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Sloane Paulcan, Anne Giersch, Virginie van Wassenhove, Valérie Doyère. Temporal order processing in rats depends on the training protocol.. *Journal of Experimental Psychology: Animal Learning and Cognition*, 2023, 49 (1), pp.31-45. 10.1037/xan0000347 . hal-04170258

HAL Id: hal-04170258

<https://hal.science/hal-04170258>

Submitted on 25 Jul 2023

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Temporal Order Processing in Rats depends on the Training

Protocol

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Data are available upon reasonable request to the corresponding authors.

Author contributions

All authors contributed to the study design. Data collection and analyses were performed by S. Paulcan under the supervision of V. Doyère and V. van Wassenhove. All authors contributed to the data interpretation. S. Paulcan drafted the manuscript. A. Giersch, van Wassenhove and V. Doyère provided critical revisions. All authors approved the final version of the manuscript for submission.

Acknowledgments

The authors thank Lucille Tallot for her help on the Python script; Jennifer Coull, Bruce Brown, Marion Noulhiane and Srdjan Ostojic for their insightful comments; Guy Sandner and Marie-José Angst for having shared their preliminary experience on temporal order experiment in rats and the animal caretakers at NeuroPSI.

Abstract

The perception of temporal order can help infer the causal structure of the world. By investigating the perceptual signatures of audiovisual temporal order in rats, we demonstrate the importance of the protocol design for reliable order processing. Rats trained with both reinforced audiovisual trials and non-reinforced unisensory trials (two consecutive tones or flashes) learned the task surprisingly faster than rats trained with reinforced multisensory trials only. They also displayed signatures of temporal order perception, such as individual biases and sequential effects that are well described in humans, and impaired in clinical populations. We conclude that an experimental protocol requiring individuals to process all stimuli in a sequence is compulsory to ensure temporal order processing.

Keywords: Multisensory, audiovisual, sequence, individual bias, psychometric curve

Statement of relevance

Lashley (1951) discussed the importance of serial order and its representation in neural systems, a topic that fed a large body of work in humans. In the few studies exploring temporal order judgments in rats, none ensured that the animal effectively used both stimuli to solve the task. Here, we designed two learning protocols: two groups of rats learned to press a spatialized lever (left or right) in response to reinforced audiovisual trials (beep first or flash first). One of the groups was trained with additional non-reinforced trials (two beeps or two flashes). While both groups showed accurate order judgements, rats exposed to unisensory trials displayed large inter-individual variability and sequential effects, comparable to studies performed in humans. Our findings open new perspectives on the study of (subjective) time perception in animals and applications to translational studies.

Temporal Order Processing in Rats depends on the Training

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Individuals live in complex multisensory surroundings, in which they must integrate and order information to take appropriate actions and decisions for survival. In humans, the processing of temporal order shows large, but stable, inter-individual variability in healthy individuals (Grabot & Wassenhove, 2017) and is impaired in various pathologies: abnormal timing is associated with a wide array of cognitive impairments, such as dyslexia (Rey et al., 2002; Jaśkowski & Rusiak, 2008), autism spectrum disorder (de Boer-Schellekens et al., 2012), or schizophrenia (Foucher et al., 2007; Martin et al., 2013; Capa et al., 2014; De Boer-Schellekens et al., 2014). It is noteworthy that, while many studies in animals have focused on memorizing the position of stimuli presented in a sequence (e.g., Dusek & Eichenbaum, 1997), very few have studied the bases of perceptual discrimination of temporal order, and this remains a matter of debate in non-human animals (Ghirlanda et al., 2017). Our goal is to validate a behavioral task for studying temporal order perception in rodents, as it has become essential as a pre-clinical tool and for neuroscientific investigation.

Herein, we explored a novel behavioral protocol that characterizes and validates the selectivity of rats' responses to temporal order, and provides several signatures of temporal order processing. One of our main concerns was to ensure that rats effectively processed the order of stimuli, and did not use alternative heuristics such as attention (Spence & Parise, 2010) or stimulus-action association that would not require order processing *per se* (e.g., the identity of the first stimulus is associated with a given action, independent of the second stimulus) (Holland, 2008; Randerath et al., 2015). For instance, Schormans et al. (2017) designed an audiovisual temporal order judgement (TOJ) task using appetitive operant conditioning in rats. In TOJ tasks,

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individuals typically report which stimulus in a pair occurred first and in this task, rats had to nose poke a spatialized feeder (located on the left or on the right) as a function of their perceived temporal order of a pair of audiovisual stimuli. The authors systematically varied the audiovisual delays, or Stimulus Onset Asynchronies (SOAs), and reported psychophysical results in rats similar to humans (Schormans et al., 2017). Specifically, they reported a sigmoidal response as a function of SOA so that the larger the SOAs, the better rats discriminated the temporal order of audiovisual stimuli. However, a parsimonious stimulus-action association would be just as effective in accounting for rats' behavior: a direct association between the first stimulus of the pair and the correct feeder could have been sufficient for accurate performance (i.e., poking the left feeder when detecting the first stimulus as auditory, poking the right feeder when detecting the first stimulus as visual). This association does not necessarily require both stimuli and their order to be processed. Specifically, this alternative explanation is consistent with a seminal study (Kosiba & Logan, 1978) suggesting that the first stimulus dominantly drives the response: using a temporal order learning task, rats had to associate pairs of stimuli in different orders with either a behavioral response or a no-response. When testing single stimuli, the authors reported that while the rats learned the temporal order task, their performance was mostly driven by the first stimulus.

In this context, the aim of our study was to validate that rats can perceptually discriminate temporal order. For this, we adapted Schormans's TOJ task, with rats trained to press a spatialized lever to be reinforced after being presented with an auditory stimulus leading a visual one (e.g., AV - left lever press) or with a visual stimulus leading an auditory one (e.g., VA - right lever press) trials. We compared two protocols: rats in the first group (G1) were free to solve the task without specific

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instructions; rats in the second group (G2) were exposed to unreinforced AA and VV trials (repetition of same sensory modality) in addition to the multisensory trials. This experimental manipulation encouraged the G2 rats to learn to process both stimuli and their order, and not solely respond on the basis of the first stimulus as suggested by previous studies. In G2, inasmuch as AV and VA trials were reinforced, and not AA and VV, the processing of the first stimulus was insufficient to produce a correct response. G2 rats had to process both stimuli to differentiate reinforced trials from unreinforced ones, whereas G1 rats that were exposed to AV and VA trials only were free to process the first stimulus, the second, or the order.

We expected that G2 rats would be slower learners than G1 rats due to their more complex protocol. After reaching an equivalent level of performance, we quantified perceptual signatures of temporal order previously reported in humans. We investigated the effect of the previous trial (trial $n-1$) on the response of the current trial (trial n), known as sequential effects (Recio, Cravo et al., 2019; Roseboom, 2019; Keane et al., 2019; Vroomen & De Gelder, 2004; Elliott et al., 2007; Giersch et al., 2009; Poncelet & Giersch, 2015; Marques-Carneiro et al., 2020). We also looked at individual biases (Grabot & Wassenhove, 2017) and whether rats displayed idiosyncratic response biases to the sensory modality (test of response bias to a single A or a single V) which could influence rats' order perception when the judgment is harder (i.e., for shorter SOAs). Finally, a psychometric test was performed with additional SOAs to compare the 50% threshold (the point of subjective simultaneity or PSS, corresponding to the SOA at which rats respond at random), and the sensitivity (just-noticeable-difference or JND, corresponding to the smallest SOA needed to detect the temporal order of stimuli). PSS and JND are extensively used in human literature (see Vroomen & Keetels, 2010; Bausenhardt et al., 2018) because they allow

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measuring two distinct properties. PSS quantifies how close a psychological feature (here, simultaneity) is to a physical property (here, SOA=0). Another is the sensitivity (JND) to the perceptual discrimination (here, order) which can be influenced by additional factors such as perceptual and decisional biases, distinct from the SOA. All tests combined show that rats can perceptually process and discriminate temporal order beyond a stimulus-action association, albeit in a manner that depends on the learning paradigm.

Materials and methods

Subjects

Subjects were 32 adult male Sprague-Dawley rats (Envigo, France), weighing 290 ± 20 g at their arrival in the laboratory. Rats were housed by four under a 12/12 h light-dark cycle in a temperature ($21 \pm 2^\circ\text{C}$) and humidity-controlled ($55 \pm 5\%$) environment. The housing environment was enriched with wood chip and sawdust, which allows digging behavior, tunnels, and wooden sticks to gnaw on. Animals were handled and weighed daily. Behavioral assessment took place during the light cycle.

Animals had free access to food and water until one week prior to the start of the behavioral training, when food restriction reduced animals' weight to 85% of their free-feeding body weight. Food restriction lasted throughout the duration of the experiment according to the Growth data guideline of Sprague Dawley rats provided by Envigo, with water available *ad libitum* in the home cage. All procedures were in accordance with the European Community Council Directives (2010/63/EU) and approved by the French Ministry of Research and the French National Ethical Committee (2013/6).

Apparatus

Eight identical conditioning chambers (30 x 25 x 30 cm, Coulbourn Instruments, USA) were used, enclosed within a sound-isolation cubicle with a ventilation fan (65 dB background noise). Behavioral protocols were designed using Graphic State 4 software. Each chamber was equipped with a red house light (15 Lux) located at the top right corner of the right wall. The house light, which changed the ambient brightness, served as a warning signal for the beginning of a trial. On the left wall, two

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levers were on either side (16 cm apart and 3 cm above the ground) of a magazine where rats could receive a pellet (dustless precision pellets, grain based, 45mg, Phymep) as a reinforcement. The visual stimulus "V" (green LED, 20 Lux) and the speaker for delivering the auditory stimulus "A" (4 kHz tone, 75 dB) were placed above the right lever (**Fig. 1b**).

Procedure

Training.

All rats were first habituated to reinforcement delivery in the magazine over a single session that lasted 30 minutes. During this session, 30 pellets were given every 40, 60, or 80 seconds. The next day, in two sessions, the rats were trained to press the levers for receiving a pellet under continuous reinforcement. Each session lasted 35 minutes or ended when the rat produced 50 lever presses, whichever came first. Two rats were eliminated because the association lever press/reinforcement was not acquired.

Two experimental groups of 15 rats each were then formed, G1 ($N = 15$) and G2 ($N = 15$). All rats were trained to associate the temporal order of audiovisual stimuli (AV for audio preceding visual, or VA for visual preceding audio) with the side of lever to press (temporal order judgment task; TOJ). To instruct for processing both the first and second stimuli, the rats in G2 received additional non-reinforced AA (two successive sounds) and VV (two successive flashes) trials. The rationale for AA and VV trials was the following: if the rat's response was determined by the processing of only the first stimulus, similar responses should be observed for AA and AV trials (or for VV and VA trials). Similarly, if the rat's response was driven by the second stimulus,

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VV and AV trials (or AA and VA trials) should yield the same response. Responses will differ only if both the first and the second stimuli were taken into consideration. The absence of reinforcement for the AA and VV trials aimed at inciting the rats to distinguish between AA and AV trials (in case of a response driven by the first stimulus, or between AA and VA trials in case of a response driven by the second stimulus), and thus to process the two successive stimuli, rather than only the first (or second) one only. The controlled assignment of rats was done to ensure maximum counterbalancing: 2 out of the 4 rats that shared the same housing cage in each group, assignment to all the conditioning chambers equilibrated between both groups, and the order/lever assignment (i.e., VA associated with left or right lever) was counterbalanced within each group.

Each trial started with the house light turned off during 1s as a warning signal followed by the house light back on for 1s before the first stimulus was presented (**Fig. 1a and 1b**). The training started with stimuli lasting 500 ms and a stimulus-onset asynchrony (SOA) of 1200 ms (P1: Protocol 1). Over the course of training, the duration of stimuli and the SOAs were progressively reduced following three different protocols (P2 to P4, **Fig. 1c**). The duration of the stimuli was first reduced to 100 ms (P2) and then to 50 ms (P3), keeping the 700 ms inter-stimulus interval constant; this caused a decrease of the SOA to 800 ms and 750 ms, respectively. In the final protocol P4, the SOA was decreased to 200 ms while keeping the stimuli duration at 50ms. A group success criterion was enforced to move from one protocol to another: 80% of correct responses and, for G2, a decrease of the response rate to AA and VV trials, indicating rats processed both stimuli and learned that these trials were not reinforced.

During all training protocols (P1 to P4), rats from both groups were exposed to 60 AV trials, and 60 VA trials. Rats from G2 had in addition 60 AA and 60 VV trials

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(i.e., a total of 120 trials per session for G1 and 240 trials per session for G2). All trials were pseudo-randomly distributed. Each rat performed two sessions per day. The first step of P1 training started with a forced-choice paradigm during which only the correct lever was deployed after the associated AV or VA stimuli, and no lever was presented after AA and VV for G2. The first day of the forced-choice paradigm, the lever was deployed after the end of the second stimulus for a maximum of 10 s (two sessions), and retracted as soon as a lever press was recorded (and reinforcement delivered), followed by an inter-trial interval (ITI) of 10 s. The maximum duration of lever availability was then reduced the next day to 5 s and 3 s (one session each). Then, during the second step of P1, the sessions were 50% forced-choice (with only the correct lever available) and 50% free-choice trials (with the two levers available), randomly distributed, until the success criterion was reached. A correct response triggered the immediate delivery of reinforcement. Then, the P1 sessions became 100% free-choice. Once the success criterion was reached for both groups (each rat was maintained on the same protocol as long as the group did not reach the criterion), i.e. after a total of twenty five sessions (6,480 AV/VA trials for both groups), the stimuli duration and SOA were reduced (P2 to P4), in 100% free-choice conditions. Several tests were run (see below), with retraining in-between with this 100% free-choice paradigm to maintain rats' performances (**Fig. 1c**).

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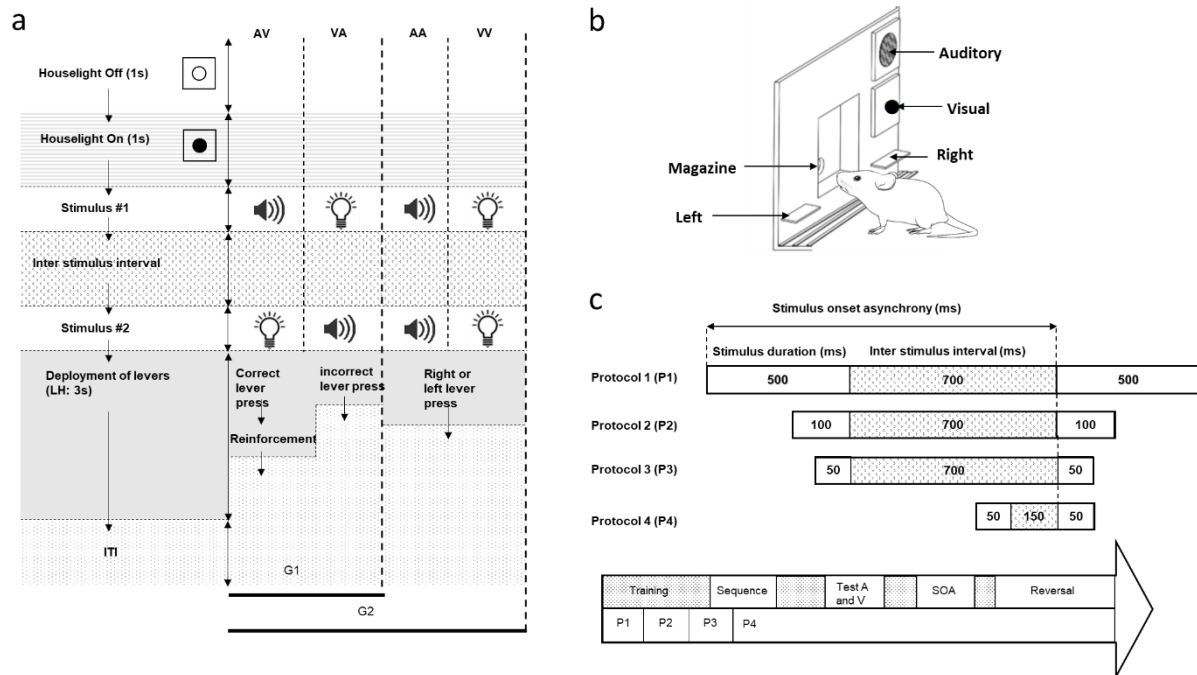


Figure 1: description of the behavioural task and protocol chronology. Description of the AV/VA trials performed by G1 and AV/VA/AA and VV trials performed by G2 during training sessions (a). Rats learned through reinforcement the association between audiovisual temporal order and lever assignment (b). Description of the different stages of reduction of stimulus duration and then of Stimulus Onset Asynchrony (SOA) applied from protocol 1 to protocol 4. Timeline of the training phase (grey frame) followed by behavioral tests carried out with each protocol (c). During the reversal, the lever/order assignment was switched.

Test for Sequential effects.

The aim of this test was to investigate to what extent the preceding trial (trial $n-1$) impacted the response on the ongoing trial (trial n). This test was included in P3 (SOA = 750 ms) and lasted 20 sessions. The same test continued in P4 (SOA = 200 ms) for 44 sessions, to let the animals adapt to the new SOA and ensure 20 sessions with stable behavior. To assess a sequential effect, a controlled distribution of the sequence of trials over one session was necessary. We designed sets of trials by creating four

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lists, the order of which differed over sessions (list 1-2-3-4, or 1-4-2-3, etc.). Each list contained the precise AV and VA sequence (e.g., list 2 = VA, VA, AV, AV, VA, AV, VA...) The trials sequences in lists 2 and 4 were mirrored replicas of lists 1 and 3. The lists were first designed for G1 (32 or 33 trials per list). Adding AA and VV trials (i.e., non-reinforced trials) in the design of the G2's sessions while keeping the number of AV and VA (reinforced) trials constant caused a reduction of the reinforced trials preceded by reinforced trials. Therefore, we added AV and VA trials, and reduced the proportion of AA and VV trials to create G2's sets (58 trials per list) ensuring as much as possible that trials preceded by AV or VA were presented as often in G2 as in G1 (e.g., list 2 = VA, VV, VA, AV, VV, AV, VA, AA, AV, VA, AV VA, ...). As a result, for G1, there were 130 trials per session, 65 AV and 65 VA, with half of them as repeated sequence of the same trial type (AV preceding AV, or VA preceding VA; "same"), and the other half as an alternate sequence (VA preceding AV or AV preceding VA; "different"). For G2, there were 232 trials per session, 98 AV and 98 VA, with 160 preceded by a reinforced trial (82 "same" sequence and 78 "different" sequence), and 36 trials AA and VV, 18 preceding AV and 18 preceding VA (9 AA and 9 VV). No more than three successive repetitions of the same trial and no successive repetitions of AA or VV were allowed. The responses during all of the 20 sessions of P3 and the last 20 sessions of P4 were analyzed.

Test for unisensory biases towards sound or vision.

Rats were retrained with the 100% free-choice paradigm for 16 sessions in P4. Then, we tested how rats responded to the presentation of a unique stimulus when no order could be processed. For this, 30 single A and V trials were added within the session. Hence, we tested a total of 120 audiovisual trials (60 AV and 60 VA) and 60 unisensory

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trials (30 A and 30 V) for both G1 and G2, and 60 trials of repeated unisensory trials (30 AA and 30 VV) for G2 only. Each experimental session began with two reinforced trials to promote the motivation of the rats immediately after the start. Only two sessions were used because rats could learn very quickly to not respond to a single stimulus, as they were not reinforced.

Rats' responses to A and V alone were characterized as AV(A) (i.e., "AV if A") or VA(A) (i.e., "VA if A") and AV(V) for "AV if V" or VA(V) for "VA if V", based on the lever press they made, and depending on the association temporal order-lever side assignment. For example, if a rat had learned the association "AV"-left lever and pressed the left lever following the presentation of A alone, we considered that the rat responded AV(A). Otherwise, if the rat had pressed the right lever after being presented with A alone, the response was categorized as VA(A). The same rationale was adopted for V alone.

Psychometric fits with variable SOAs.

After retraining for 6 sessions, a psychometric test was performed in order to examine each rat's perception of temporal order. For this, we added trials with six new SOAs (± 100 , ± 40 , ± 20 ms) to P4. The stimuli duration was kept at 50 ms for all trials, resulting in partially overlapping stimuli for the ± 40 and ± 20 ms SOAs, as done in psychometric assessments in humans (Dean et al., 2017; Grabot & Wassenhove, 2017; Recio, Cravo et al., 2019). Only the ± 200 ms trials were reinforced after a correct response, with a proportion of reinforced trials higher than the non-reinforced to prevent rats from biasing their responses during this test (2 sessions). Each session began with two reinforced trials to motivate the rats immediately after the start. In both groups, a total

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of 120 reinforced trials AV/VA with SOA = ± 200 ms, and 10 non-reinforced trials for each other SOAs of ± 100 / ± 40 / ± 20 ms (total of 60 unreinforced trials) were given. For G2, 60 AA/VV non-reinforced trials with SOA of ± 200 ms were also given randomly.

Rats' performances, pooled over the 2 sessions, were analyzed as the proportion of "visual first" responses, with negative SOAs indicating that the sound preceded the visual stimulus and positive SOAs that the flash led the sound. Individual responses were fitted with a sigmoid function (**Fig. S8**) to calculate the point of subjective simultaneity (PSS), namely the SOA for which the order is no longer perceived (50% of correct response), and the just noticeable difference (JND), calculated as the difference of SOA between 25% and 75% of correct response on the sigmoid function divided by 2 ($(SOA_{75\%} - SOA_{25\%}) / 2$), which corresponds to the smallest SOA needed to detect temporal order (sensitivity in the detection of the order).

Reversal procedure.

After one day of retraining, animals were submitted to a reversal procedure. The lever/order assignment was switched (i.e., if rats had learned to press the left lever after AV, they have to press the right lever during the reversal, and vice versa). Thirteen rats in G2 succeeded to learn the reversal, whereas only nine rats did in G1 (**Fig. 2a bottom**) within 68 sessions. The test of reversal provided us with an additional sorting criterion to equate as much as possible the quality of learning between the two groups.

Statistical analyses.

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The statistical analyses included one sample t-test for the comparison of the percentage of correct responses, percentage of no-response and response latencies against the basal level, which corresponded to the average obtained the first day of training for the corresponding variables for each animal. The normal distribution and variance homogeneity were systematically verified. As a result, the non-parametric Wilcoxon tests were performed for the comparison of responses to the single A and V against 50%, and for the percentage of responses “VA” during AA/VV the day before the test of response bias to A and V compared to the day of the test. Paired sample t-tests were used for the comparison of “same” and “different” responses type during AA/VV trials of the sequential test and the G2 individual’s responses to single A and V compared to AA/VV trials. Between groups comparisons were performed using an independent t-test for the PSS and the JNDs. As the number of days to criterion did not respect the normal distribution and variance homogeneity, the non-parametric test of Mann-Whitney was used.

Two- or three-way repeated measures analyses of variance (ANOVA) were used for the sequential effects and the psychometric tests. In the sequential effect analysis, the Group was the between subject factor and the within subject factors were the Trial Type (trial n: AV or VA) and the Sequence (trial n-1: same or different from trial n). During the psychometric test, when the percentage of correct responses for the 8 SOAs were analyzed, Mauchly’s test indicated that the assumption of sphericity was violated ($\chi^2(27) = 82.921, p < .001$): degrees of freedom were then corrected using the Greenhouse-Geisser ($\epsilon = .42$).

A custom-made Python program was used to fit the individual rats’ responses during the psychometric test using a sigmoid function (logistic). The fit was optimized

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with no minimum or maximum values to ensure a better prediction quality ($R^2 > 0.8$) of the PSS and JND.

Principal Component Analysis (PCA) was also performed, using XLStat software, to sort out the behavioral data in two orthogonal principal components (varimax rotation). Bartlett test of sphericity revealed that variables in the two components respected the homogeneity of variances ($\chi^2 (21) = 65.690$, $p < .0001$). Linear regressions were made to define the coefficients of correlation between variables that belonged to one component. For all statistics, the alpha level was set at .05.

Results

Learning and reversal of the temporal order rule

During training, all 30 rats in G1 ($N = 15$) and G2 ($N = 15$) learned to associate a given audiovisual temporal order with a lever (**Figs. 2a top and S1a – S1b**). However, during the reversal, only 22 rats (G1, $N = 9$; G2, $N = 13$) reached the success criterion (**Fig. 2a bottom**), suggesting that some rats may have solved the task in a different way than the others. To equate the comparability between the two groups as much as possible, only the results of rats that were successful during reversal are presented in the main manuscript. However, for full disclosure, the results of all animals are reported in the supplementary material (**Figs. S1, S3, S6 and S8**), indicating similar results when all rats were considered.

At the end of training, no statistical differences were found in the percentage of correct responses, or in the latency of these responses between the two groups ($F(1, 20) = .002, p = .967$; $F(1, 20) = 2.133, p = .160$, respectively). Using a one sample t-test, we found that correct responses on the last day of training were significantly above the basal level (i.e., the average obtained the first day of training) ($t(21) = 49.319, p < .001$; basal level = 52.42%), and that their latencies were significantly slower than at the outset of training ($t(21) = 7.514, p < .001$; basal level = 0.54s) (**Fig. 2c**). As expected, G2 rats responded less frequently to non-reinforced trials AA and VV with time. A one sample t-test showed, compared to the basal level (1.25), an increase of the number of trials for which rats did not respond (AA, $t(12) = 3.676, p = .003$ and VV, $t(12) = 3.280, p = .007$), as well as a slowdown of their responses in AA ($t(12) = 8.232, p < .001$) and in VV ($t(12) = 7.175, p < .001$) trials (basal level = 0.57s) (**Figs. 2d and S2a** for the time course of performance during the first 25 days). We found no significant differences in the latencies between AA and VV ($t(12) = -1.381, p = .192$).

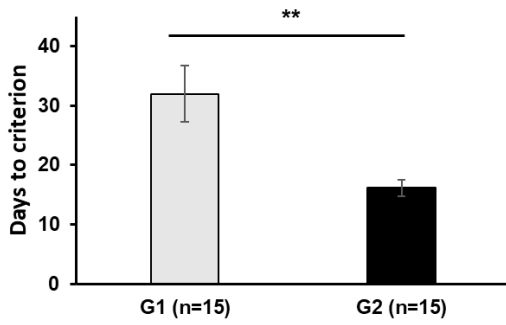
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Interestingly, and contrary to our expectations, G2 rats reached the success criterion of 80% correct response significantly faster ($M = 12.84$ days, $SD = 3.31$) than rats in G1 ($M = 24.55$ days, $SD = 12.96$), $U (G1 = 9, G2 = 13) = 107.00, p = .001$ (**Fig. 2a top**).

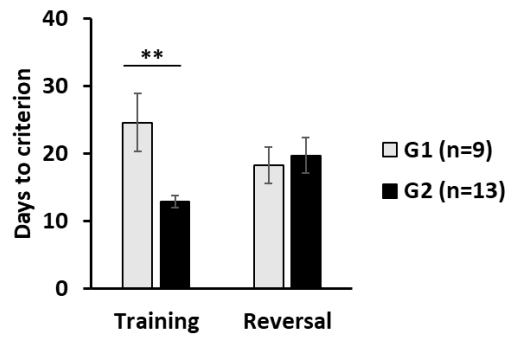
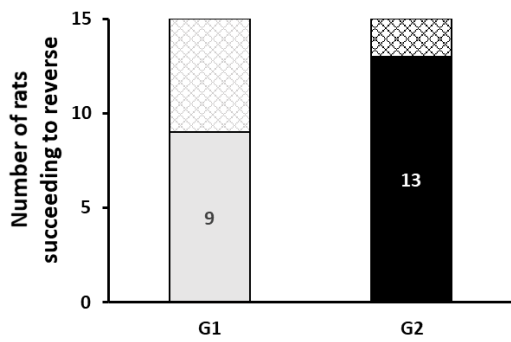
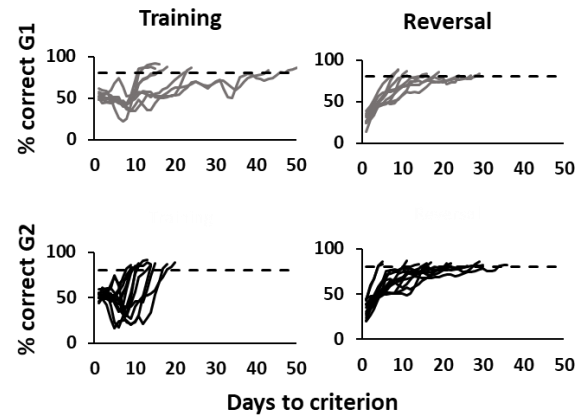
The performance in both groups remained high throughout the different phases of training, with slower responses as the task demands became more difficult, i.e., when decreasing the duration of the stimuli or when decreasing the SOA (see **Fig. S1**). When the rule order-lever side was reversed at the end of all testing, the percentage of correct responses for both groups dropped below the basal level ($t(21) = -11.803, p < .001$), with no more differences with the basal level for the latency ($t(21) = .547, p = .590$). The percentage of AA or VV trials without a response in G2 dropped back to basal level ($t(12) = 1.633, p = .128$), but when responding to AA and to VV trials, the response latency remained statistically longer than the basal level ($t(12) = 4.520, p < .001$ and $t(12) = 3.709, p < .001$, respectively). All performances in both groups improved during the sessions of reversal until reaching the same level as in the last day of training (**Figs. 2c and 2d. See Fig. S2b** for G2's performance time course). In contrast to what was observed during the initial learning of the task, G1 and G2 rats reached the success criterion during reversal after a similar number of days, (G1: $M = 18.22$ days, $SD = 8.07$; G2: $M = 19.69$ days, $SD = 9.33$), $U (G1 = 9, G2 = 13) = 52.00, p = .688$ (**Fig. 2b**).

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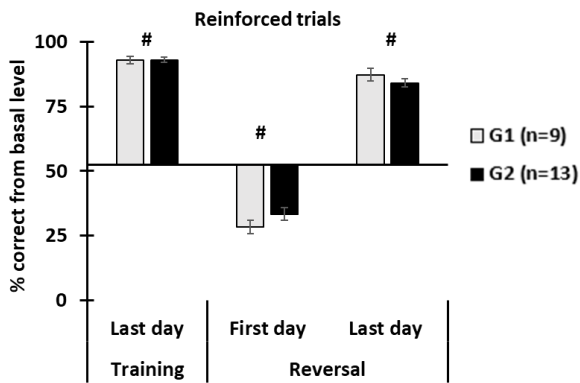
a



b



c



d

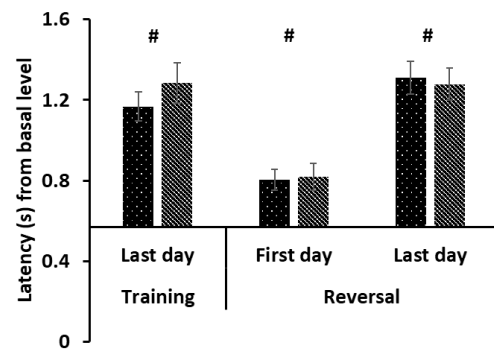
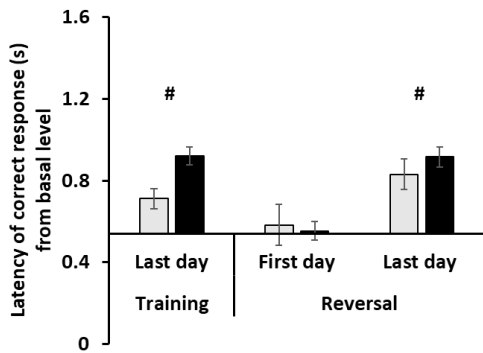
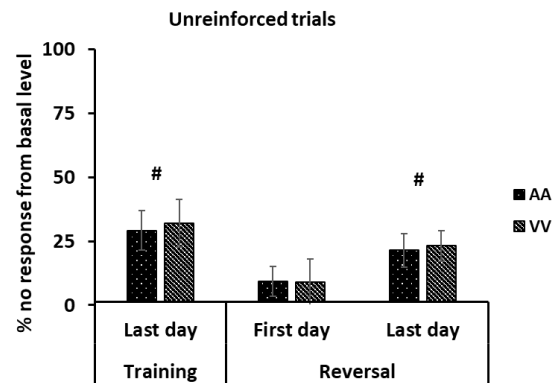


Figure 2: Audiovisual temporal order task: G2 reached the success criterion faster than G1 during training, with a tendency to better learn the reversal. Rats in G2 (G2; N = 15) reached the criterion faster than those in G1 (G1; N = 15) (**a**; top) under P1, **, U = 195, $p < 0.001$, $rpb = .733$, 95% CI [.475, .875]. Only 9 rats in G1 and 13 rats in G2 succeeded to learn the new rule during reversal (**a**; bottom). Performance of individual rats until they reached the criterion during training and reversal (**b**; four upper graphs), and mean (\pm SEM) number of days to criterion during both phases for the animals that succeeded to reverse (histograms). **, unpaired $p < 0.01$. The mean (\pm SEM) percentage of correct responses (**c**, top) and their latencies (**c**, bottom) from basal level for each group during the last day of training, and the first and last day of reversal. Mean (\pm SEM) percentage of no-response (**d**, top) and latency of responses (**d**, bottom) from basal level during unreinforced (AA and VV) trials for G2. #, $p < 0.05$, as compared to the basal level corresponding to the first day of training for **c** and **d**.

Sequential effects

Rats' temporal order judgments during trial n appeared to depend on the stimuli order of the $n-1$ trial in P4 (**Fig. 3**). A three-way ANOVA was conducted with factors of group (2: G1, G2), sequence order at trial $n-1$ (2: same or different) and percentage of correct responses at trial n for each trial type (2: AV or VA). Results showed a main effect of group ($F(1, 20) = 43.331, p < .001, MSE = 135.233$), a main effect of trial type ($F(1, 20) = 7.142, p = .015, MSE = 24.463$) and a main effect of the sequence order at trial $n-1$ ($F(1, 20) = 33.534, p < .001, MSE = 4.164$). We also found a significant interaction of group \times sequence order at trial $n-1$ ($F(1, 20) = 36.716, p < .001, MSE = 4.164$) (**Fig. S4a left**), and a significant interaction sequence order \times the order type at trial n ($F(1, 20) = 13.989, p = .001, MSE = 3.645$) (**Fig. S4a right**). No significant group \times sequence order \times trial type interaction was found ($F(1, 20) = 0.572, p = .458$).

Post-hoc comparisons (Bonferroni) indicated that only the G2 rats displayed a sequential effect: on trials preceded by a different stimulus order, animals were significantly less accurate than on trials preceded by the same stimulus order ($p < .001$; **Fig. S4a left**). Furthermore, a significant sequential effect was observed when the ongoing trial was VA (but not AV), with a poorer performance when the preceding trial was a different order (i.e., AV) ($p < .001$; **Fig. S4a right**). A more detailed analysis indicated that the sequence effect depending on the trial type was mainly driven by effects observed in G2 (**Fig. 3a left**). Interestingly, these sequence effects in G2 were accompanied by an increase in the latency for correct responses, when the previous trials were different ($F(1, 20) = 23.366, p < 0.001, MSE = .003$; post hoc for G1, $p = 0.343$; G2, $p < 0.001$) (**Figs. 3a right, S4b**).

We wondered whether the response emitted on the trial $n-1$ may have been responsible for the sequential effect observed in G2, rather than the actual order of the

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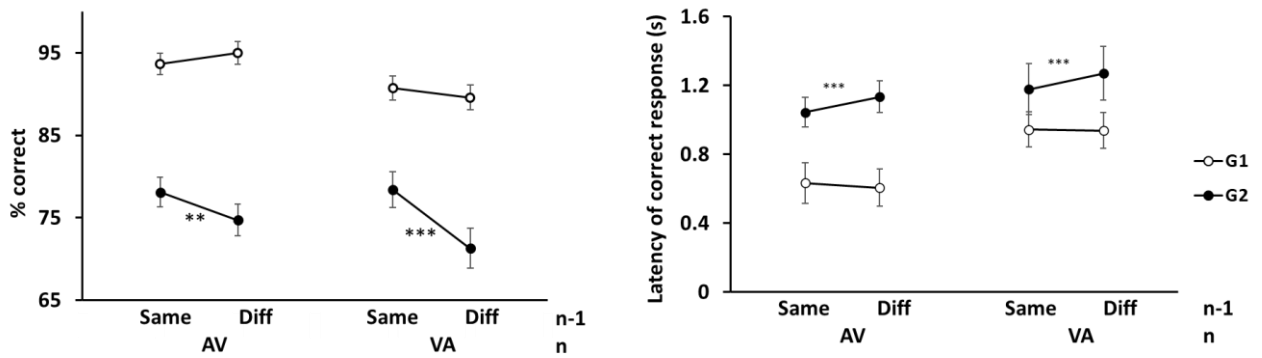
stimuli processed during trial $n-1$. To investigate this possibility, we analyzed the response type (“same” as on trial $n-1$, “different” compared to trial $n-1$, or no-response) during the unreinforced AA and VV trials depending on the previous order (AV or VA). Using a paired sample t-test to compare “same” vs. “different” response type, we found that the response type to unreinforced trials n was biased toward the first stimulus, and not the response given (correct or incorrect), on the previous reinforced trial $n-1$ (**Fig. 3b left**). That is, when presented with AA trials, rats responded significantly more “same” (e.g., left) than “different” (e.g., right) if the preceding trial had been AV (i.e., A first associated with ‘left’; $t(12) = 6.045, p < .001$), and more “different” than “same” if the preceding trial $n-1$ had been VA (i.e., V first associated with right, $t(12) = -6.386, p < .001$). The same applied for VV trials: more “same” response type when the $n-1$ trial had been VA (i.e., V first, $t(12) = 3.597, p = .004$), and more “different” when the $n-1$ trial was AV (i.e., A first, $t(12) = -2.218, p = .047$). Response latencies to these non-reinforced trials were not informative (**Fig. 3b right**). Thus, the sequence effect may have been driven by the first stimulus of trial $n-1$ and n , but not by the response on $n-1$.

The same analyses conducted on data collected during protocol 3 (**Fig. S5**) showed essentially the same differential sequence effect depending on the group and on the order type, as well as the results in AA trials (see legend of **Fig. S5** for statistics). The slight differences may have been due to more training and/or the smaller inter-stimulus interval (150 ms instead of 700 ms) during protocol 4.

Considering the difficulty of distinguishing a perceptual from a decisional bias in the sequential effects, we turned to the analysis of unisensory biases.

AUDIOVISUAL TEMPORAL ORDER

a



b

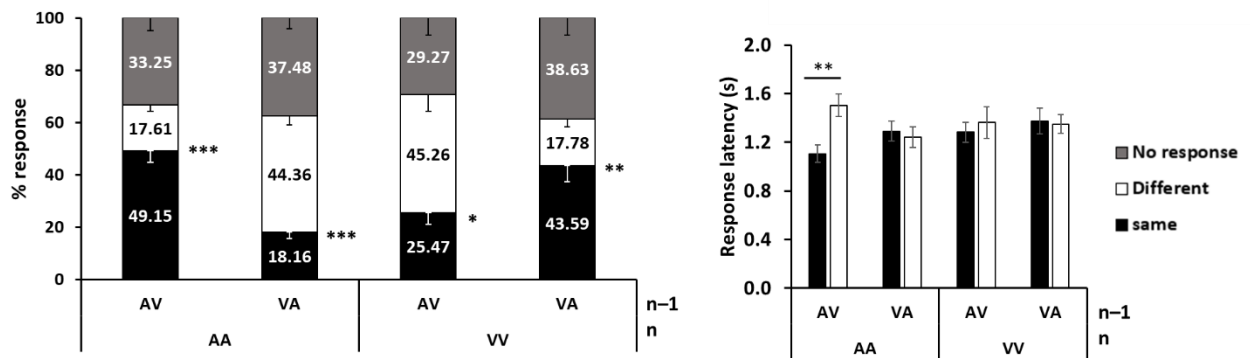


Figure 3 : Sequential effects: G2 rats displayed serial dependency of the preceding trial (n-1) on the current trial (n). Results from P4. The mean (\pm SEM) percentage of correct responses (a, left) and latency (a, right) plotted separately for G1 and G2 as a function of the type of trials (n) and the same or different (Diff) trial type of the sequence (n-1). The percentage of correct responses depended on the order presented on the previous trial in G2, but not in G1. For G2, the mean (\pm SEM) percentage of response type (b) during AA and VV trials depended on the first stimulus of the previous sequence (AV or VA). The tendency to respond to the first stimulus aligns well with the sequential effect in (a): if G2 rats optimize their responses on the basis of the first stimulus, when the order presented in n-1 and n is identical, so are the first stimuli and G2 rats will tend to persist; when the order in n and n-1 trials differ, the first stimuli also differ and G2 rats must correct their tendency to respond to the first stimulus. The mean (\pm SEM) latency of response (d) during unreinforced trials AA and VV as a

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function of the previous trial (AV or VA) for G2. Post-hoc (a) and paired t-test (b): **, $p = 0.01$
; ***, $p < 0.001$.

Test for unisensory biases toward A and V

Responses to the unisensory stimuli A and V were characterized as if the trial had been AV or VA, and compared to random responding by using a one sample t-test. On average, G1 rats were responding VA above chance level (VA(A), $U = 45.00$, $p = .009$, and VA(V), $U = 44.00$, $p = .012$, **Fig. 4a histograms**). Looking at individual responses indicated that only 3 rats responded “VA” to both A and V, whereas the other 6 rats were close to random responding to at least one of the stimuli (**Fig. 4a lower**). In contrast, for G2 rats, no specific response type was found on average after presentation of A, $U = 72.00$, $p = .069$, or V alone, $U = 30.00$, $p = .294$ (**Fig. 4b histograms**). Individual response analysis highlighted a range of selecting the “VA” or “AV” levers (from AV to both A and V, to VA to both A and V, to different “AV” or “VA” response depending on the single stimulus, **Fig. 4b lower**). No significant differences were found in the total responses and latencies to A, V, AA and VV trials (**Figs. S7a and S7b**), although G2 rats distinguished unimodal stimulus during A and V from double presentation of the same stimulus (AA and VV trials) as shown by the paired sample t-test, $t(12) = 2.925$, $p = .013$, and $t(12) = -3.480$, $p = .005$, respectively (**Fig. 4c**), while their responses to AA and VV were stable over sessions (i.e., when compared with the day before, $U = 40.00$, $p = .735$, and $U = 55.00$, $p = .542$, respectively, **Fig. 4d**). Thus, G1 animals showed a “VA” bias (which cannot be a response side bias, as the associated lever side was counterbalanced between animals within the group), whereas no specific bias was observed for G2 animals which exhibited AV or VA type responses, but also no bias (responding VA to A and AV to V, or at random).

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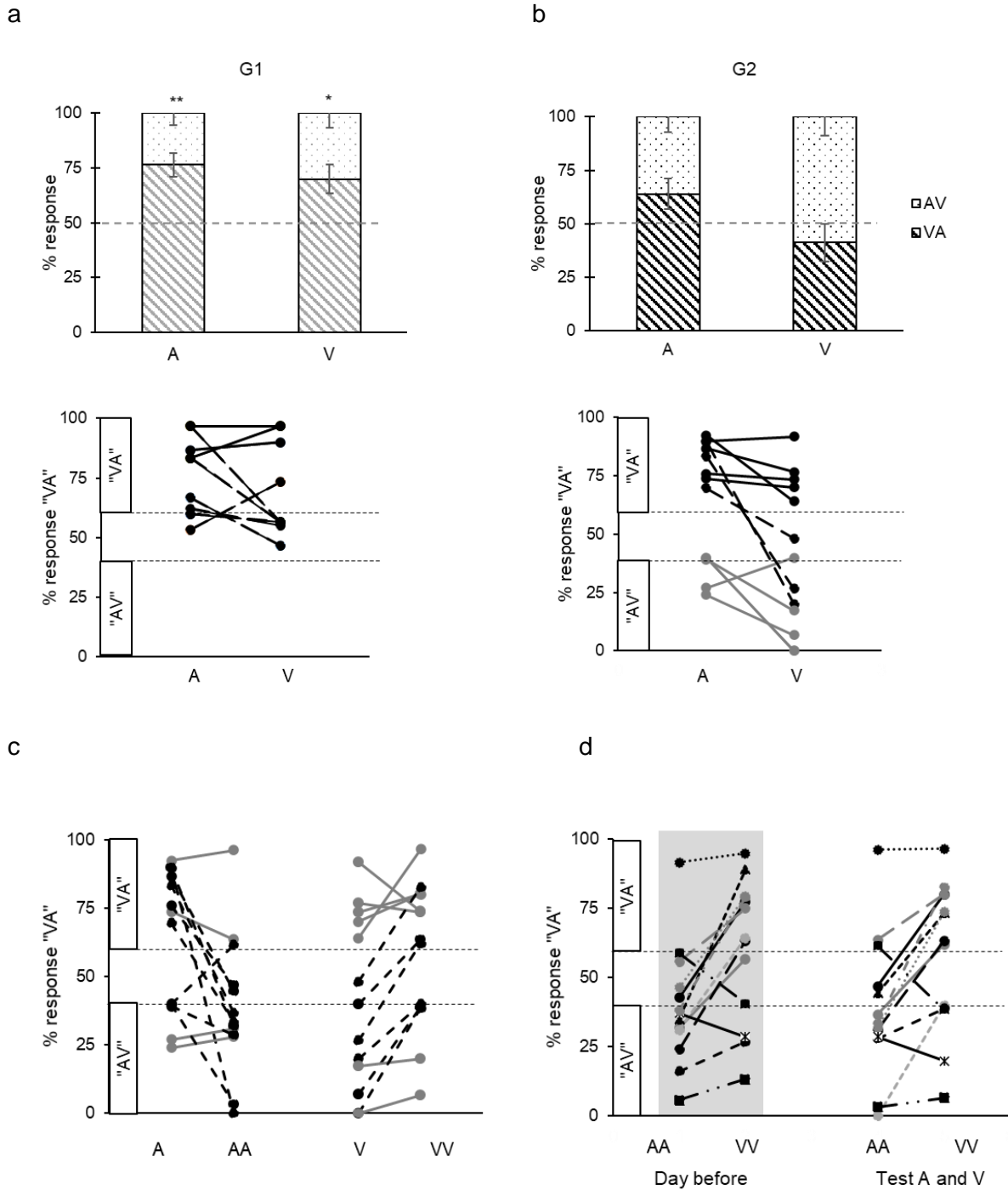


Figure 4: Temporal order bias in G1, but not in G2. G1 rats were biased towards responding VA, as demonstrated by their responses after the presentation of a single V and a single A. The mean (\pm SEM) (top) or individual (bottom) percentage of responses categorized as “AV” or “VA” after the presentation of A and V stimulus alone in G1 (a) and G2 (b). Solid black lines represent “VA” responses, solid grey lines represent “AV” responses, and the dotted lines are random responses that could not be characterized consistently as “AV” or “VA” across

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A and V tests. *, $p = 0.01$; **, $p = 0.009$ compared to 50%. Rats in G2 did not respond to A and V tests as if they were AA or VV (**c**) while their pattern of responding to AA and VV trials was stable over sessions (**d**). G2 distinguished single stimulus presentation from AA and VV trials.

Psychometric fits, PSS and JNDs

The data obtained during the psychometric tests are shown in **Fig. 5**. This figure shows the proportion of “visual first” responses, i.e. the correct response associated to VA type of trial, with negative SOAs representing the “AV” order and positive SOAs the “VA” order. While both groups of rats responded correctly for sensory stimuli with long SOAs (e.g., ~94% of correct responses for SOA of 200 ms), the performance expectedly decreased as SOAs became shorter. A two-way ANOVA with factors of group (2: G1, G2), SOA (8) and dependent variable of order judgment (here, “V first” responses) showed a main effect of SOA ($F(2.976, 59.519) = 178.385, p < .001, MSE = 329.019$) and of Group ($F(1, 20) = 4.362, p = .050, MSE = 658.249$). The main group effect consisted in a general negative shift of the curves between G1 and G2: while both groups were biased towards responding “V first”, G1 rats were more biased towards responding “V first” than G2 were (**Fig. 5a**). No interaction between Group and SOA was found ($F(2.976, 59.519) = 1.304, p = .281$).

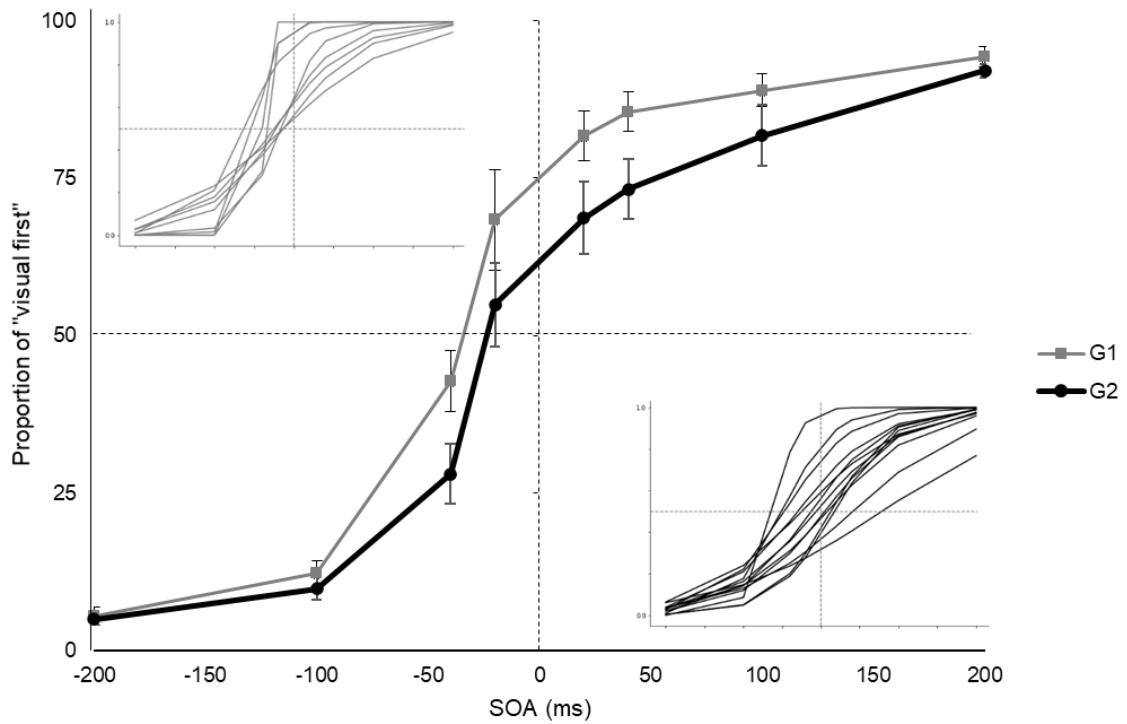
The response curves of individual rats were fitted (**Fig. S8**) to extract the Point of Subjective Simultaneity (PSS: SOA at which the rats perform at chance level) and the Just-Noticeable Difference (JND: minimal necessary SOA for the rats to perform above chance level). G1 rats uniformly displayed negative PSS indicating that all rats were overall biased towards responding “V first” irrespective of the SOAs, meaning that they required the sound to be presented much earlier than the visual flash to consider them simultaneous. The G2 rats showed larger inter-individual variability covering both negative and positive PSS values. Due to this large inter-individual variability, no significant differences of PSS were found between the two groups ($t(20) = -1.865, p = .077$) (**Fig. 5b**). A one sample t-test was conducted to compare the PSS of each group with the physical simultaneity of the stimuli (SOA = 0 ms). Results

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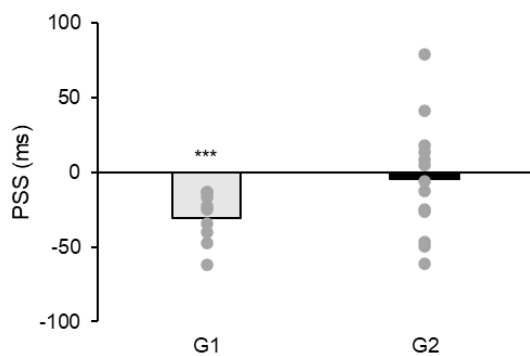
showed a better accuracy for G2, as the PSS was not significantly different from the veridical synchrony ($t(12) = -0.444, p = .665$), contrary to a significant bias to 'V first' in G1 rats ($t(8) = -5.461, p < .001$). The JND were significantly different between groups ($t(20) = -2.179, p = .041$), showing that G2 was less sensitive in order detection than G1 (**Fig. 5c**).

AUDIOVISUAL TEMPORAL ORDER

a



b



c

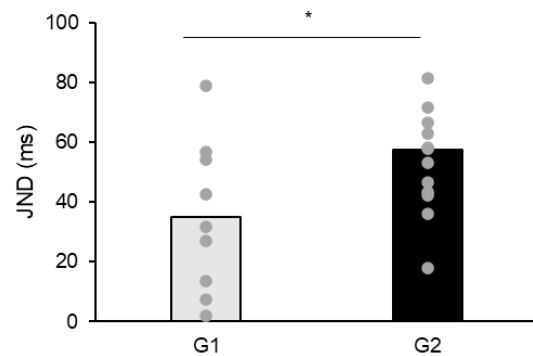


Figure 5: Psychophysics of temporal order: G2 rats showed large interindividual variability, better accuracy but less sensitivity to order detection than G1. The mean (\pm SEM) proportion of "visual-first" response, i.e. the correct response associated to VA type of trials (a) during the presentation of the 8 Stimulus Onset Asynchronies (SOAs) for both groups and the individuals' psychophysical fits plotted for G1 (inset top left) and G2 (inset

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bottom right). Negative SOAs are associated with AV order and positive SOAs with VA order. The mean (histograms) and individual (grey dots) of the point of subjective simultaneity (PSS) for both groups (**b**). The mean (histograms) and individual (grey dots) of the just noticeable difference (JND) for both groups (**c**). *, $p = 0.04$.

Principal component analysis and linear regressions

To explore whether it was genuine inter-individual differences in the animals or differences in the protocols that drove the observed differences in sequential effects and temporal order judgments, we performed a Principal Component Analysis. PCA is a statistical procedure that regroups observations from different variables into orthogonal factors called principal components. We decided to select the group membership as a qualitative variable reflecting differences in protocol ("Group": G1 and G2), and six quantitative variables for each animal, which reflect inter-individual differences: the number of days to reach the criterion during training (Criterion), the ratio of correct responses between same/different sequences of VA trials (Sequence VA), the response type to the test of response bias to A and V (i.e., VA(V) and VA(A)), and the PSS and JND during the psychometric test. Results are reported in the correlation table (**Fig. 6a upper**) and visualized in the biplot graph, which displays the observations in the PCA space (**Fig. 6a lower**). Variables that share small angles are positively correlated (JND and PSS, or sequence VA and G2), whereas variables that are opposite, with 180° angle, are negatively correlated (PSS and VA(A)) and variables separated by 90° angle are uncorrelated (sequence VA, groups G1 and G2 and criterion with the JND, PSS, VA(A) and VA(V)). The two orthogonal/independent components represented 73.1% of the total variance. Thereby, in the first component (F1), which represents 53.31% of the variability, the negative correlation between the PSS and VA(A) (-0.842) was highest. The PSS was positively correlated with the JND (0.741). VA(A) showed a correlation of 0.661 with VA(V). Linear regressions were performed to refine direct correlations between variables, as for the PSS and VA(A) which were highly correlated $r(20) = .709$, $p < .001$, and for PSS and JND, $r(20) = .550$, $p < .001$ (**Fig. 6b upper**). The second component (F2), which represents 19.82%

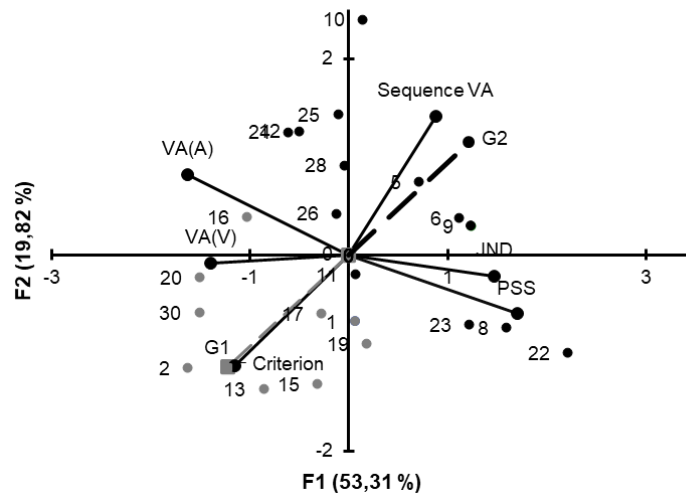
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of the variability, was composed of the Group variable which showed a strong correlation with the Criterion (0.716, positive for G1 and negative for G2) and with the Sequence VA (0.751, negative for G1 and positive for G2). The linear regression analysis showed high correlations between Group variable with Criterion, $r(20) = .512$, $p < .001$, and with Sequence VA, $r(20) = .565$, $p < .001$ (**Fig. 6b lower**). These results indicate that variables of F1 represent the inter-individual variability of the rats, while the variables of F2 are dependent on the behavioral protocol.

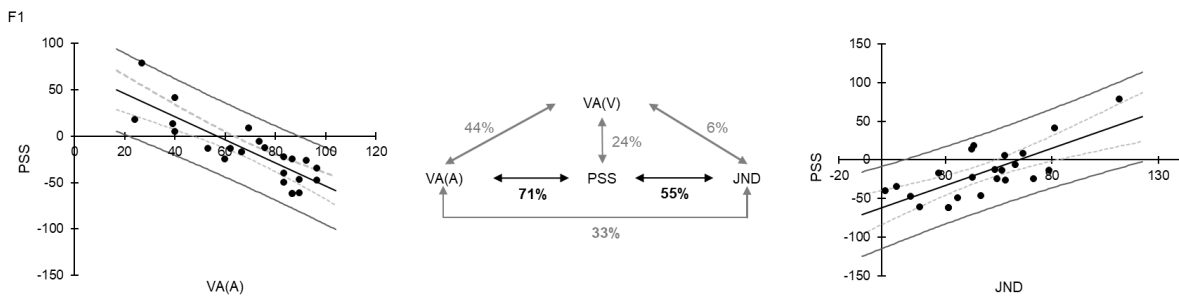
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a

| Variables | | Group | PSS | JND | Criterion | Sequence VA | VA(V) | VA(A) |
|-------------|----|-------|--------|--------|-----------|-------------|--------|--------|
| Group | G1 | 1 | -0.385 | -0.438 | 0.716 | -0.751 | 0.473 | 0.278 |
| | G2 | 1 | 0.385 | 0.438 | -0.716 | 0.751 | -0.473 | -0.278 |
| PSS | | | 1 | 0.741 | -0.336 | 0.223 | -0.494 | -0.842 |
| JND | | | | 1 | -0.336 | 0.338 | -0.254 | -0.578 |
| Criterion | | | | | 1 | -0.465 | 0.462 | 0.257 |
| Sequence VA | | | | | | 1 | -0.273 | -0.090 |
| VA(V) | | | | | | | 1 | 0.661 |
| VA(A) | | | | | | | | 1 |



b



c

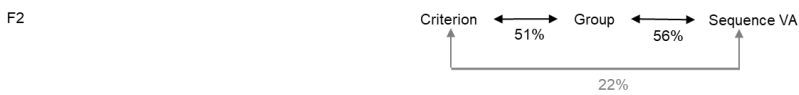


Figure 6: The behavioral protocol determines the sequential effect and the number of days to criterion. Two PCA orthogonal factors accounted well for behavioral variance capturing individual variables and protocol manipulations. The correlation matrix (**a**) obtained after PCA between one qualitative (group) and 7 quantitative variables characterizing behavioral performances (PSS and JND extracted from psychometric tests; number of days to learning Criterion; Same-Different ratio for sequence effects to VA trials; VA(V) or VA(A) categorization to V and A single stimulus presentation, respectively). Bold values indicate the significant correlations. Only correlations greater than 0.6 were retained (grey frames). Biplot of the observations (as points) and the variables (as vectors) represented in the two principal component spaces. Factor 1 (F1) and Factor 2 (F2) explained 73.13 % of the total variance in the behavioral data. Dash lines represented the qualitative variables and solid lines the quantitative variables. The most significant correlation between F1 variables (**b**) (PSS, JND, VA(A) and VA(V)) calculated via a simple linear regression (percentage of correlation (middle)). Linear regression between the PSS and VA(A) (left) and between the PSS and JND (right). The black central solid line represents the model, the two grey solid lines, the observed interval of confidence and the two dotted grey lines the 95% interval of confidence. The most significant correlation between F2 variables (**c**) (Group, Sequence VA and learning Criterion), calculated via a simple linear regression (percentage of correlation). Black percentages represent the most significant relations for **b** and **c** ($p < 0.0001$).

Discussion

We investigated temporal order judgments in rats comparing two training protocols: one in which the processing of both stimuli and their serial order was encouraged by the paradigm (G2), and one in which the rats could develop alternative strategies to respond correctly (G1). Interestingly, G2 rats reached the success criterion faster than the G1 rats. Unlike the G1 rats, the G2 rats showed a sequential effect with a reduced accuracy when the order of stimuli was different on trial n than on trial $n-1$. The two groups learned to process audiovisual stimuli differently: G2 rats distinguished single stimuli (A or V) from both multisensory pairs (AV or VA) and repeated unisensory stimuli (AA or VV), showing they effectively processed the two stimuli in the pair. This contrasts with G1 rats, which seemed strongly biased towards answering “VA”. The psychometrics showed that G2 rats were more accurate, but less sensitive, in their TOJ as compared to G1. Hence, while both groups could perform TOJ, only rats trained in G2 displayed robust behavioral signatures similar to those reported in humans, as discussed below.

In line with these general observations, the PCA showed two orthogonal principal factors. Interestingly, the factor F2 grouped the speed to reach the success criterion and the sequential effect, two variables controlled by the training protocol. Both variables affected the results as a function of the group: G2 were faster learners and displayed a sequential effect. The faster learning was not expected, as G2 animals were under a partial reinforcement schedule (50% of trials unreinforced) and a more complex discriminative task. In effect, these results contrast with Kosiba & Logan, 1978 who reported that groups exposed to more complex temporal order protocols reached a maximum learning index later than groups exposed to simpler protocols. The

difference with our results may originate from a higher level of complexity in their behavioral protocol, in which they used unreinforced single stimuli in addition to unreinforced pairs of stimuli and reinforced temporal order stimuli. Here, we did not add single stimuli during the training, but we tested them separately from the main task. Furthermore, our learning criterion was mainly based on performance to reinforced VA and AV order stimuli, and was not as stringent with regard to unreinforced AA and VV trials (reaching on average 25% of no response, but with increased response latency when responding, thus indicating discrimination). Interestingly, the progression of performance at the outset of training indicates that G2 rats first learned discriminations between bi-sensory (AV, VA) and unisensory (AA, VV) modality compounds before learning a conditional lever choice discrimination between the AV and VA stimuli (see Fig. S2). This successive discrimination acquisition may have helped G2 rats to learn the task faster, by learning to take into account both the first and the second stimulus and extinguishing biases that may exist towards the first stimulus. Another, not mutually exclusive, possibility may lie in the difference in reinforcement schedules between the two groups, as the greater the inter-reinforcement interval, the faster the acquisition of the conditioned response (Deisig et al., 2007; Gibbon & et al, 1977; Kirkpatrick & Church, 2000; Ward & Gallistel, 2013). In our study, we used a fixed inter-trial interval of 10 s in both groups, leading to a larger inter-reinforcement interval for G2, due to the unreinforced trials. Thereby, the informativeness of the reinforced trials for G2 was greater than for G1, which could explain the G2's faster reaching of the criterion. One way to test this hypothesis would be to train rats with a protocol similar to G1 but with longer inter-trial intervals, and determine whether they would reach the criterion sooner. Another possibility could relate to differences in level of attention, as proposed by Pearce & Hall, 1980, in which a cue predicting a consequence 100% of

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the time would decrease its associability (e.g. in G1, the stimulus A always predicts V as a second stimulus and a given side for a correct response), whereas a partially predictable index (the stimulus A predicts either **AV** or **AA** in G2) would maintain the attentional level of the subject. As a result, the associability of the first stimulus would be stronger for G2 compared to G1, leading to faster learning of G2 than of G1. G2 rats also showed a tendency to produce more correct responses than G1 during all protocols except during the sequential effect sessions where there was a reduction in the number of unreinforced trials, suggesting that G2 performance may depend on the proportion of AA and VV trials. These observations are consistent with Wasserman et al., 1984, who tested the discrimination and retention of temporal order in pigeons. In this study, pigeons were trained to peck a stimulus after the presentation of a pair of visual stimuli (A and B) followed by a test stimulus (C and D). Similarly to our study, the AA and BB stimuli were added to ensure that the order of successive stimuli was processed. Among the trial types presented, only one condition was rewarded (**AB-C / BA-D**). Pigeons learned to discriminate those trials from the reverse ones (**AB-D / BA-C**) and stimuli repetition trials (**AA-C/D** and **BB-C/D**) which were unreinforced. The authors showed that pigeons were able to distinguish temporal order of visual stimuli since the response rate to unreinforced trials (reverse and repetition trial) decreased drastically compared to reinforced trials. Trials with the repetition of the same stimulus were more easily discriminated compared to the reverse trials, in particular when the interval between the pair of stimuli and the test stimulus was increased. In agreement with our observation of successive discrimination acquisition, these results suggest that the addition of trials with a repetition of the same stimulus may foster temporal order discrimination. In our experiment, the poorer performance when the proportion of AA and VV trials was reduced during sequential effect sessions further confirms that

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the repeated stimuli did promote the processing of both stimuli by the animals to correctly respond to the order. However, the decrease in performance suggests that reducing the amount of AA / VV trials during the sequential test sessions may have also increased the task difficulty for G2 animals, and this may have promoted the use of the previous trial in order to compensate for the loss of information, thus leading to a sequential effect. Whether task difficulty is a critical parameter for observing sequential effects in animals remains an open question.

The sequential effects indicated that G2 rats kept the tendency to report the same order in trial n as in trial $n-1$. This effect was presumably due to the first stimulus of the $n-1$ trial biasing the rat in the same direction in trial n . This measurement is, therefore, an additional indicator for understanding how rats process the order of stimuli. Weisman et al., 1980, found that, when pigeons were trained to discriminate between a reinforced ordered pair of visual stimuli and combinations of these stimuli, either in a different order or unique stimulus presentation, their behavior showed initially a strong recency effect (bias toward the second stimulus in the pair), then some primacy effect (bias toward the first stimulus in the pair), before mastering the order processing as training progressed. Whether similar recency and primacy effects play a role in the sequential effects observed here remains an open question. Sequential effects described in G2 are in accordance with several studies in humans, including those using unisensory stimuli and asynchronous discrimination tasks (Giersch et al., 2009; Marques-Carneiro et al., 2020; Elliott et al., 2007). In a study by Recio, Cravo et al. (2019), human participants were biased towards reporting the same perception as in the previous trial in a TOJ task, and similar results were reported by Roseboom (2019), and Keane et al. (2019). The presence of sequential effects in rats strongly

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indicate that only G2 may have actually processed temporal order with sequential effects imputed to perceptual representations, not response repetition.

The other factor (F1) groups together variables related to individual differences, rather than training rules. The response types during the test of single stimuli (A alone and V alone) were correlated with the individuals' PSS. Rats in G1 were homogeneously biased towards responding Visual first, but a large heterogeneity was observed in G2 rats. Within-individual variability in rats has previously been reported (Schormans et al., 2017) but fully accounted for by the intensity (hence presumed saliency) of the stimuli. Inter-individual variability has been reported in humans, and interpreted as a structural bias in perceptual systems affecting both auditory, visual, audiovisual temporal order, as well as spatialized order (Grabot & Wassenhove, 2017). Inter-individual variability may drive differences in multisensory processing (Powers et al., 2009; Stevenson et al., 2008). Ultimately, such intrinsic biases affect the functional interpretability of an individual's ongoing neural activity towards representing a temporal order or another (Grabot et al., 2018; Kösem et al., 2014). In our study, the intensities of the stimuli were kept constant within and across groups; it is thus unlikely that the 'visual-first' bias seen in G1 was induced by a strong difference in stimulus salience, as the bias was not observed in G2 with the same parameters. For a given set of auditory and visual intensities, we observed, as for humans, consistent inter-individual biases towards reporting a particular order.

An alternative hypothesis would be that rats used the 1st stimulus to direct their spatial position and then adapted their response (or inhibition of response) according to the second stimulus. If this were the case, no sequential effects would have been observed for G2. Each trial would be independent of the previous trial and only the first

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stimulus of a trial would bias the response. Thus, the correct response rate should have been equivalent between the AV (or VA) trials preceded by AV or VA. Concerning G1, the result during the test of single A and V stimuli should have shown a bias toward “AV” after the presentation of A alone, and a bias towards “VA” after presentation of V alone. However, a “VA” response bias was observed during A and V alone. The results of the two groups, therefore, contradict the hypothesis of a spatial positioning dependent upon the first stimulus followed by a response dependent upon the second stimulus.

The disruption of temporal order processing has been described in a variety of pathologies. Individuals in autism spectrum disorders (ASD) and with schizophrenia have difficulties discriminating events in time in general, whether in unisensory and multisensory modalities (Foucher et al., 2007; Martin et al., 2013; Noel, 2018; Wallace & Stevenson, 2014). Individuals with schizophrenia also show difficulties in ordering stimuli (Capa et al., 2014), and patients with ASD and dyslexia show a reduced sensitivity to temporal order (de Boer-Schellekens et al., 2012; Jaśkowski & Rusiak, 2008). Ordering difficulties are especially crucial given the relationship between ordering and causality (Woods et al., 2014; Bramley et al., 2018). In turn, difficulties to judge causality may originate delusions (Maeda et al., 2012; Tschacher & Kupper, 2006). However, the mechanisms of the ordering difficulties in patients are still unknown. A behavioral task in animals may be a precious pre-clinical tool for the investigation of the neural properties of the structures involved in the treatment of temporal order, the associated subjectivity of temporal experience, and the development of potential therapeutic treatments.

One may wonder, however, to what extent rodents process the order similarly as to humans, and whether they have an explicit concept of order when solving the

task. In particular, some authors have suggested that animals discriminate between stimulus sequences by using memory traces, rather than processing order (Ghirlanda et al., 2017). In other words, G2 animals would discriminate AV trials from VV trials because there remains a trace of A when V appears. Under this hypothesis, the animal could have associated one lever with V as last stimulus and the other lever with A as last, and learned not to respond when the traces of the first and second stimuli are coming from the same sensory modality. If this were the case, we would expect a synchronous increase between the percentage of no-response and/or increased response latencies to AA/VV trials and the percentage of correct responses to AV/VA during the first days of learning. This is not what we observed (see Fig. S2), but the training paradigm with mixed forced and free choice trials may have modified differentially the learning dynamics between the two groups. Further studies are needed to address specifically this question.

Taken together, our results demonstrate that the addition of non-reinforced pairs of unisensory trials is a simple, but highly efficient, instruction to encourage rats to process temporal order, as manifested by its perceptual signatures. The patterns of responses were similar to those found in humans for whom the temporal order rule can simply be stated verbally. Among the perceptual signatures selectively found for G2, and not G1, we report inter-individual variability and sequential effects. Altogether, we thus conclude that the processing of individual stimuli and of their order requires well-tuned instructional protocols.

Declaration of conflicting interests

The authors declare that they had no conflicts of interest with respect to their authorship or the publication of this article.

Funding sources: ANR AutoTime

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Figure Captions

Figure 1: description of the behavioural task and protocol chronology. Description of the AV/VA trials performed by G1 and AV/VA/AA and VV trials performed by G2 during training sessions (**a**). Rats learned through reinforcement the association between audiovisual temporal order and lever assignment (**b**). Description of the different stages of reduction of stimulus duration and then of Stimulus Onset Asynchrony (SOA) applied from protocol 1 to protocol 4. Timeline of the training phase (grey frame) followed by behavioral tests carried out with each protocol (**c**). During the reversal, the lever/order assignment was switched.

Figure 2: Audiovisual temporal order task: G2 reached the success criterion faster than G1 during training, with a tendency to better learn the reversal. Rats in G2 (G2; N = 15) reached the criterion faster than those in G1 (G1; N = 15) (**a**; top) under P1, **, U = 195, $p < 0.001$, $rpb = .733$, 95% CI [.475, .875]. Only 9 rats in G1 and 13 rats in G2 succeeded to learn the new rule during reversal (**a**; bottom). Performance of individual rats until they reached the criterion during training and reversal (**b**; four upper graphs), and mean (\pm SEM) number of days to criterion during both phases for the animals that succeeded to reverse (histograms). **, unpaired $p < 0.01$. The mean (\pm SEM) percentage of correct responses (**c**, top) and their latencies (**c**, bottom) from basal level for each group during the last day of training, and the first and last day of reversal. Mean (\pm SEM) percentage of no-response (**d**, top) and latency of responses (**d**, bottom) from basal level during unreinforced (AA and VV) trials for G2. #, $p < 0.05$, as compared to the basal level corresponding to the first day of training for **c** and **d**.

Figure 3 : Sequential effects: G2 rats displayed serial dependency of the preceding trial (n-1) on the current trial (n). Results from P4. The mean (\pm SEM) percentage of correct responses (**a**, left) and latency (**a**, right) plotted separately for G1 and G2 as a function of the type of trials (n) and the same or different (Diff) trial type of the sequence (n-1). The percentage

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of correct responses depended on the order presented on the previous trial in G2, but not in G1. For G2, the mean (\pm SEM) percentage of response type (**b**) during AA and VV trials depended on the first stimulus of the previous sequence (AV or VA). The tendency to respond to the first stimulus aligns well with the sequential effect in (**a**): if G2 rats optimize their responses on the basis of the first stimulus, when the order presented in $n-1$ and n is identical, so are the first stimuli and G2 rats will tend to persist; when the order in n and $n-1$ trials differ, the first stimuli also differ and G2 rats must correct their tendency to respond to the first stimulus. The mean (\pm SEM) latency of response (**d**) during unreinforced trials AA and VV as a function of the previous trial (AV or VA) for G2. Post-hoc (a) and paired t-test (b): **, $p = 0.01$; ***, $p < 0.001$.

Figure 4 : Temporal order bias in G1, but not in G2. G1 rats were biased towards responding VA, as demonstrated by their responses after the presentation of a single V and a single A. The mean (\pm SEM) (top) or individual (bottom) percentage of responses categorized as “AV” or “VA” after the presentation of A and V stimulus alone in G1 (**a**) and G2 (**b**). Solid black lines represent “VA” responses, solid grey lines represent “AV” responses, and the dotted lines are random responses that could not be characterized consistently as “AV” or “VA” across A and V tests. *, $p = 0.01$; **, $p = 0.009$ compared to 50%. Rats in G2 did not respond to A and V tests as if they were AA or VV (**c**) while their pattern of responding to AA and VV trials was stable over sessions (**d**). G2 distinguished single stimulus presentation from AA and VV trials.

Figure 5: Psychophysics of temporal order: G2 rats showed large interindividual variability, better accuracy but less sensitivity to order detection than G1. The mean (\pm SEM) proportion of “visual-first” response (**a**) during the presentation of the 8 Stimulus Onset Asynchronies (SOAs) for both groups and the individuals’ psychophysical fits plotted for G1 (inset top left) and G2 (inset bottom right). Negative SOAs are associated with AV order and positive SOAs with VA order. The mean (histograms) and individual (grey dots) of the point of

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subjective simultaneity (PSS) for both groups (**b**). The mean (histograms) and individual (grey dots) of the just noticeable difference (JND) for both groups (**c**). *, $p = 0.04$.

Figure 6: The behavioral protocol determines the sequential effect and the number of days to criterion. Two PCA orthogonal factors accounted well for behavioral variance capturing individual variables and protocol manipulations. The correlation matrix (**a**) obtained after PCA between one qualitative (group) and 7 quantitative variables characterizing behavioral performances (PSS and JND extracted from psychometric tests; number of days to learning Criterion; Same-Different ratio for sequence effects to VA trials; VA(V) or VA(A) categorization to V and A single stimulus presentation, respectively). Bold values indicate the significant correlations. Only correlations greater than 0.6 were retained (grey frames). Biplot of the observations (as points) and the variables (as vectors) represented in the two principal component spaces. Factor 1 (F1) and Factor 2 (F2) explained 73.13 % of the total variance in the behavioral data. Dash lines represented the qualitative variables and solid lines the quantitative variables. The most significant correlation between F1 variables (**b**) (PSS, JND, VA(A) and VA(V)) calculated via a simple linear regression (percentage of correlation (middle)). Linear regression between the PSS and VA(A) (left) and between the PSS and JND (right). The black central solid line represents the model, the two grey solid lines, the observed interval of confidence and the two dotted grey lines the 95% interval of confidence. The most significant correlation between F2 variables (**c**) (Group, Sequence VA and learning Criterion), calculated via a simple linear regression (percentage of correlation). Black percentages represent the most significant relations for **b** and **c** ($p < 0.0001$).