R2 inhibition compared to the control group (repeated-measures ANOVA MSA-controls, effect of group, $F(1, 20)= 15.0$. $p=0.001$).

-- Please insert fig. 1 about here --

Conditioned eyeblink responses

All MSA patients showed few random blinks as assessed by the UCS only trials (3.0±6.7 % across both paradigms). The conditioned responses (CRs) were first analyzed for block 1-6 excluding the tone alone trials (see below). In either paradigm MSA patients showed significantly fewer CRs than the control group (**figure 2;** repeated-measures ANOVA MSA-control**,** effect of group, $F(1, 39)= 37.1$, $p<0.0001$; effect of block, $F(3.4, 39)= 7.0$, $p<0.0001$; interaction of group by block, $F(3.4, 266)= 3.325$, $p=0.017$, no main effect of paradigm). Adding the rate of alpha blinks as covariate to the ANOVA did not abolish the effect of group ($F(1, 38)= 31.5$, $p<0.0001$). These results were supported by a separate analysis of the tone alone trials (trial 11, block 1-6), in which the MSA group yielded an average number of CRs of 14±17 % in the delay and 12±17 % in the trace paradigm, which was significantly less than the control group with 73±23 % and 55±27 % of CRs respectively (ANOVA MSA-control, effect of group, $F(1,37)=59.1$, $p<0.0001$). There was again no main effect of paradigm, i.e. trace and delay paradigm did not differ from each other.

Considering the MSA patients only, there was no difference in the occurrence of CRs between MSA-P and MSA-C patients (ANOVA, effect of MSA subtype, F(1,17)=2.5, p=0.13).

-- Please insert fig. 2 about here -

Alpha Blinks

In either paradigm MSA patients tended to show less alpha blinks than controls (repeated measures ANOVA, effect of group F(1,38)=4.0, p=0.054; **figure 3**.). The mean percentage of alpha blinks across all blocks in MSA patients was 17.6±4.6 % in the delay and 14.4±4.1 % in the trace paradigm, for control subjects 31.5±11.1 % and 35.2±11.3 % respectively. There were significantly more alpha blinks in earlier blocks than in late blocks for both paradigms (effect of block, F(4.3, 163.7)=8.5, p<0.0001). Considering the MSA patients only, there was no statistically significant difference in the occurrence of alpha blinks between MSA-P and MSA-C patients.

-- Please insert fig. 3 about here --

Serial reaction time task (SRTT)

Reaction time

MSA patients showed longer reaction times compared with controls (repeated measures ANOVA MSA-control, effect of group, F(1,18)=20.2, p<0.0001; and a trend for an interaction of group by block, F(1.52, 27.34)=2.77, p=0.10, **figure 4A**). In both groups reaction times decreased from block 1 to the sequence blocks 3, 4, 5 and 6 (effect of block**,** F(1.52, 27.34)=5.5, p=0.016). The reaction time increase from sequence block 6 to random block 7, which is considered a measure of implicit learning, was significant in the control group only (t-test p<0.01; MSA p=0.1).

-- Please insert fig. 4 about here --

Accuracy errors

The average error rate of MSA patients across blocks was 19.7±4.2 %, which is significantly higher compared to controls with a rate of 2.6±0.8 % (repeated measures ANOVA MSA-control, effect of group, $F(1,18)=10.1$, $p=0.005$). In both groups error rates decreased from the first random to the sequence blocks (effect of block, $F(3.37, 42.66)=3.9$, $p=0.022$) and tended to increase between the last sequence block and the random block 7 without being significant.

Retrieval of sequence

There was no significant difference between MSA patients and controls in the measures of sequence detection (manual sequence retrieval, ANOVA MSA-control, effect of group, $F(1,18)=0.7$, $p=0.42$). Both groups detected an increasing amount of the sequence during the course of the experiment (ANOVA, effect of block, $F(3.58, 64.48)=31.0$, $p<0.001$). A small percentage of repetition was seen even before the sequence was presented, which indicates the baseline guessing rate (**figure 4B)**.

Correlation analyses for MSA patients

We did not find a significant correlation between the average number of CRs across block 3-6 (steady state) and the duration of disease, the Hamilton scale score, the MMST, the motor examination of the UPDRS, the cerebellar or autonomic impairment as assessed by the scores (see table 1) for either paradigm. The percentage of manual retrieval in the SRTT did not correlate with any of these parameters either.

Retrospective comparison of all patient groups (MSA, PSP, IPD) and the control group

In the blink reflex recovery cycle, the R2 inhibition was not different between the patient groups (figure 1). For the EBCC paradigms, the lack of eyeblink conditioning in MSA patients was similar to the PSP group and differed significantly from the increasing occurrence of CRs in IPD patients and controls (ANOVA, effect of group, $F(3,63)=23.2$, $p<0.0001$; interaction of group by block, $F(11.1, 233.0)=3.6$, $p<0.0001$, post-hoc t-test with Bonferroni correction; figure 2). Adding the rate of alpha blinks as covariate did not abolish the effect of group ($F(1, 32)= 16.7$, $p<0.0001$). Also in the tone alone trials, MSA and PSP patients showed fewer CRs than the IPD and control groups (ANOVA, effect of group, $F(3,64)=19.0$, $p<0.0001$; interaction of group by block, $F(15,320)=1.8$, $p=0.04$). MSA and PSP groups both showed fewer alpha blinks than IPD patients and controls (ANOVA, effect of group, $F(3,61)=3.5$, $p=0.02$; interaction of group by block ($F(12.73, 259.0)=2.0$, $p=0.025$; see figure 3). However, the post-hoc t-test analysis indicated these differences to be non-significant.

For the differentiation of MSA and PSP from IPD we considered the mean percentage of CRs in the steady state part of the EBCC learning curve (blocks 3-6). The mean percentage of CRs allows a non-overlapping separation of the MSA and PSP groups from IPD in the trace paradigm with a cutoff at 26% (**figure 5).** In the delay paradigm, the separation between groups was less complete. Whereas all PSP patients exhibited less than 26% of CRs, the fraction of MSA patients with values equal or below 26% was 0.91. This corresponds to sensitivity values for the separation of PSP and MSA from IPD of 100% and 91%, respectively. If the specificity is defined as the fraction of IPD patients with CRs values above 26%, the specificity in the delay paradigm is 75%. As reported above, the sensitivity and specificity values for the trace paradigm are 100%. The slightly better performance of IPD patients[13] as compared to controls was not significant.

-- Please insert fig. 5 about here --

In the SRTT, MSA patients showed significantly longer reaction times than the IPD patients, but no significant difference compared to the PSP group (ANOVA: effect of group F(3,30)=7.4, p=0.001; see figure 4). With regard to the error rate, MSA patients performed again very similar to the PSP patients, who showed 19.5±1.8 % accuracy errors, but significantly worse than the IPD patients (error rate 4.8±1.7 %; ANOVA, effect of group, F(3,32)=6.1, p=0.002). The sequence recall measurements revealed no statistically significant differences between groups.

DISCUSSION

The differentiation of IPD and atypical Parkinsonian syndromes (MSA, PSP) constitutes a challenge for neurologists, as the motor symptoms often present very similarly, in particular in the early stages. Additional markers such as imaging have been evaluated,[24 25] but these provide insufficient sensitivity values or are technically challenging. In addition, macroscopically discernible structural changes as detectable by MRI are likely to occur some time after functional loss has begun. Therefore functional tests might be better suited because they reveal deficits before discernible structural changes occur. In this study we focus on the differential leaning abilities tested by eyeblink conditioning (EBCC) and a serial reaction time task (SRTT). First, the results of the MSA patients will be discussed, followed by a comparison with PSP and the putative impact for differentiation from IPD.

The MSA patients showed severely impaired implicit learning in the trace as well as in the delay eyeblink conditioning paradigm, with standard deviations in the range of other studies,[10 26] whereas non-learning associated blink reflex latencies as indicators of oculomotor pathways were normal.

Histopathological alterations in both subtypes of MSA include the substantia nigra, putamen, descending and ascending fiber tracts of the motor system, olivary and pontine nuclei, brainstem-cerebellar circuits as well as cerebellar structures (hemispheres and vermis).[27-29] This has been confirmed *in vivo* by diffusion tensor imaging of white matter microstructure.[30] We suggest that

damage of cerebellar structures and associated brainstem-cerebellar circuits are responsible for the failure of EBCC learning in MSA patients. This assumption is supported by the findings of impaired EBCC in patients with cerebellar damage,[22 31-33] positron-emission tomography (PET) measurements in healthy humans showing changes in glucose metabolism in the cerebellum and pons during EBCC[21 34] as well as in experiments studying the influence of selective pharmacological blockade of cerebellar input on EBCC in rabbits.[35] Most patients in our study were clinically characterized as MSA-P and not MSA-C. However, the failure in blink reflex acquisition in both groups points to a subclinical cerebellar involvement in MSA-P, which is in accordance with the histopathological studies.[28 29] EBCC therefore seems to detect cerebellar involvement at a subclinical stage.

In addition to the cerebellum, several studies indicate that acquisition of CR in the trace paradigm depends on forebrain areas such as the hippocampus or prefrontal cortex, in particular for longer interstimulus intervals.[36 37] In our study, the failure of CR acquisition in MSA patients was slightly more pronounced in the trace compared to the delay paradigm. Therefore, lesions of the frontal lobe, which have been suggested by neuropsychological testing[6 38] and confirmed histopathologically in a variety of MSA cases,[39 40] may have contributed to impaired EBCC acquisition in the trace paradigm.

An alternative explanation that was brought up by an anonymous reviewer is that the tone may be a less salient CS to the MSA patients than to the control group. The reduced number of alpha blinks would support this assumption. Following that very elegant line of thought, the EBCC group difference between MSA patients and control subjects would have to do less with implicit learning and more with responsiveness and associative processes related to external stimuli. While this may have some relevance, adding the number of alpha blinks to the ANOVAs on conditioned eyeblink responses did not abolish the between-group differences.

In the SRTT the MSA patients showed severe impairment in terms of prolonged reaction time, high error rate and, in some patients, early discontinuation due to fatigue and exhaustion. In contrast

to the control group they showed no significant reaction time increase between block 6 (random) and 7 (sequence) and this is indicative of implicit learning deficits. On the other hand they showed good performance on the parameters of sequence recall (explicit learning). This preservation of SRTT explicit learning parts may be explained by the relative preservation of posterior association (temporal and parietal) cortex and hippocampus in MSA. However, the validity of the SRTT learning results is limited by the discontinuation of patients and our "last observation carried forward approach" (see Methods). In addition, the patients' motor impairment, which may interfere with the motor part of the task, and the fact that sequence learning and movement preparation seem to share similar attentional and working memory resources[41] have to be considered. Therefore the SRTT seems to be inappropriate to assess learning abilities in MSA patients. This is in contrast to the EBCC, which is independent of the motor performance of patients. Furthermore, EBCC circuits are located anatomically closer to the affected brainstem regions.

With all the limitations of such a retrospective comparison of data acquired in different patient groups by the same authors, the implicit learning impairment in MSA patients as clearly revealed by the EBCC and with some limitation by the SRTT parallels the previously reported findings in PSP patients.[14] Interestingly, MSA and PSP are characterized by different histopathological alterations, α-synuclein positive inclusions versus tau-positive aggregations, which lead to presume different pathophysiological mechanisms. However, the common involvement of cerebellar structures in both diseases[27 42] seems to be responsible for the clinical phenomenology independent of the cellular mechanism.

In contrast to MSA and PSP, IPD patients show normal[12] or even enhanced[13] acquisition of conditioned responses in EBCC. EBCC may therefore contribute to distinguish IPD from these atypical Parkinsonian syndromes. As the development of cerebellar and related neuropathology in MSA or PSP often occurs prior to or even without clinical manifestation,[29 42] we propose impaired EBCC particularly in the trace paradigm as an early indicator of neurodegeneration of areas beyond those typically affected in IPD. However, as the retrospective synopsis is clearly limited, the pivotal

questions of whether EBCC can serve as predictor for the development of typical or atypical disease and whether EBCC is a useful addition to imaging techniques in establishing an early differential diagnosis are unanswered yet and require further prospective investigation.

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Contributors

All authors listed above fulfill all three International Committee of Medical Journal Editors (ICMJE) guidelines for authorship which are (1) substantial contributions to conception and design, acquisition of data or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be published. MSo, CT and WP conceived the study. MSch, FvL and MSo were responsible for obtaining the data. FvL and MSo conducted the data analysis. All authors contributed to decisions on the interpretation of results. FvL and MSo contributed to the drafting of the manuscript. CT and WP were responsible for editing and providing guidance on the paper. All authors were responsible for critically revising the paper. All authors approved the final version of the manuscript prior to submission.

Competing interests None

Ethics approval Ethics committee of the Medical Faculty of the University of Goettingen.

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Legends to tables and figures

Table 1: Characteristics of the MSA patients. Type: Parkinsonian (MSA-P) or cerebellar (MSA-C) predominance; LED = L-Dopa equivalent dose, + indicates additional budipine medication, # additional anticholinergic medication; UPDRS = Unified Parkinson's disease rating scale, motor examination only (high number of points indicates high disability); MMS= Mini Mental State (30 points are normal, ≤26 is usually considered as cognitive impairment). Cerebellar impairment was evaluated for ataxia (arm, leg), saccades and intention tremor (finger-nose test), autonomic impairment for postural faintness, syncopes, urinary incontinence or retention, faecal incontinence, and impotence (males only). Pyramidal tract impairment was scored for hyperreflexia and Babinski sign. Each item was scored if present with 1, otherwise 0. In the Hamilton scale a low score indicates few depressive symptoms. *not investigated.

Table 2: Characteristics of controls, IPD and PSP patients in part taken from earlier publications.[12] [14] Dementia had been ruled out using the Mattis Dementia Rating scale (MDRS)[43 44], where higher scores out of a maximum of 144 indicate better performance, with a cut-off ≤123 considered as cognitive impairment[45]. Depression had been assessed using the Beck Depression Inventory (BDI), where higher scores out of a maximum of 63 points indicate a more severe depressed state, and a score of 15 is regarded as cut off for a self report of mild depression.[46 47] *not investigated. The MDRS was not available at the German study sites.

Figure 1: Blink reflex recovery cycle with interstimulus intervals of 100, 300 and 600 ms. In MSA patients, the inhibition of the ipsilateral R2 response to the second stimulus is weaker than in the

control group. Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 2: Conditioned eyeblink responses in two different paradigms: left, delay (interstimulus interval 0 ms), right, trace paradigm (interstimulus interval 600 ms). In both paradigms, the number of conditioned responses was significantly lower in MSA and PSP patients than in the control and IPD groups indicating impaired implicit learning. CS, conditioned stimulus (tone), UCS, unconditioned stimulus (electrical stimulation to the supraorbital nerve). Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 3: Occurrence of 'alpha-blinks'. These bursts are a startle reaction to the tone (CS) and are less frequent in MSA and PSP patients than in the control and IPD groups. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Data are indicated as average value and single standard deviation and were pooled for both paradigms.

Figure 4: A Reaction time in a serial reaction time task (SRTT). An implicit learning effect is indicated by the reaction time increase between the last sequence block (6) and the following random block (7). **B** Explicit learning in the SRTT was tested after each block by manual retrieval of the sequence (repetition of the last 10 key presses) and revealed no significant difference between groups. Data are indicated as average value and single standard deviation. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Asterisks indicate a significant difference for the comparison of blocks 6 and 7 ($p<0.05$).

Figure 5: Overview of the mean percentage of conditioned eyeblink responses (CRs) across blocks 3-6 in the EBCC paradigms in MSA patients and control subjects from this study and in IPD and PSP patients from earlier studies.[12 14] With the trace paradigm a complete separation between IPD and the atypical Parkinsonian syndromes MSA and PSP is achieved (cutoff 26%), whereas in the delay paradigm there is a small overlap between these groups. Overall, IPD patients perform slightly better than control subjects,[13] further enhancing the group distinction between IPD and atypical syndromes.

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Impairment of brainstem implicit learning paradigms differentiates multiple system atrophy (MSA) from idiopathic Parkinson syndrome

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Impairment of brainstem implicit learning paradigms differentiates multiple system atrophy (MSA) from idiopathic Parkinson syndrome

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ABSTRACT

Objectives: Learning as measured by eyeblink classical conditioning is preserved in patients with idiopathic Parkinson's disease, but severely affected in patients with progressive supranuclear palsy. We here sought to clarify whether procedural learning is impaired in multiple system atrophy, and whether it may be helpful for the differentiation of Parkinsonian syndromes.

Design: We investigated learning using (1) eyeblink classical conditioning with a delay (interstimulus interval 0 ms) and a trace (600 ms) paradigm and (2) a serial reaction time task.

Setting: Participants were recruited from academic research centers.

Participants: 11 patients with multiple system atrophy and 11 healthy controls.

Results: Implicit learning in eyeblink classical conditioning (acquisition of conditioned responses) as well as the serial reaction time task measures of implicit learning (reaction time change) are impaired in multiple system atrophy patients as compared to controls, whereas explicit learning as measured by the sequence recall of the serial reaction time task is relatively preserved.

Analysis: We hypothesize that the MSA patients' learning deficits are due to lesions of cerebellar and connected brainstem areas.

Conclusions: A retrospective synopsis of these novel data on multiple system atrophy patients and groups of idiopathic Parkinson's disease patients and progressive supranuclear palsy patients studied earlier suggests that eyeblink classical conditioning may contribute to the early differentiation of atypical Parkinson syndromes from idiopathic Parkinson's disease. This hypothesis should be tested in a prospective trial.

ARTICLE SUMMARY:

Article focus:

- We tested if the non-motor feature of procedural learning is impaired in patients afflicted with multiple system atrophy.
- We studied the eyeblink classical conditioning and a serial reaction time task in 11 MSA patients and matched control subjects.

Key messages:

- Blink reflex pathways were intact, but eyeblink conditioning was significantly impaired in MSA patients. By contrast, the serial reaction time task was difficult and inconclusive in these patients due to motor constraints impairing finger tapping.
- A retrospective comparison with previously studied groups patients with idiopathic Parkinson's disease or Progressive Supranuclear Palsy points to a putative role of eyeblink conditioning in distinguishing typical from atypical Parkinsonian disorders.

Strength and limitations:

- The study differentiates feasible and non-feasible assessments of procedural learning in multiple system atrophy.
- The comparison to other patient groups is clearly retrospective and needs to be validated by a prospective trial.

INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurodegenerative disorder characterized by an absent or poorly levodopa responsive Parkinsonism, cerebellar dysfunction and autonomic failure.[1] A predominantly Parkinsonian (MSA-P) and a cerebellar subtype (MSA-C) are recognized. Despite the development of consensus criteria,[2] the differential diagnosis between MSA and other hypokinetic rigid syndromes, such as idiopathic Parkinson's disease (IPD) or progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome, PSP), remains a clinical challenge.[3 4]

Recent years have seen an increasing focus on non-motor symptoms such as cognitive deficits in Parkinsonian disorders. Different patterns of cognitive impairment including deficits in executive function and learning abilities have been described.[5-9]

A well established task to study associative, procedural learning[10] is eyeblink classical conditioning (EBCC), which some regard as a model of implicit learning[11]. Previous studies have shown that learning as measured by EBCC is normal in patients with IPD, but, severely affected in patients with PSP.[12-14] In contrast to tracking or pointing tasks,[15 16] EBCC has the advantage not to depend on manual motor skills. Learning assessed by the serial reaction time task (SRTT) showed the implicit motor skill close to normal in IPD patients, whereas PSP patients were markedly impaired; in contrast, the SRTT sequence recall component as measure of explicit learning was largely preserved in both groups.[12 14] We sought to investigate whether implicit learning deficits are specific for PSP or present in MSA as well, thereby comparing the clinical feasibility of SRTT and of EBCC in this patient group.

METHODS

Subjects

11 MSA patients were recruited from the outpatient clinics in Munich and Göttingen between 1999 and 2008 (**table 1**). The clinical diagnosis of "probable MSA" was established following consensus criteria.[2] 7 MSA patients were taking L-Dopa, two dopamine agonists (ropinirole, pramipexole),

one amantadine, one budipine, one biperiden, another metixen, and two were taking antidepressants. L-Dopa equivalent doses (LEDs) were calculated according to Tomlinson and colleagues[17] except for budipine, biperiden and metixen, where no conversion factor was given.

To rule out an immediate impact of medication on the patients' memory performance, the anti-parkinsonian medication was discontinued on the morning of the day of the study. MSA patients were compared with 11 healthy control subjects, matched for age (t-test), and chosen for the absence of neurodegenerative or any other neurological disease, and for the absence of intake of CNS-active medication (mean age 59.5±10.0 years, 6 male, 5 female). A subgroup was already involved in our earlier published study (numbers 2,3,5,6,8,9,11,12,14 according to Table 2 in[12]). All participants gave written informed consent; the research protocol was approved by the local ethics committee. Neither the patients nor the control subjects had any sign of cranial nerve impairment or auditory deficits in routine neurological examination.

Table 1

Pat Nr.	MSA Type	Age [year]	Sex	Duration [year]	L-Dopa response	LED [mg]	UPDRS Max=108	Cerebellar Max=4	Autonomic Max=5[f], 6[m]	Pyramidal Max=2	Hamilton Max=69	MMS Max=30
1	P	66	F	9	Poor	0+	50	0	1	0	11	27
2	P	69	M	4.5	Poor	125	20	0	2	0	20	30
3	P	73	M	8	Absent	255	16	0	3	0	15	28
4	P	59	F	1.5	Poor	125#	30	0	2	1	16	26
5	P	71	M	4	Absent	150	35	0	4	0	6	29
6	P	75	M	5	Modest	524	38	0	3	0	6	29
7	P	75	F	3	Poor	375	40	0	3	0	10	28
8	P	58	M	3	Poor	105#	18	0	1	0	2	30
9	C	64	M	2	Poor	900	69	2	2	0	22	27
10	C	56	M	2.5	*	0	5	3	1	0	16	28
11	C	60	F	8	*	0	30	3	3	1	14	26
Mean		66.0		4.6			31.9	0.7	2.3	0.2	12.5	28.0
S.D.		7.1		2.6			17.7	1.3	1.0	0.4	6.2	1.4

Clinical testing procedures

The Hamilton rating scale for depression[18] and the Mini-Mental state examination[19] were used to quantify the affective and general cognitive status, respectively, with pragmatic and established

tests. Motor features in patients were evaluated with the Unified Parkinson Disease Rating Scale (UPDRS, part III).[20] Further clinical assessments are listed in table 1.

To investigate eyeblink function we used the blink reflex (BR) and its R2 recovery cycle detailed elsewhere.[12 21 22] In brief, a single electrical stimulation of the supraorbital nerve (duration: 0.2 ms) elicits an early unilateral (R1) and a late bilateral (R2) blink response. Paired-pulse supraorbital stimulation probes brainstem interneuronal excitability and yields an inhibition of the R2 of the second stimulus. We used interstimulus intervals (ISIs) of 100 ms, 300 ms and 600 ms on both sides. For EBCC we stimulated the side in which amplitudes and latencies were within or close to the normal range or arbitrarily the right supraorbital nerve if no side-differences were found.

EBCC-implicit learning

The procedures were virtually identical to and detailed in the earlier studies from our group.[12 14] In brief, an unconditioned stimulus, i.e. en electric pulse over the supraorbital nerve, invariably induces an eyeblink reflex. It is paired with a preceding conditioned stimulus, i.e. a tone, which by itself may elicit early startle responses (so called alpha blinks, within 200 ms after the tone). With repeated presentation of the tone and the electric pulse, a conditioned eyeblink response is expected to occur before the onset of the electrical stimulus. In the current study, the tone (conditioned stimulus, CS, 400 ms duration) was generated by a custom built sound generator (Department of Electronic Engineering, University of Goettingen) and presented via earphones (Cherry Inc., Japan) at an intensity perceived as very loud by each subject, usually 80 dB. A brief electrical stimulus to the supraorbital nerve eliciting a blink reflex served as unconditioned stimulus (UCS). We tested the EBCC with a two different interstimulus intervals between the end of the tone and the beginning of the electric pulse, either ISI 0 ms (delay paradigm) or 600 ms (trace paradigm), in randomized order. For each paradigm we administered six learning blocks, each with CS and UCS in trials 1-9, UCS only in trial 10 (to control for random blinks) and CS only in trial 11 (to test for a persistent learning

effect). To control for extinction of learned responses a seventh block consisted of 11 trials with CS only.[23] The intertrial interval was randomized between 10 and 30 seconds.

Electromyographic (EMG) activity was recorded using silver-silverchloride cup electrodes fixed with adhesive tape over the lower eyelid (active electrode) and over the ipsilateral temple (reference electrode); with a sampling rate of 10 kHz.[12 14] EMG signals were fed into a recording device and filtered at 100 Hz and 5 kHz (SynAmps, Neuroscan Inc, Virginia, USA). To detect any ongoing muscular activity we recorded 400 ms before and 1600 ms after CS onset.

Serial reaction time task (SRTT)

The SRTT is established as a test of implicit learning.[12 24] Subjects were sitting in front of a computer screen, and were told that single asterisks would appear in one out of four positions on a computer screen. They were instructed to press a marked key on a computer keyboard that was underneath the position of the asterisk on the screen. The asterisks were presented in three random blocks (blocks 1, 2, 7) and four identical sequence blocks (blocks 3-6), in each of which a sequence of 10 elements (CBDABCDCBA) was presented 10 times. After each block subjects were asked to repeat the last 10 asterisk positions manually on the computer keyboard, which may have accentuated explicit aspects of the task.[25 26] We analyzed reaction time, errors and number of correctly repeated parts of the sequence. This test was difficult for many patients: Only 6 patients completed the test as required, one patient discontinued after block 1 and was excluded from the analysis. Two others discontinued after block 4, one after block 3. To enable some kind of statistical analysis, the result that these patients reached in their last sequence block was carried forward to the following sequence blocks, and the result of the second random block was assumed for block 7. One patient apparently responded with random typing to the letters presented and was therefore excluded from the analysis.

Comparison of MSA patients with PSP and IPD patients studied earlier

While clearly retrospective, we have taken the liberty to compare the MSA patients results obtained here with the group of PSP patients that we studied in 2001[14] and a subgroup of IPD patients studied in 1999 with identical electrophysiological methods (numbers 1-4 and 6-11 according to Table 1 in[12], selected to match as good as possible the current MSA group with regard to the disease severity (according to UPDRS part III), even though retrospective matching based in part on different scales used in different laboratories is certainly not perfect. Demographical data are cited in table 2. In figure 1, 2 and 4, data from these IPD and PSP patients are given in dashed lines.

Table 2

Nr.	group	Age [year]	Sex	Dura-tion [year]	UPDRS Max=108	BDI Max=63	MMS Max=30	MDRS Max=144
1	C	57	m	-	-	2		144
2	C	60	f	-	-	9		142
3	C	50	m	-	-	0		141
4	C	64	f	-	-	0		142
5	C	58	m	-	-	1		138
6	C	73	m	-	-	6		134
7	C	49	f	-	-	0		143
8	C	45	m	-	-	1		144
9	C	53	m	-	-	1		142
10	C	73	f	-	-	11	30	
11	C	72	f	-	-	0	30	
Mean		59.5				2.6		
S.D.		10.0				3.6		
1	IPD	69	f	2	45	11		138
2	IPD	64	f	6	39	15		143
3	IPD	62	m	5	21	9		132
4	IPD	45	m	6	28	5		141
5	IPD	47	m	7	31	6		139
6	IPD	49	m	7	25	6		140
7	IPD	64	m	9	47	11		135
8	IPD	63	m	8	16	11		141
9	IPD	61	m	5	33	8		143
10	IPD	50	m	3	44	11		143
Mean		57.4		5.8	32.9	9.3		139.5
S.D.		8.7		2.1	10.7	3.1		3.7
1	PSP	54	m	2	22	6	30	127
2	PSP	69	m	9	34	0	28	110
3	PSP	65	f	2	44	13	28	107
4	PSP	57	f	3	30	35	30	135
5	PSP	66	m	2	30	13	28	135
6	PSP	59	m	6	50	4	22	100
7	PSP	68	f	4	43	50	18	116
8	PSP	63	m	5	54	*	23	112
Mean		62.6		4.1	38.4	17.3	25.9	117.8
S.D.		5.4		2.5	11.1	18.4	4.4	13.1

Data analysis

UPDRS scores in the patient groups, and age in all four groups, were compared using factorial ANOVAs with group (three or four levels) as between-subject factor. R2 latencies were measured off-line. For analysis of the blink reflex recovery cycle, we entered the R2 amplitudes of the second pulse normalized to the R2 amplitudes of the first pulse into a repeated measures analysis of variance (ANOVA) with "interstimulus interval" (three levels: 100; 300, 600 ms) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) as

between subject factor.[12 21 22] In the EBCC, EMG bursts were regarded as present if their peak-to-peak amplitude exceeded baseline noise by at least 1.5 fold and reached at least 50 μV. They were counted as alpha-blinks, i.e. startle responses, or conditioned responses (CRs) if they occurred within the appropriate time window (alpha blinks: within 200ms after onset of tone (CS); CRs: within 200 ms before electrical stimulus (UCS)). For the tone-alone-trials we extended the time window until 300 ms after the end of the UCS to detect delayed CRs.[27] Random blinks were counted as EMG bursts occurring in the CR time window in the absence of a CS, i.e. in the UCS only trials. Their occurrence rate was reported numerically. We analyzed the percentage of conditioned eyeblink responses repeated measures ANOVAs with "block" (six levels: blocks 1-6) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors. In addition, we repeated the ANOVAs for conditioned eyeblink responses with the individual average alpha blink rate across blocks 1-6 as covariate. We calculated separate repeated measures ANOVAs for the tone alone trials (trial 11, block 1-6), with "block" (six levels: blocks 1-6) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors. For alpha blink rate, we calculated a repeated-measures ANOVA with "block" (seven levels: blocks 1-6 and CS only block) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors.

For SRTT, we analyzed reaction time, accuracy errors and retrieval using separate repeated measures ANOVAs with "block" (seven levels: blocks 1-7) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) as between subject factor. Post-hoc, we compared the effect change between from the last sequence block 6 to random block 7, which is considered a measure of implicit learning, within group and with uncorrected, two-tailed t-tests.

In all analyses, Mauchly's sphericity test was performed and Greenhouse–Geisser correction was applied when necessary. The level of significance was set at $p<0.05$. Post-hoc t-tests were calculated for the four-group comparisons and Bonferroni-corrected. A correlation between two parameters was determined by calculating Pearson's correlation coefficient and was reported if it was higher than 0.75 or lower than -0.75. The results are given as mean values ± one standard deviation.

RESULTS

Rating scales

Details of age, sex, disease duration, L-Dopa response and rating scales of MSA patients are displayed in **table 1**. UPDRS scores for motor impairment placed the patients in an intermediately impaired range. The Hamilton depression score revealed mild depressive symptoms (mean 12.5±6.2 out of 69 points) and the Mini-Mental State scored between 26 and 30 points (mean 28.0±1.4) indicating mild cognitive impairment in more than half of the patients. These results are comparable to the IPD and PSP groups reported earlier.[12 14] The UPDRS score did not differ between the three patient groups (factorial ANOVA; no effect of group, no post-hoc difference on Bonferroni-corrected t-tests); in addition, all four groups did not differ with regard to age.

Blink reflex pathways

Blink reflex responses in MSA patients showed normal latencies (ipsilateral R1: 11.6±1.0 ms, ipsilateral R2: 31.5±4.9 ms contralateral R2: 34.4±4.1 ms). An R2 recovery cycle could be obtained in all patients (**figure 1**) with no significant side difference between the ipsi- and contralatral R2 recovery. MSA patients showed significantly less R2 inhibition compared to the control group (repeated-measures ANOVA MSA-controls, effect of group, $F(1, 20)= 15.0$. $p=0.001$.

-- Please insert fig. 1 about here --

Conditioned eyeblink responses

All MSA patients showed few random blinks as assessed by the UCS only trials (3.0±6.7 % across both paradigms). The conditioned responses (CRs) were first analyzed for block 1-6 excluding the tone alone trials (see below). In either paradigm MSA patients showed significantly fewer CRs than the control group (**figure 2;** repeated-measures ANOVA MSA-control**,** effect of group, $F(1, 39)= 37.1$, $p<0.0001$; effect of block, $F(3.4, 39)= 7.0$, $p<0.0001$; interaction of group by block, $F(3.4, 266)= 3.325$, $p=0.017$, no main effect of paradigm). Adding the rate of alpha blinks as covariate to the ANOVA did not abolish the effect of group ($F(1, 38)= 31.5$, $p<0.0001$).

These results were supported by a separate analysis of the tone alone trials (trial 11, block 1-6), in which the MSA group yielded an average number of CRs of 14±17 % in the delay and 12±17 % in the trace paradigm, which was significantly less than the control group with 73±23 % and 55±27 % of CRs respectively (ANOVA MSA-control, effect of group, $F(1,37)=59.1$, $p<0.0001$). There was again no main effect of paradigm, i.e. trace and delay paradigm did not differ from each other.

~~Considering the MSA patients only, there was no difference in the occurrence of CRs between MSA-P and MSA-C patients (ANOVA, effect of MSA subtype, $F(1,17)=2.5$, $p=0.13$).~~

-- Please insert fig. 2 about here -

Alpha Blinks

In either paradigm MSA patients tended to show less alpha blinks than controls (repeated measures ANOVA, effect of group $F(1,38)=4.0$, $p=0.054$; **figure 3**.). The mean percentage of alpha blinks across all blocks in MSA patients was 17.6±4.6 % in the delay and 14.4±4.1 % in the trace paradigm, for control subjects 31.5±11.1 % and 35.2±11.3 % respectively. There were significantly more alpha blinks in earlier blocks than in late blocks for both paradigms (effect of block, $F(4.3,$

163.7)=8.5, p<0.0001). ~~Considering the MSA patients only, there was no statistically significant difference in the occurrence of alpha blinks between MSA-P and MSA-C patients~~.

-- Please insert fig. 3 about here --

Serial reaction time task (SRTT)

Reaction time

MSA patients showed longer reaction times compared with controls (repeated measures ANOVA MSA-control, effect of group, F(1,18)=20.2, p<0.0001; and a trend for an interaction of group by block, F(1.52, 27.34)=2.77, p=0.10, **figure 4A**). In both groups reaction times decreased from block 1 to the sequence blocks 3, 4, 5 and 6 (effect of block**,** F(1.52, 27.34)=5.5, p=0.016). The reaction time increase from sequence block 6 to random block 7, which is considered a measure of implicit learning, was significant in the control group only (t-test p<0.01; MSA p=0.1).

-- Please insert fig. 4 about here --

Accuracy errors

The average error rate of MSA patients across blocks was 19.7±4.2 %, which is significantly higher compared to controls with a rate of 2.6±0.8 % (repeated measures ANOVA MSA-control, effect of group, F(1,18)=10.1, p=0.005). In both groups error rates decreased from the first random to the sequence blocks (effect of block, F(3.37, 42.66)=3.9, p=0.022) and tended to increase between the last sequence block and the random block 7 without being significant.

Retrieval of sequence

There was no significant difference between MSA patients and controls in the measures of sequence detection (manual sequence retrieval, ANOVA MSA-control, effect of group, F(1,18)=0.7, p=0.42).

Both groups remembered more items of the sequence in post block reproduction of the last 10 items during the course of the experiment (ANOVA, effect of block, $F(3.58, 64.48)=31.0$, $p<0.001$). A small percentage of repetition was seen even before the sequence was presented, which indicates the baseline guessing rate (**figure 4B)**.

Correlation analyses for MSA patients

We did not find a significant correlation between the average number of CRs across block 3-6 (steady state) and the duration of disease, the Hamilton scale score, the MMST, the motor examination of the UPDRS, the cerebellar or autonomic impairment as assessed by the scores (see table 1) for either paradigm. The percentage of manual retrieval in the SRTT did not correlate with any of these parameters either.

Retrospective comparison of all patient groups (MSA, PSP, IPD) and the control group

In the blink reflex recovery cycle, the R2 inhibition was not different between the patient groups (figure 1). For the EBCC paradigms, the lack of eyeblink conditioning in MSA patients was similar to the PSP group and differed significantly from the increasing occurrence of CRs in IPD patients and controls (ANOVA, effect of group, $F(3,63)=23.2$, $p<0.0001$; interaction of group by block, $F(11.1, 233.0)=3.6$, $p<0.0001$; figure 2). Post-hoc t-test with Bonferroni correction confirmed a difference between MSA patients and PD patients, and between MSA patients and control subjects. Adding the rate of alpha blinks as covariate did not abolish the effect of group ($F(1, 32)= 16.7$, $p<0.0001$). Also in the tone alone trials, MSA and PSP patients showed fewer CRs than the IPD and control groups (ANOVA, effect of group, $F(3,64)=19.0$, $p<0.0001$; interaction of group by block, $F(15,320)=1.8$, $p=0.04$). MSA and PSP groups both showed fewer alpha blinks than IPD patients and controls (ANOVA, effect of group, $F(3,61)=3.5$, $p=0.02$; interaction of group by block ($F(12.73, 259.0)=2.0$, $p=0.025$; see figure 3). However, with post-hoc, Bonferroni-corrected t-tests these differences were not significant.

For the differentiation of MSA and PSP from IPD we considered the mean percentage of CRs in the steady state part of the EBCC learning curve (blocks 3-6). The mean percentage of CRs allows a non-overlapping separation of the MSA and PSP groups from IPD in the trace paradigm with a cutoff at 26% (**figure 5).** In the delay paradigm, the separation between groups was less complete. Whereas all PSP patients exhibited less than 26% of CRs, the fraction of MSA patients with values equal or below 26% was 0.91. This corresponds to sensitivity values for the separation of PSP and MSA from IPD of 100% and 91%, respectively. If the specificity is defined as the fraction of IPD patients with CRs values above 26%, the specificity in the delay paradigm is 75%. As reported above, the sensitivity and specificity values for the trace paradigm are 100%. The slightly better performance of IPD patients[13] as compared to controls was not significant.

-- Please insert fig. 5 about here --

In the SRTT, MSA patients showed significantly longer reaction times than the IPD patients, but no significant difference compared to the PSP group (ANOVA: effect of group $F(3,30)=7.4$, $p=0.001$; see figure 4). With regard to the error rate, MSA patients performed again very similar to the PSP patients, who showed 19.5±1.8 % accuracy errors, but significantly worse than the IPD patients (error rate 4.8±1.7 %; ANOVA, effect of group, $F(3,32)=6.1$, $p=0.002$). The sequence recall measurements revealed no statistically significant differences between groups.

DISCUSSION

The differentiation of IPD and atypical Parkinsonian syndromes (MSA, PSP) constitutes a challenge for neurologists, as the motor symptoms often present very similarly, in particular in the early stages. Additional markers such as imaging have been evaluated,[28 29] but these provide insufficient sensitivity values or are technically challenging. In addition, macroscopically discernible structural changes as detectable by MRI are likely to occur some time after functional loss has begun.

Therefore functional tests might be better suited because they reveal deficits before discernible structural changes occur. In this study we focus on the differential leaning abilities tested by eyeblink conditioning (EBCC) and a serial reaction time task (SRTT). First, the results of the MSA patients will be discussed, followed by a comparison with PSP and the putative impact for differentiation from IPD.

The MSA patients showed severely impaired implicit learning in the trace as well as in the delay eyeblink conditioning paradigm, with standard deviations in the range of other studies,[10 30] whereas non-learning associated blink reflex latencies as indicators of oculomotor pathways were normal.

Histopathological alterations in both subtypes of MSA include the substantia nigra, putamen, descending and ascending fiber tracts of the motor system, olivary and pontine nuclei, brainstem-cerebellar circuits as well as cerebellar structures (hemispheres and vermis).[31-33] This has been confirmed *in vivo* by diffusion tensor imaging of white matter microstructure.[34] We suggest that damage of cerebellar structures and associated brainstem-cerebellar circuits are responsible for the failure of EBCC learning in MSA patients. This assumption is supported by the findings of impaired EBCC in patients with cerebellar damage,[27 35-37] positron-emission tomography (PET) measurements in healthy humans showing changes in glucose metabolism in the cerebellum and pons during EBCC[23 38] as well as in experiments studying the influence of selective pharmacological blockade of cerebellar input on EBCC in rabbits.[39] Most patients in our study were clinically characterized as MSA-P and not MSA-C. However, the failure in blink reflex acquisition in both groups points to a subclinical cerebellar involvement in MSA-P, which is in accordance with the histopathological studies.[32 33] EBCC therefore seems to detect cerebellar involvement at a subclinical stage.

In addition to the cerebellum, several studies indicate that acquisition of CR in the trace paradigm depends on forebrain areas such as the hippocampus or prefrontal cortex, in particular for longer interstimulus intervals.[40 41] In our study, the failure of CR acquisition in MSA patients was

slightly more pronounced in the trace compared to the delay paradigm. Therefore, lesions of the frontal lobe, which have been suggested by neuropsychological testing[6 42] and confirmed histopathologically in a variety of MSA cases,[43 44] may have contributed to impaired EBCC acquisition in the trace paradigm.

An alternative explanation that was brought up by an anonymous reviewer is that the tone may be a less salient CS to the MSA patients than to the control group. The reduced number of alpha blinks would support this assumption. Following that very elegant line of thought, the EBCC group difference between MSA patients and control subjects would have to do less with implicit learning and more with responsiveness and associative processes related to external stimuli. While this may have some relevance, adding the number of alpha blinks to the ANOVAs on conditioned eyeblink responses did not abolish the between-group differences.

In the SRTT the MSA patients showed severe impairment in terms of prolonged reaction time, high error rate and, in some patients, early discontinuation due to fatigue and exhaustion. In contrast to the control group they showed no significant reaction time increase between block 6 (random) and 7 (sequence) and this is indicative of implicit learning deficits. On the other hand they showed good performance on the parameters of sequence recall (explicit learning). This preservation of SRTT explicit learning parts may be explained by the relative preservation of posterior association (temporal and parietal) cortex and hippocampus in MSA. It has to be interpreted with some caution, though, given limitations of spatial working memory in MSA.[42] However, the validity of the SRTT learning results is limited by the discontinuation of patients and our "last observation carried forward approach" (see Methods). In addition, the patients' wide range of motor impairment, which may interfere with the motor part of the task, and the fact that sequence learning and movement preparation seem to share similar attentional and working memory resources[45] have to be considered. Therefore the SRTT seems to be inappropriate to assess learning abilities in MSA patients. This is in contrast to the EBCC, which is independent of the motor performance of

patients. Furthermore, EBCC circuits are located anatomically closer to the affected brainstem regions.

With all the limitations of such a retrospective comparison of data acquired in different patient groups by the same authors, the implicit learning impairment in MSA patients as clearly revealed by the EBCC and with some limitation by the SRTT parallels the previously reported findings in PSP patients.[14] Interestingly, MSA and PSP are characterized by different histopathological alterations, α-synuclein positive inclusions versus tau-positive aggregations, which lead to presume different pathophysiological mechanisms. However, the common involvement of cerebellar structures in both diseases[31 46] seems to be responsible for the clinical phenomenology independent of the cellular mechanism.

In contrast to MSA and PSP, IPD patients show normal[12] or even enhanced[13] acquisition of conditioned responses in EBCC. EBCC may therefore contribute to distinguish IPD from these atypical Parkinsonian syndromes. As the development of cerebellar and related neuropathology in MSA or PSP often occurs prior to or even without clinical manifestation,[33 46] we propose impaired EBCC particularly in the trace paradigm as an early indicator of neurodegeneration of areas beyond those typically affected in IPD. However, as the retrospective synopsis is clearly limited, the pivotal questions of whether EBCC can serve as predictor for the development of typical or atypical disease and whether EBCC is a useful addition to imaging techniques in establishing an early differential diagnosis are unanswered yet and require further prospective investigation.

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Contributors

All authors listed above fulfill all three International Committee of Medical Journal Editors (ICMJE) guidelines for authorship which are (1) substantial contributions to conception and design, acquisition of data or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be published. MSo, CT and WP conceived the study. MSch, FvL and MSo were responsible for obtaining the data. FvL and MSo conducted the data analysis. All authors contributed to decisions on the interpretation of results. FvL and MSo contributed to the drafting of the manuscript. CT and WP were responsible for editing and providing guidance on the paper. All authors were responsible for critically revising the paper. All authors approved the final version of the manuscript prior to submission.

Competing interests None

Ethics approval Ethics committee of the Medical Faculty of the University of Goettingen.

Data sharing statement There are no additional data available.

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Legends to tables and figures

Table 1: Characteristics of the MSA patients. Type: Parkinsonian (MSA-P) or cerebellar (MSA-C) predominance; LED = L-Dopa equivalent dose, + indicates additional budipine medication, # additional anticholinergic medication; UPDRS = Unified Parkinson's disease rating scale, motor examination only (high number of points indicates high disability); MMS= Mini Mental State (30 points are normal, ≤26 is usually considered as cognitive impairment). Cerebellar impairment was evaluated for ataxia (arm, leg), saccades and intention tremor (finger-nose test), autonomic impairment for postural faintness, syncopes, urinary incontinence, urinary retention, faecal incontinence and impotence (males only). Pyramidal tract impairment was scored for hyperreflexia and Babinski sign. Each item was scored if present with 1, otherwise 0. In the Hamilton scale a low score indicates few depressive symptoms. *not investigated.

Table 2: Characteristics of controls, IPD and PSP patients in part taken from earlier publications.[12] [14] Dementia had been ruled out using the Mattis Dementia Rating scale (MDRS)[47 48], where higher scores out of a maximum of 144 indicate better performance, with a cut-off ≤123 considered as cognitive impairment[49]. Depression had been assessed using the Beck Depression Inventory (BDI), where higher scores out of a maximum of 63 points indicate a more severe depressed state, and a score of 15 is regarded as cut off for a self report of mild depression.[50 51] *not investigated. The MDRS was not available at the German study sites.

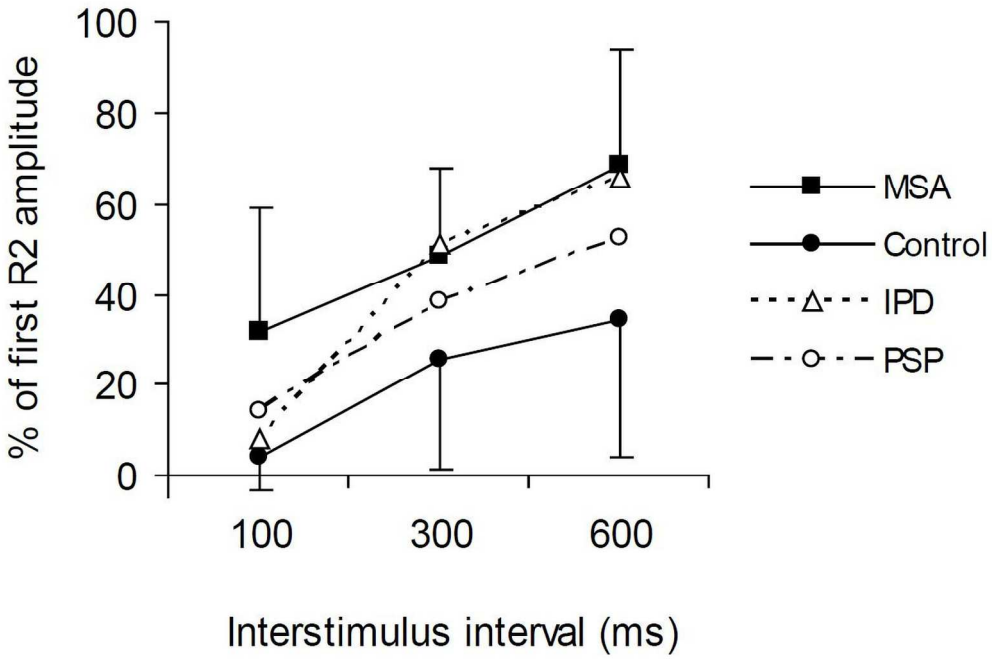
Figure 1: Blink reflex recovery cycle with interstimulus intervals of 100, 300 and 600 ms. In MSA patients, the inhibition of the ipsilateral R2 response to the second stimulus is weaker than in the control group. Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 2: Conditioned eyeblink responses in two different paradigms: left, delay (interstimulus interval 0 ms), right, trace paradigm (interstimulus interval 600 ms). In both paradigms, the number of conditioned responses was significantly lower in MSA and PSP patients than in the control and IPD groups indicating impaired implicit learning. CS, conditioned stimulus (tone), UCS, unconditioned stimulus (electrical stimulation to the supraorbital nerve). Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 3: Occurrence of 'alpha-blinks'. These bursts are a startle reaction to the tone (CS) and are less frequent in MSA and PSP patients than in the control and IPD groups. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Data are indicated as average value and single standard deviation and were pooled for both paradigms.

Figure 4: A Reaction time in a serial reaction time task (SRTT). An implicit learning effect is indicated by the reaction time increase between the last sequence block (6) and the following random block (7). **B** Explicit learning in the SRTT was tested after each block by manual retrieval of the sequence (repetition of the last 10 key presses) and revealed no significant difference between groups. Data are indicated as average value and single standard deviation. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Asterisks indicate a significant difference for the comparison of blocks 6 and 7 ($p<0.05$, post-hoc t-test).

Figure 5: Overview of the mean percentage of conditioned eyeblink responses (CRs) across blocks 3-6 in the EBCC paradigms in MSA patients and control subjects from this study and in IPD and PSP patients from earlier studies.[12 14] With the trace paradigm a complete separation between IPD and the atypical Parkinsonian syndromes MSA and PSP is achieved (cutoff 26%), whereas in the delay paradigm there is a small overlap between these groups. Overall, IPD patients perform slightly better than control subjects,[13] further enhancing the group distinction between IPD and atypical syndromes.



Blink reflex recovery cycle with interstimulus intervals of 100, 300 and 600 ms. In MSA patients, the inhibition of the ipsilateral R2 response to the second stimulus is weaker than in the control group. Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.
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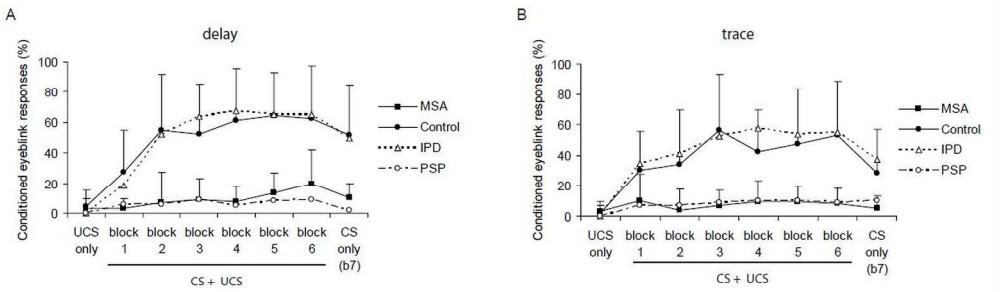

Figure 2, von Lewinski et al.

Conditioned eyeblink responses in two different paradigms: left, delay (interstimulus interval 0 ms), right, trace paradigm (interstimulus interval 600 ms). In both paradigms, the number of conditioned responses was significantly lower in MSA and PSP patients than in the control and IPD groups indicating impaired implicit learning. CS, conditioned stimulus (tone), UCS, unconditioned stimulus (electrical stimulation to the supraorbital nerve). Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.
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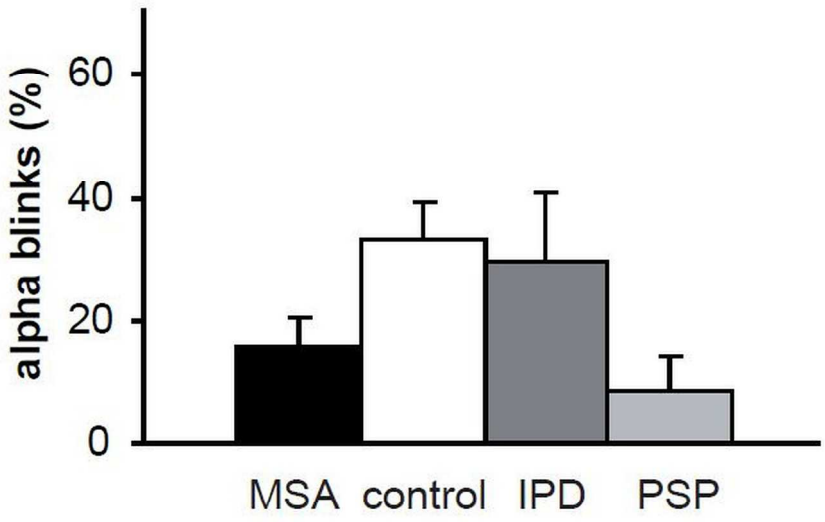

Figure 3, von Lewinski et al.

Occurrence of 'alpha-blinks'. These bursts are a startle reaction to the tone (CS) and are less frequent in MSA and PSP patients than in the control and IPD groups. Data for IPD and PSP patients were taken from our earlier studies using identical methods. Data are indicated as average value and single standard deviation and were pooled for both paradigms.
193x205mm (300 x 300 DPI)

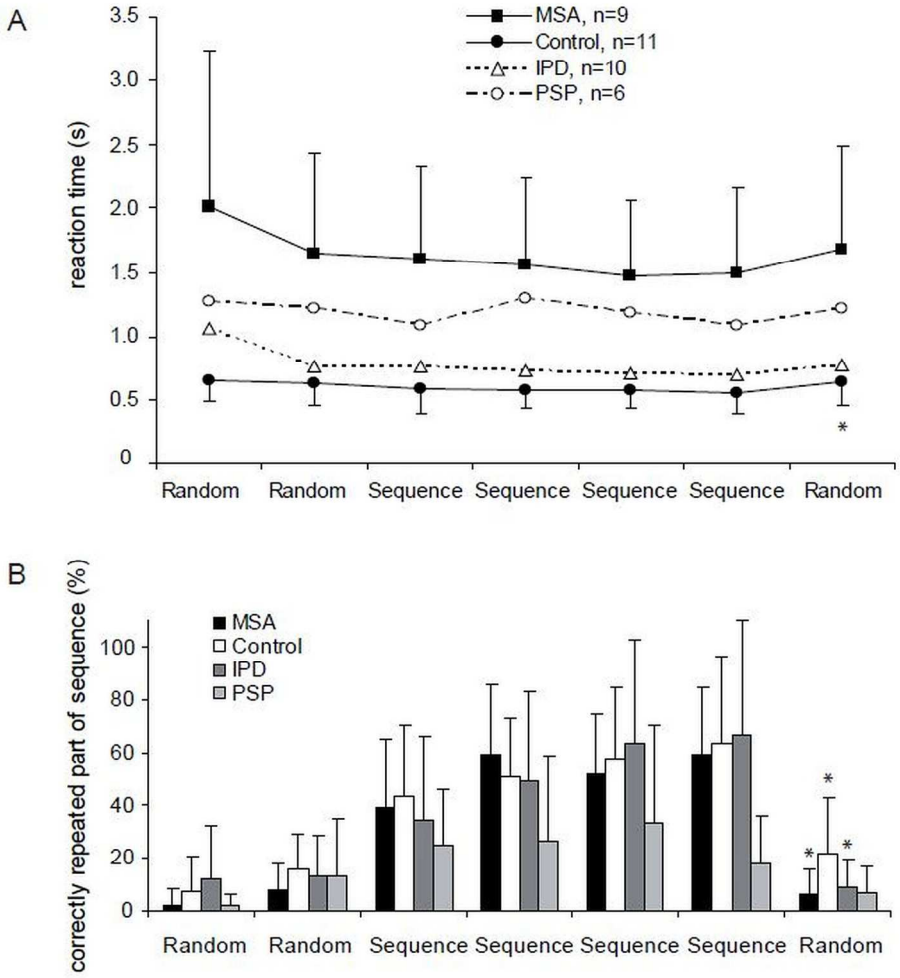

Figure 4, von Lewinski et al.

A Reaction time in a serial reaction time task (SRTT). An implicit learning effect is indicated by the reaction time increase between the last sequence block (6) and the following random block (7). B Explicit learning in the SRTT was tested after each block by manual retrieval of the sequence (repetition of the last 10 key presses) and revealed no significant difference between groups. Data are indicated as average value and single standard deviation. Data for IPD and PSP patients were taken from our earlier studies using identical methods.12 14 Asterisks indicate a significant difference for the comparison of blocks 6 and 7 ($p<0.05$)
189x226mm (300 x 300 DPI)

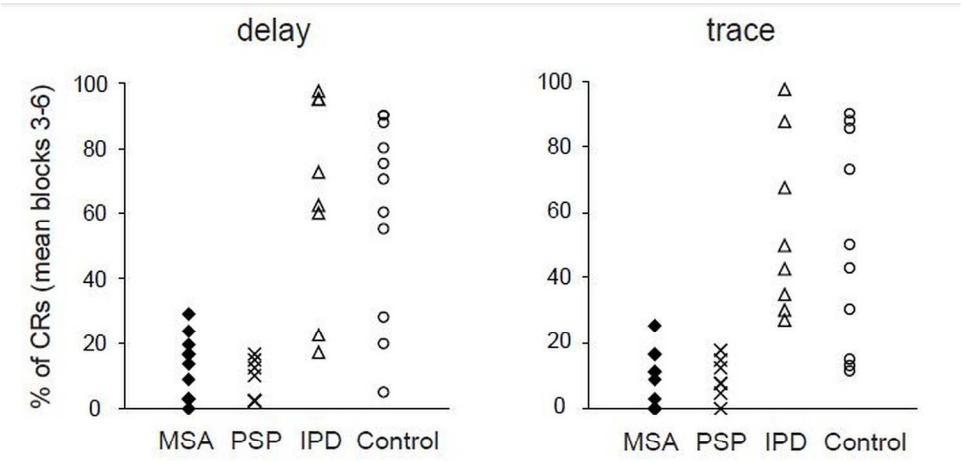

Figure 5, von Lewinski et al.

Overview of the mean percentage of conditioned eyeblink responses (CRs) across blocks 3-6 in the EBCC paradigms in MSA patients and control subjects from this study and in IPD and PSP patients from earlier studies. With the trace paradigm a complete separation between IPD and the atypical Parkinsonian syndromes MSA and PSP is achieved (cutoff 26%), whereas in the delay paradigm there is a small overlap between these groups. Overall, IPD patients perform slightly better than control subjects, further enhancing the group distinction between IPD and atypical syndromes.
187x234mm (300 x 300 DPI)

Impairment of brainstem implicit learning paradigms differentiates multiple system atrophy (MSA) from idiopathic Parkinson syndrome

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Key words: Eyeblink classical conditioning (EBCC), multiple system atrophy (MSA), implicit and explicit learning, serial reaction time task (SRTT), non-motor symptoms

Word count (excl. Title page, Abstract, References and Figures/Tables): 3757

ABSTRACT

Objectives: Learning as measured by eyeblink classical conditioning is preserved in patients with idiopathic Parkinson's disease, but severely affected in patients with progressive supranuclear palsy. We here sought to clarify whether procedural learning is impaired in multiple system atrophy, and whether it may be helpful for the differentiation of Parkinsonian syndromes.

Design: We investigated learning using (1) eyeblink classical conditioning with a delay (interstimulus interval 0 ms) and a trace (600 ms) paradigm and (2) a serial reaction time task.

Setting: Participants were recruited from academic research centers.

Participants: 11 patients with multiple system atrophy and 11 healthy controls.

Results: Implicit learning in eyeblink classical conditioning (acquisition of conditioned responses) as well as the serial reaction time task measures of implicit learning (reaction time change) are impaired in multiple system atrophy patients as compared to controls, whereas explicit learning as measured by the sequence recall of the serial reaction time task is relatively preserved.

Analysis: We hypothesize that the MSA patients' learning deficits are due to lesions of cerebellar and connected brainstem areas.

Conclusions: A retrospective synopsis of these novel data on multiple system atrophy patients and groups of idiopathic Parkinson's disease patients and progressive supranuclear palsy patients studied earlier suggests that eyeblink classical conditioning may contribute to the early differentiation of atypical Parkinson syndromes from idiopathic Parkinson's disease. This hypothesis should be tested in a prospective trial.

ARTICLE SUMMARY:

Article focus:

- We tested if the non-motor feature of procedural learning is impaired in patients afflicted with multiple system atrophy.
- We studied the eyeblink classical conditioning and a serial reaction time task in 11 MSA patients and matched control subjects.

Key messages:

- Blink reflex pathways were intact, but eyeblink conditioning was significantly impaired in MSA patients. By contrast, the serial reaction time task was difficult and inconclusive in these patients due to motor constraints impairing finger tapping.
- A retrospective comparison with previously studied groups patients with idiopathic Parkinson's disease or Progressive Supranuclear Palsy points to a putative role of eyeblink conditioning in distinguishing typical from atypical Parkinsonian disorders.

Strength and limitations:

- The study differentiates feasible and non-feasible assessments of procedural learning in multiple system atrophy.
- The comparison to other patient groups is clearly retrospective and needs to be validated by a prospective trial.

INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurodegenerative disorder characterized by an absent or poorly levodopa responsive Parkinsonism, cerebellar dysfunction and autonomic failure.[1] A predominantly Parkinsonian (MSA-P) and a cerebellar subtype (MSA-C) are recognized. Despite the development of consensus criteria,[2] the differential diagnosis between MSA and other hypokinetic rigid syndromes, such as idiopathic Parkinson's disease (IPD) or progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome, PSP), remains a clinical challenge.[3 4]

Recent years have seen an increasing focus on non-motor symptoms such as cognitive deficits in Parkinsonian disorders. Different patterns of cognitive impairment including deficits in executive function and learning abilities have been described.[5-9]

A well established task to study associative, procedural learning[10] is eyeblink classical conditioning (EBCC), which some regard as a model of implicit learning[11]. Previous studies have shown that learning as measured by EBCC is normal in patients with IPD, but, severely affected in patients with PSP.[12-14] In contrast to tracking or pointing tasks,[15 16] EBCC has the advantage not to depend on manual motor skills. Learning assessed by the serial reaction time task (SRTT) showed the implicit motor skill close to normal in IPD patients, whereas PSP patients were markedly impaired; in contrast, the SRTT sequence recall component as measure of explicit learning was largely preserved in both groups.[12 14] We sought to investigate whether implicit learning deficits are specific for PSP or present in MSA as well, thereby comparing the clinical feasibility of SRTT and of EBCC in this patient group.

METHODS

Subjects

11 MSA patients were recruited from the outpatient clinics in Munich and Göttingen between 1999 and 2008 (**table 1**). The clinical diagnosis of "probable MSA" was established following consensus criteria.[2] 7 MSA patients were taking L-Dopa, two dopamine agonists (ropinirole, pramipexole),

one amantadine, one budipine, one biperiden, another metixen, and two were taking antidepressants. L-Dopa equivalent doses (LEDs) were calculated according to Tomlinson and colleagues[17] except for budipine, biperiden and metixen, where no conversion factor was given.

To rule out an immediate impact of medication on the patients' memory performance, the anti-parkinsonian medication was discontinued on the morning of the day of the study. MSA patients were compared with 11 healthy control subjects, matched for age (t-test), and chosen for the absence of neurodegenerative or any other neurological disease, and for the absence of intake of CNS-active medication (mean age 59.5±10.0 years, 6 male, 5 female). A subgroup was already involved in our earlier published study (numbers 2,3,5,6,8,9,11,12,14 according to Table 2 in[12]). All participants gave written informed consent; the research protocol was approved by the local ethics committee. Neither the patients nor the control subjects had any sign of cranial nerve impairment or auditory deficits in routine neurological examination.

Table 1

Pat Nr.	MSA Type	Age [year]	Sex	Duration [year]	L-Dopa response	LED [mg]	UPDRS Max=108	Cerebellar Max=4	Autonomic Max=5[f], 6[m]	Pyramidal Max=2	Hamilton Max=69	MMS Max=30
1	P	66	F	9	Poor	0+	50	0	1	0	11	27
2	P	69	M	4.5	Poor	125	20	0	2	0	20	30
3	P	73	M	8	Absent	255	16	0	3	0	15	28
4	P	59	F	1.5	Poor	125#	30	0	2	1	16	26
5	P	71	M	4	Absent	150	35	0	4	0	6	29
6	P	75	M	5	Modest	524	38	0	3	0	6	29
7	P	75	F	3	Poor	375	40	0	3	0	10	28
8	P	58	M	3	Poor	105#	18	0	1	0	2	30
9	C	64	M	2	Poor	900	69	2	2	0	22	27
10	C	56	M	2.5	*	0	5	3	1	0	16	28
11	C	60	F	8	*	0	30	3	3	1	14	26
Mean		66.0		4.6			31.9	0.7	2.3	0.2	12.5	28.0
S.D.		7.1		2.6			17.7	1.3	1.0	0.4	6.2	1.4

Clinical testing procedures

The Hamilton rating scale for depression[18] and the Mini-Mental state examination[19] were used to quantify the affective and general cognitive status, respectively, with pragmatic and established

tests. Motor features in patients were evaluated with the Unified Parkinson Disease Rating Scale (UPDRS, part III).[20] Further clinical assessments are listed in table 1.

To investigate eyeblink function we used the blink reflex (BR) and its R2 recovery cycle detailed elsewhere.[12 21 22] In brief, a single electrical stimulation of the supraorbital nerve (duration: 0.2 ms) elicits an early unilateral (R1) and a late bilateral (R2) blink response. Paired-pulse supraorbital stimulation probes brainstem interneuronal excitability and yields an inhibition of the R2 of the second stimulus. We used interstimulus intervals (ISIs) of 100 ms, 300 ms and 600 ms on both sides. For EBCC we stimulated the side in which amplitudes and latencies were within or close to the normal range or arbitrarily the right supraorbital nerve if no side-differences were found.

EBCC-implicit learning

The procedures were virtually identical to and detailed in the earlier studies from our group.[12 14] In brief, an unconditioned stimulus, i.e. en electric pulse over the supraorbital nerve, invariably induces an eyeblink reflex. It is paired with a preceding conditioned stimulus, i.e. a tone, which by itself may elicit early startle responses (so called alpha blinks, within 200 ms after the tone). With repeated presentation of the tone and the electric pulse, a conditioned eyeblink response is expected to occur before the onset of the electrical stimulus. In the current study, the tone (conditioned stimulus, CS, 400 ms duration) was generated by a custom built sound generator (Department of Electronic Engineering, University of Goettingen) and presented via earphones (Cherry Inc., Japan) at an intensity perceived as very loud by each subject, usually 80 dB. A brief electrical stimulus to the supraorbital nerve eliciting a blink reflex served as unconditioned stimulus (UCS). We tested the EBCC with a two different interstimulus intervals between the end of the tone and the beginning of the electric pulse, either ISI 0 ms (delay paradigm) or 600 ms (trace paradigm), in randomized order. For each paradigm we administered six learning blocks, each with CS and UCS in trials 1-9, UCS only in trial 10 (to control for random blinks) and CS only in trial 11 (to test for a persistent learning

effect). To control for extinction of learned responses a seventh block consisted of 11 trials with CS only.[23] The intertrial interval was randomized between 10 and 30 seconds.

Electromyographic (EMG) activity was recorded using silver-silverchloride cup electrodes fixed with adhesive tape over the lower eyelid (active electrode) and over the ipsilateral temple (reference electrode); with a sampling rate of 10 kHz.[12 14] EMG signals were fed into a recording device and filtered at 100 Hz and 5 kHz (SynAmps, Neuroscan Inc, Virginia, USA). To detect any ongoing muscular activity we recorded 400 ms before and 1600 ms after CS onset.

Serial reaction time task (SRTT)

The SRTT is established as a test of implicit learning.[12 24] Subjects were sitting in front of a computer screen, and were told that single asterisks would appear in one out of four positions on a computer screen. They were instructed to press a marked key on a computer keyboard that was underneath the position of the asterisk on the screen. The asterisks were presented in three random blocks (blocks 1, 2, 7) and four identical sequence blocks (blocks 3-6), in each of which a sequence of 10 elements (CBDABCDCBA) was presented 10 times. After each block subjects were asked to repeat the last 10 asterisk positions manually on the computer keyboard, which may have accentuated explicit aspects of the task.[25 26] We analyzed reaction time, errors and number of correctly repeated parts of the sequence. This test was difficult for many patients: Only 6 patients completed the test as required, one patient discontinued after block 1 and was excluded from the analysis. Two others discontinued after block 4, one after block 3. To enable some kind of statistical analysis, the result that these patients reached in their last sequence block was carried forward to the following sequence blocks, and the result of the second random block was assumed for block 7. One patient apparently responded with random typing to the letters presented and was therefore excluded from the analysis.

Comparison of MSA patients with PSP and IPD patients studied earlier

While clearly retrospective, we have taken the liberty to compare the MSA patients results obtained here with the group of PSP patients that we studied in 2001[14] and a subgroup of IPD patients studied in 1999 with identical electrophysiological methods (numbers 1-4 and 6-11 according to Table 1 in[12], selected to match as good as possible the current MSA group with regard to the disease severity (according to UPDRS part III), even though retrospective matching based in part on different scales used in different laboratories is certainly not perfect. Demographical data are cited in table 2. In figure 1, 2 and 4, data from these IPD and PSP patients are given in dashed lines.

Table 2

Nr.	group	Age [year]	Sex	Dura-tion [year]	UPDRS Max=108	BDI Max=63	MMS Max=30	MDRS Max=144
1	C	57	m	-	-	2		144
2	C	60	f	-	-	9		142
3	C	50	m	-	-	0		141
4	C	64	f	-	-	0		142
5	C	58	m	-	-	1		138
6	C	73	m	-	-	6		134
7	C	49	f	-	-	0		143
8	C	45	m	-	-	1		144
9	C	53	m	-	-	1		142
10	C	73	f	-	-	11	30	
11	C	72	f	-	-	0	30	
Mean		59.5				2.6		
S.D.		10.0				3.6		
1	IPD	69	f	2	45	11		138
2	IPD	64	f	6	39	15		143
3	IPD	62	m	5	21	9		132
4	IPD	45	m	6	28	5		141
5	IPD	47	m	7	31	6		139
6	IPD	49	m	7	25	6		140
7	IPD	64	m	9	47	11		135
8	IPD	63	m	8	16	11		141
9	IPD	61	m	5	33	8		143
10	IPD	50	m	3	44	11		143
Mean		57.4		5.8	32.9	9.3		139.5
S.D.		8.7		2.1	10.7	3.1		3.7
1	PSP	54	m	2	22	6	30	127
2	PSP	69	m	9	34	0	28	110
3	PSP	65	f	2	44	13	28	107
4	PSP	57	f	3	30	35	30	135
5	PSP	66	m	2	30	13	28	135
6	PSP	59	m	6	50	4	22	100
7	PSP	68	f	4	43	50	18	116
8	PSP	63	m	5	54	*	23	112
Mean		62.6		4.1	38.4	17.3	25.9	117.8
S.D.		5.4		2.5	11.1	18.4	4.4	13.1

Data analysis

UPDRS scores in the patient groups, and age in all four groups, were compared using factorial ANOVAs with group (three or four levels) as between-subject factor. R2 latencies were measured off-line. For analysis of the blink reflex recovery cycle, we entered the R2 amplitudes of the second pulse normalized to the R2 amplitudes of the first pulse into a repeated measures analysis of variance (ANOVA) with "interstimulus interval" (three levels: 100; 300, 600 ms) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) as

between subject factor.[12 21 22] In the EBCC, EMG bursts were regarded as present if their peak-to-peak amplitude exceeded baseline noise by at least 1.5 fold and reached at least 50 µV. They were counted as alpha-blinks, i.e. startle responses, or conditioned responses (CRs) if they occurred within the appropriate time window (alpha blinks: within 200ms after onset of tone (CS); CRs: within 200 ms before electrical stimulus (UCS)). For the tone-alone-trials we extended the time window until 300 ms after the end of the UCS to detect delayed CRs.[27] Random blinks were counted as EMG bursts occurring in the CR time window in the absence of a CS, i.e. in the UCS only trials. Their occurrence rate was reported numerically. We analyzed the percentage of conditioned eyeblink responses repeated measures ANOVAs with "block" (six levels: blocks 1-6) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors. In addition, we repeated the ANOVAs for conditioned eyeblink responses with the individual average alpha blink rate across blocks 1-6 as covariate. We calculated separate repeated measures ANOVAs for the tone alone trials (trial 11, block 1-6), with "block" (six levels: blocks 1-6) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors. For alpha blink rate, we calculated a repeated-measures ANOVA with "block" (seven levels: blocks 1-6 and CS only block) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) and "paradigm" (two levels: delay versus trace) as between subject factors.

For SRTT, we analyzed reaction time, accuracy errors and retrieval using separate repeated measures ANOVAs with "block" (seven levels: blocks 1-7) as within subject factor and "group" (two levels: control and MSA; or four levels: control, MSA, IPD, PSP) as between subject factor. Post-hoc, we compared the effect change between from the last sequence block 6 to random block 7, which is considered a measure of implicit learning, within group and with uncorrected, two-tailed t-tests.

In all analyses, Mauchly's sphericity test was performed and Greenhouse–Geisser correction was applied when necessary. The level of significance was set at $p<0.05$. Post-hoc t-tests were calculated for the four-group comparisons and Bonferroni-corrected. A correlation between two parameters was determined by calculating Pearson's correlation coefficient and was reported if it was higher than 0.75 or lower than -0.75. The results are given as mean values ± one standard deviation.

RESULTS

Rating scales

Details of age, sex, disease duration, L-Dopa response and rating scales of MSA patients are displayed in **table 1**. UPDRS scores for motor impairment placed the patients in an intermediately impaired range. The Hamilton depression score revealed mild depressive symptoms (mean 12.5±6.2 out of 69 points) and the Mini-Mental State scored between 26 and 30 points (mean 28.0±1.4) indicating mild cognitive impairment in more than half of the patients. These results are comparable to the IPD and PSP groups reported earlier.[12 14] The UPDRS score did not differ between the three patient groups (factorial ANOVA; no effect of group, no post-hoc difference on Bonferroni-corrected t-tests); in addition, all four groups did not differ with regard to age.

Blink reflex pathways

Blink reflex responses in MSA patients showed normal latencies (ipsilateral R1: 11.6±1.0 ms, ipsilateral R2: 31.5±4.9 ms contralateral R2: 34.4±4.1 ms). An R2 recovery cycle could be obtained in all patients (**figure 1**) with no significant side difference between the ipsi- and contralatral R2 recovery. MSA patients showed significantly less R2 inhibition compared to the control group (repeated-measures ANOVA MSA-controls, effect of group, $F(1, 20)= 15.0$. $p=0.001$.

-- Please insert fig. 1 about here --

Conditioned eyeblink responses

All MSA patients showed few random blinks as assessed by the UCS only trials (3.0±6.7 % across both paradigms). The conditioned responses (CRs) were first analyzed for block 1-6 excluding the tone alone trials (see below). In either paradigm MSA patients showed significantly fewer CRs than the control group (**figure 2;** repeated-measures ANOVA MSA-control**,** effect of group, $F(1, 39)= 37.1$, $p<0.0001$; effect of block, $F(3.4, 39)= 7.0$, $p<0.0001$; interaction of group by block, $F(3.4, 266)= 3.325$, $p=0.017$, no main effect of paradigm). Adding the rate of alpha blinks as covariate to the ANOVA did not abolish the effect of group ($F(1, 38)= 31.5$, $p<0.0001$).

These results were supported by a separate analysis of the tone alone trials (trial 11, block 1-6), in which the MSA group yielded an average number of CRs of 14±17 % in the delay and 12±17 % in the trace paradigm, which was significantly less than the control group with 73±23 % and 55±27 % of CRs respectively (ANOVA MSA-control, effect of group, $F(1,37)=59.1$, $p<0.0001$). There was again no main effect of paradigm, i.e. trace and delay paradigm did not differ from each other.

~~Considering the MSA patients only, there was no difference in the occurrence of CRs between MSA-P and MSA-C patients (ANOVA, effect of MSA subtype, $F(1,17)=2.5$, $p=0.13$).~~

-- Please insert fig. 2 about here -

Alpha Blinks

In either paradigm MSA patients tended to show less alpha blinks than controls (repeated measures ANOVA, effect of group $F(1,38)=4.0$, $p=0.054$; **figure 3**.). The mean percentage of alpha blinks across all blocks in MSA patients was 17.6±4.6 % in the delay and 14.4±4.1 % in the trace paradigm, for control subjects 31.5±11.1 % and 35.2±11.3 % respectively. There were significantly more alpha blinks in earlier blocks than in late blocks for both paradigms (effect of block, $F(4.3,$

163.7)=8.5, p<0.0001). ~~Considering the MSA patients only, there was no statistically significant difference in the occurrence of alpha blinks between MSA-P and MSA-C patients~~.

-- Please insert fig. 3 about here --

Serial reaction time task (SRTT)

Reaction time

MSA patients showed longer reaction times compared with controls (repeated measures ANOVA MSA-control, effect of group, F(1,18)=20.2, p<0.0001; and a trend for an interaction of group by block, F(1.52, 27.34)=2.77, p=0.10, **figure 4A**). In both groups reaction times decreased from block 1 to the sequence blocks 3, 4, 5 and 6 (effect of block**,** F(1.52, 27.34)=5.5, p=0.016). The reaction time increase from sequence block 6 to random block 7, which is considered a measure of implicit learning, was significant in the control group only (t-test p<0.01; MSA p=0.1).

-- Please insert fig. 4 about here --

Accuracy errors

The average error rate of MSA patients across blocks was 19.7±4.2 %, which is significantly higher compared to controls with a rate of 2.6±0.8 % (repeated measures ANOVA MSA-control, effect of group, F(1,18)=10.1, p=0.005). In both groups error rates decreased from the first random to the sequence blocks (effect of block, F(3.37, 42.66)=3.9, p=0.022) and tended to increase between the last sequence block and the random block 7 without being significant.

Retrieval of sequence

There was no significant difference between MSA patients and controls in the measures of sequence detection (manual sequence retrieval, ANOVA MSA-control, effect of group, F(1,18)=0.7, p=0.42).

Both groups remembered more items of the sequence in post block reproduction of the last 10 items during the course of the experiment (ANOVA, effect of block, $F(3.58, 64.48)=31.0$, $p<0.001$). A small percentage of repetition was seen even before the sequence was presented, which indicates the baseline guessing rate (**figure 4B)**.

Correlation analyses for MSA patients

We did not find a significant correlation between the average number of CRs across block 3-6 (steady state) and the duration of disease, the Hamilton scale score, the MMST, the motor examination of the UPDRS, the cerebellar or autonomic impairment as assessed by the scores (see table 1) for either paradigm. The percentage of manual retrieval in the SRTT did not correlate with any of these parameters either.

Retrospective comparison of all patient groups (MSA, PSP, IPD) and the control group

In the blink reflex recovery cycle, the R2 inhibition was not different between the patient groups (figure 1). For the EBCC paradigms, the lack of eyeblink conditioning in MSA patients was similar to the PSP group and differed significantly from the increasing occurrence of CRs in IPD patients and controls (ANOVA, effect of group, $F(3,63)=23.2$, $p<0.0001$; interaction of group by block, $F(11.1, 233.0)=3.6$, $p<0.0001$; figure 2). Post-hoc t-test with Bonferroni correction confirmed a difference between MSA patients and PD patients, and between MSA patients and control subjects. Adding the rate of alpha blinks as covariate did not abolish the effect of group ($F(1, 32)= 16.7$, $p<0.0001$). Also in the tone alone trials, MSA and PSP patients showed fewer CRs than the IPD and control groups (ANOVA, effect of group, $F(3,64)=19.0$, $p<0.0001$; interaction of group by block, $F(15,320)=1.8$, $p=0.04$). MSA and PSP groups both showed fewer alpha blinks than IPD patients and controls (ANOVA, effect of group, $F(3,61)=3.5$, $p=0.02$; interaction of group by block ($F(12.73, 259.0)=2.0$, $p=0.025$; see figure 3). However, with post-hoc, Bonferroni-corrected t-tests these differences were not significant.

For the differentiation of MSA and PSP from IPD we considered the mean percentage of CRs in the steady state part of the EBCC learning curve (blocks 3-6). The mean percentage of CRs allows a non-overlapping separation of the MSA and PSP groups from IPD in the trace paradigm with a cutoff at 26% (**figure 5).** In the delay paradigm, the separation between groups was less complete. Whereas all PSP patients exhibited less than 26% of CRs, the fraction of MSA patients with values equal or below 26% was 0.91. This corresponds to sensitivity values for the separation of PSP and MSA from IPD of 100% and 91%, respectively. If the specificity is defined as the fraction of IPD patients with CRs values above 26%, the specificity in the delay paradigm is 75%. As reported above, the sensitivity and specificity values for the trace paradigm are 100%. The slightly better performance of IPD patients[13] as compared to controls was not significant.

-- Please insert fig. 5 about here --

In the SRTT, MSA patients showed significantly longer reaction times than the IPD patients, but no significant difference compared to the PSP group (ANOVA: effect of group $F(3,30)=7.4$, $p=0.001$; see figure 4). With regard to the error rate, MSA patients performed again very similar to the PSP patients, who showed 19.5±1.8 % accuracy errors, but significantly worse than the IPD patients (error rate 4.8±1.7 %; ANOVA, effect of group, $F(3,32)=6.1$, $p=0.002$). The sequence recall measurements revealed no statistically significant differences between groups.

DISCUSSION

The differentiation of IPD and atypical Parkinsonian syndromes (MSA, PSP) constitutes a challenge for neurologists, as the motor symptoms often present very similarly, in particular in the early stages. Additional markers such as imaging have been evaluated,[28 29] but these provide insufficient sensitivity values or are technically challenging. In addition, macroscopically discernible structural changes as detectable by MRI are likely to occur some time after functional loss has begun.

Therefore functional tests might be better suited because they reveal deficits before discernible structural changes occur. In this study we focus on the differential leaning abilities tested by eyeblink conditioning (EBCC) and a serial reaction time task (SRTT). First, the results of the MSA patients will be discussed, followed by a comparison with PSP and the putative impact for differentiation from IPD.

The MSA patients showed severely impaired implicit learning in the trace as well as in the delay eyeblink conditioning paradigm, with standard deviations in the range of other studies,[10 30] whereas non-learning associated blink reflex latencies as indicators of oculomotor pathways were normal.

Histopathological alterations in both subtypes of MSA include the substantia nigra, putamen, descending and ascending fiber tracts of the motor system, olivary and pontine nuclei, brainstem-cerebellar circuits as well as cerebellar structures (hemispheres and vermis).[31-33] This has been confirmed *in vivo* by diffusion tensor imaging of white matter microstructure.[34] We suggest that damage of cerebellar structures and associated brainstem-cerebellar circuits are responsible for the failure of EBCC learning in MSA patients. This assumption is supported by the findings of impaired EBCC in patients with cerebellar damage,[27 35-37] positron-emission tomography (PET) measurements in healthy humans showing changes in glucose metabolism in the cerebellum and pons during EBCC[23 38] as well as in experiments studying the influence of selective pharmacological blockade of cerebellar input on EBCC in rabbits.[39] Most patients in our study were clinically characterized as MSA-P and not MSA-C. However, the failure in blink reflex acquisition in both groups points to a subclinical cerebellar involvement in MSA-P, which is in accordance with the histopathological studies.[32 33] EBCC therefore seems to detect cerebellar involvement at a subclinical stage.

In addition to the cerebellum, several studies indicate that acquisition of CR in the trace paradigm depends on forebrain areas such as the hippocampus or prefrontal cortex, in particular for longer interstimulus intervals.[40 41] In our study, the failure of CR acquisition in MSA patients was

slightly more pronounced in the trace compared to the delay paradigm. Therefore, lesions of the frontal lobe, which have been suggested by neuropsychological testing[6 42] and confirmed histopathologically in a variety of MSA cases,[43 44] may have contributed to impaired EBCC acquisition in the trace paradigm.

An alternative explanation that was brought up by an anonymous reviewer is that the tone may be a less salient CS to the MSA patients than to the control group. The reduced number of alpha blinks would support this assumption. Following that very elegant line of thought, the EBCC group difference between MSA patients and control subjects would have to do less with implicit learning and more with responsiveness and associative processes related to external stimuli. While this may have some relevance, adding the number of alpha blinks to the ANOVAs on conditioned eyeblink responses did not abolish the between-group differences.

In the SRTT the MSA patients showed severe impairment in terms of prolonged reaction time, high error rate and, in some patients, early discontinuation due to fatigue and exhaustion. In contrast to the control group they showed no significant reaction time increase between block 6 (random) and 7 (sequence) and this is indicative of implicit learning deficits. On the other hand they showed good performance on the parameters of sequence recall (explicit learning). This preservation of SRTT explicit learning parts may be explained by the relative preservation of posterior association (temporal and parietal) cortex and hippocampus in MSA. It has to be interpreted with some caution, though, given limitations of spatial working memory in MSA.[42] However, the validity of the SRTT learning results is limited by the discontinuation of patients and our "last observation carried forward approach" (see Methods). In addition, the patients' wide range of motor impairment, which may interfere with the motor part of the task, and the fact that sequence learning and movement preparation seem to share similar attentional and working memory resources[45] have to be considered. Therefore the SRTT seems to be inappropriate to assess learning abilities in MSA patients. This is in contrast to the EBCC, which is independent of the motor performance of

patients. Furthermore, EBCC circuits are located anatomically closer to the affected brainstem regions.

With all the limitations of such a retrospective comparison of data acquired in different patient groups by the same authors, the implicit learning impairment in MSA patients as clearly revealed by the EBCC and with some limitation by the SRTT parallels the previously reported findings in PSP patients.[14] Interestingly, MSA and PSP are characterized by different histopathological alterations, α-synuclein positive inclusions versus tau-positive aggregations, which lead to presume different pathophysiological mechanisms. However, the common involvement of cerebellar structures in both diseases[31 46] seems to be responsible for the clinical phenomenology independent of the cellular mechanism.

In contrast to MSA and PSP, IPD patients show normal[12] or even enhanced[13] acquisition of conditioned responses in EBCC. EBCC may therefore contribute to distinguish IPD from these atypical Parkinsonian syndromes. As the development of cerebellar and related neuropathology in MSA or PSP often occurs prior to or even without clinical manifestation,[33 46] we propose impaired EBCC particularly in the trace paradigm as an early indicator of neurodegeneration of areas beyond those typically affected in IPD. However, as the retrospective synopsis is clearly limited, the pivotal questions of whether EBCC can serve as predictor for the development of typical or atypical disease and whether EBCC is a useful addition to imaging techniques in establishing an early differential diagnosis are unanswered yet and require further prospective investigation.

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Contributors

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Competing interests None

Ethics approval Ethics committee of the Medical Faculty of the University of Goettingen.

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Legends to tables and figures

Table 1: Characteristics of the MSA patients. Type: Parkinsonian (MSA-P) or cerebellar (MSA-C) predominance; LED = L-Dopa equivalent dose, + indicates additional budipine medication, # additional anticholinergic medication; UPDRS = Unified Parkinson's disease rating scale, motor examination only (high number of points indicates high disability); MMS= Mini Mental State (30 points are normal, ≤26 is usually considered as cognitive impairment). Cerebellar impairment was evaluated for ataxia (arm, leg), saccades and intention tremor (finger-nose test), autonomic impairment for postural faintness, syncopes, urinary incontinence, urinary retention, faecal incontinence and impotence (males only). Pyramidal tract impairment was scored for hyperreflexia and Babinski sign. Each item was scored if present with 1, otherwise 0. In the Hamilton scale a low score indicates few depressive symptoms. *not investigated.

Table 2: Characteristics of controls, IPD and PSP patients in part taken from earlier publications.[12] [14] Dementia had been ruled out using the Mattis Dementia Rating scale (MDRS)[47 48], where higher scores out of a maximum of 144 indicate better performance, with a cut-off ≤123 considered as cognitive impairment[49]. Depression had been assessed using the Beck Depression Inventory (BDI),

where higher scores out of a maximum of 63 points indicate a more severe depressed state, and a score of 15 is regarded as cut off for a self report of mild depression.[50 51] *not investigated. The MDRS was not available at the German study sites.

Figure 1: Blink reflex recovery cycle with interstimulus intervals of 100, 300 and 600 ms. In MSA patients, the inhibition of the ipsilateral R2 response to the second stimulus is weaker than in the control group. Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 2: Conditioned eyeblink responses in two different paradigms: left, delay (interstimulus interval 0 ms), right, trace paradigm (interstimulus interval 600 ms). In both paradigms, the number of conditioned responses was significantly lower in MSA and PSP patients than in the control and IPD groups indicating impaired implicit learning. CS, conditioned stimulus (tone), UCS, unconditioned stimulus (electrical stimulation to the supraorbital nerve). Data of MSA patients and controls are indicated as average value and single standard deviation. Data for IPD and PSP patients (dashed lines) were taken from our earlier studies using identical methods.[12 14]

Figure 3: Occurrence of 'alpha-blinks'. These bursts are a startle reaction to the tone (CS) and are less frequent in MSA and PSP patients than in the control and IPD groups. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Data are indicated as average value and single standard deviation and were pooled for both paradigms.

Figure 4: A Reaction time in a serial reaction time task (SRTT). An implicit learning effect is indicated by the reaction time increase between the last sequence block (6) and the following random block (7). **B** Explicit learning in the SRTT was tested after each block by manual retrieval

of the sequence (repetition of the last 10 key presses) and revealed no significant difference between groups. Data are indicated as average value and single standard deviation. Data for IPD and PSP patients were taken from our earlier studies using identical methods.[12 14] Asterisks indicate a significant difference for the comparison of blocks 6 and 7 ($p<0.05$, post-hoc t-test).

Figure 5: Overview of the mean percentage of conditioned eyeblink responses (CRs) across blocks 3-6 in the EBCC paradigms in MSA patients and control subjects from this study and in IPD and PSP patients from earlier studies.[12 14] With the trace paradigm a complete separation between IPD and the atypical Parkinsonian syndromes MSA and PSP is achieved (cutoff 26%), whereas in the delay paradigm there is a small overlap between these groups. Overall, IPD patients perform slightly better than control subjects,[13] further enhancing the group distinction between IPD and atypical syndromes.