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Nucleus-accumbens dopamine tracks aversive-stimulus duration and prediction but not value or prediction error

AUTHORS

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ABSTRACT (< 150 words)

There is active debate on the role of dopamine in processing aversive stimuli, where inferred roles range from no involvement at all, to signaling an aversive-prediction error (APE). Here, we systematically investigate dopamine release in the nucleus-accumbens core (NAC), which is closely linked to reward-prediction errors, in rats exposed to white noise (WN, a versatile, underutilized, aversive stimulus) and its predictive cues. Both induced a negative dopamine ramp, followed by slow signal recovery upon stimulus cessation. In contrast to reward conditioning, this dopamine signal was unaffected by WN value, context valence, or probabilistic contingencies, and the WN dopamine-response shifted only partially towards its predictive cue. However, unpredicted WN provoked slower post-stimulus signal recovery than predicted WN. Despite differing signal qualities, dopamine responses to simultaneous presentation of rewarding and aversive stimuli were additive. Together, our findings demonstrate that instead of an APE, NAC dopamine primarily tracks prediction and duration of aversive events.

INTRODUCTION

The midbrain dopamine system plays critical roles in motivation, learning, and movement; specifically for learning about rewards and creating motivational states that promote reward-seeking (Berridge & Robinson, 1998; Bromberg-Martin *et al.,* 2010; Berke 2018; Schultz, 2019). One of the most prominent functions of dopamine is the encoding of a so-called reward prediction error (RPE) signal (Schultz *et al.,* 1997): When a reward is fully predicted by a cue, the increase in dopamine cell firing and terminal release of dopamine shifts backwards in time from the moment of reward delivery, to that of the cue presentation (Schultz *et al.,* 1997; Flagel *et al.,* 2011). Furthermore, dopamine neurons pause their firing when a predicted reward is omitted, and increase their firing in response to the

delivery of an unpredicted reward. Thus, dopamine neurons encode the difference between predicted and obtained reward, which is corroborated by the fact that dopamine-neuron activity scales with the relative value of reward and unexpected deviations from this value (Bromberg-Martin & Hikosaka, 2009).

Although the vast majority of studies focus on the relationship between dopamine and stimuli with a positive valence (rewards), the relevance of the dopamine system in processing stimuli with the opposite valence (aversive) has also generated great interest. In contrast to the primarily stimulatory response of rewards on dopamine activity, the reports on the effect of aversive events on the dopamine system are less consistent. For example, on the level of dopamine-neuron cell-bodies, aversive stimuli were demonstrated to result in inhibition of neuronal activity (Ungless *et al*., 2004; Mileykocskiy & Morales, 2011), excitation thereof (Anstrom *et al*., 2009; Valenti *et al*., 2011), or no effect at all (Mirenowicz & Schultz, 1996; Fiorillo, 2013). The widely-accepted explanation for these varying results is that sub-populations of dopamine neurons exhibit different response profiles to aversive stimuli (Schultz & Romo, 1987; Guarraci & Kapp, 1999; Coizet *et al*., 2006; Bromberg-Martin & Hikosaka, 2009; Zweifel *et al*., 2011; Cohen *et al*., 2012; Lammel *et al*., 2011), whereby variance is presumably introduced by different types of aversive stimuli, by the fact that some studies were performed in awake and others in anesthetized animals, and by the location and projection targets of the recorded dopamine neurons (Brischoux *et al*., 2009; Matsumoto & Hikosaka, 2009; Lammel *et al*., 2011). However, activity at the level of dopamine-neuron cell-bodies does not necessarily always translate to their projection-targets (Mohebi *et al*., 2019), as axonal-terminal release of dopamine can operate independently from cell-body activity (Threlfell *et al*., 2012). Therefore, in interrogating the entire spectrum of functions of the dopamine system, it is imperative to include projection-target measurements of extracellular dopamine concentrations.

Midbrain dopamine neurons modulate their targets via population signals: Dopamine release from a large number of extra-synaptic terminals, combined, constitutes a diffusion-based signal that is perpetuated by volume transmission (Rice & Cragg, 2008). The vast majority of projections from dopaminergic neurons target the striatum and its subregions. Inconsistent with the classic hypothesis positing that the dopamine system broadcasts a uniform signal across the striatum, it has been reported multiple times in recent years, that dopamine signals display regional heterogeneity (Willuhn *et al.,* 2012, Willuhn *et al.,* 2014a, Lammel *et al.,* 2011, DeJong *et al.,* 2019, Menegas *et al.* 2017; Klanker *et al.* 2019). This heterogeneity is reflected in dopamine responses to aversive events throughout the striatum: Whereas microdialysis studies report an increase in dopamine release in the nucleus accumbens in response to aversive events (Young *et al*., 1993; Young, 2004; Wilkinson *et al*., 1998; Bassareo *et al*., 2002; Pascucci *et al*., 2007; Ventura *et al*., 2007; Martinez *et al*., 2008 but see Mark *et al*., 1999; Liu *et al*. 2008), studies employing techniques with a higher, subsecond temporal resolution (e.g., fast-scan cyclic voltammetry (FSCV) or fluorescence fiber photometry) arrive at less consistent conclusions. For example, aversive stimuli produced an increase in dopaminergic activity in the nucleus accumbens shell (NAS) in some studies (Badrinarayan *et al.,* 2012; DeJong *et al.,* 2019), but a decrease in others (Roitman *et al.,* 2008; Wheeler *et al.,* 2011; McCutcheon *et al.,* 2012;

Twining *et al.,* 2015). Similarly, contradictory findings are also reported in the neighboring nucleus accumbens core (NAC), where studies find both increased (Budygin *et al.,* 2012; Mikhailova *et al.,* 2019) and decreased dopamine activity (Badrinarayan *et al.,* 2012; Oleson *et al.,* 2012; DeJong *et al.,* 2019; Stelly *et al.,* 2019). In contrast, in the tail of the striatum, aversive events exclusively result in increased dopaminergic activity (Menegas *et al.,* 2017; Menegas *et al.,* 2018). Overall, it can be concluded that most studies observe a change in dopaminergic activity in response to aversive stimuli, that there are substantial differences between striatal regions in this response, and that it remains unclear what determines whether aversive events provoke an increase or a decrease in dopaminergic activity within striatal regions.

Delineating the role of dopamine in processing aversive events crucially requires understanding what the above-described changes in dopamine signaling encode specifically; or in other words, whether these changes reflect aversive-prediction errors (APEs, in which the dopamine response would reflect the discrepancy between expected and received aversive events) or merely individual aspects of aversive conditioning (such as the presence of aversive stimuli, and/or their prediction). A thorough analysis by Fiorillo (2013) concluded that dopaminergic midbrain neurons do not encode aversive stimuli, but other studies have observed aspects of a dopamine APE, such as the predictive cue adopting the dopamine response of an aversive stimulus (Guaracci & Kapp, 1998; Oleson *et al*., 2012; Badrinarayan *et al*., 2012), or an APE-like response when the aversive stimulus was unpredicted or omitted (Matsumoto & Hikosaka, 2009; Matsumoto *et al*. 2016; Menegas *et al*. 2017; Salinas-Hernández *et al*., 2018; DeJong *et al*., 2019). However, it should be kept in mind that in case of omission or early termination of an expected aversive event, rewarding aspects of a milder-than-expected aversive event (RPE) may be mixed with an APE (Oleson *et al*., 2012; Salinas-Hernández *et al*., 2018; Stelly *et al*., 2019).

To consolidate these contradictory findings, we systematically evaluated whether dopamine truly signals an APE through a series of behavioral experiments in rats, in which we varied the value of the aversive stimulus, context valence, and probabilistic contingencies, and compared aversive and appetitive conditioning. Using FSCV, we measured the real-time dopamine response to these conditions in the NAC, since this striatal region is a hot spot for RPE-like signals (Flagel *et al*., 2011; Papageorgiou *et al*., 2016) and is also tightly linked to motivational processes related to aversion avoidance (Badrinarayan *et al.,* 2012; Oleson *et al.,* 2012; Stelly *et al.,* 2019). We employed loud white noise (WN) as the aversive stimulus. WN provides several advantages over more commonly-used aversive stimuli (such as electric shocks or air puffs), as it is well-tolerated by rats and not painful, precisely-controllable (intensity and duration can be effortlessly titrated), aversive without inducing freezing (most pertinent to the current study, as this might interfere with the dopamine signal), does not jeopardize the recording equipment, does not introduce artefacts to the recordings, and can be administered reliably (see Discussion). Based on the above-described findings, we hypothesized that NAC dopamine would exhibit an APE. However, we find that NAC dopamine concentration ramps down in response to both WN exposures and its predicting cue, ramps back

upwards upon stimulus cessation, that these ramps were qualitatively different from appetitive conditioning, and were inconsistent with a full APE signal.

RESULTS

White noise (WN) is aversive

We established the aversiveness of loud WN using a real-time place-preference test, in which rats were exposed to 90-dB WN upon entry into one of the four quadrants of an open field (Figure 1A). An example path traversed by a rat over the course of 30 minutes is depicted in Figure 1A (left panel), where the dark shaded area represents the quadrant paired with WN. On average, rats (n = 10) spent a significantly smaller percentage of time in the WN quadrant (9.71 ± 1.65) compared to chance level ($t(9) = 9.585$, $p < 0.0001$; Figure 1A, right panel). Next, we validated that rats can discern between different magnitudes of WN in an operant choice task. Here, rats could choose between pressing one of two extended levers, both of which prompted immediate delivery of a food pellet, but one of which additionally presented 5s of 70, 90, or 96 dB WN. Unsurprisingly, rats (n = 6) preferred the non-WN lever, as indicated by a significant main effect of WN intensity using a Friedman test ($\chi^2(3) = 11.57$, $p = 0.0003$). Importantly, when comparing the WN-paired lever presses, rats significantly preferred the 70-dB WN to the 96-dB WN (post-hoc Dunn's tests,$p = 0.0027$; Figure 1B). Interestingly, exposure to WN stimulated locomotor activity: Rats in an operant box significantly increased locomotion speed in response to semi-random presentation of 6s-WN bouts ($t(13) = 7.059$, $p < 0.0001$; Figure 1C).

WN suppresses dopamine release in the nucleus accumbens core (NAC)

All FSCV recordings (and all behavioral data presented from here on out) were conducted in operant boxes equipped with a food magazine, a multiple-tone generator (cue speaker), and WN generators (WN speaker) (Med-Associates; Figure 1D). Electrolytic lesions at the FSCV electrode tip were used to histologically verify electrode placement (Figure 1E). Histological analysis confirmed for all animals included in this study that the sensing end of their FSCV electrodes was consistently placed in the NAC (Figure 1F). Unexpected exposure to 6s of 90-dB WN strongly and reliably decreased extracellular concentrations of dopamine in the NAC (Figure 1G).

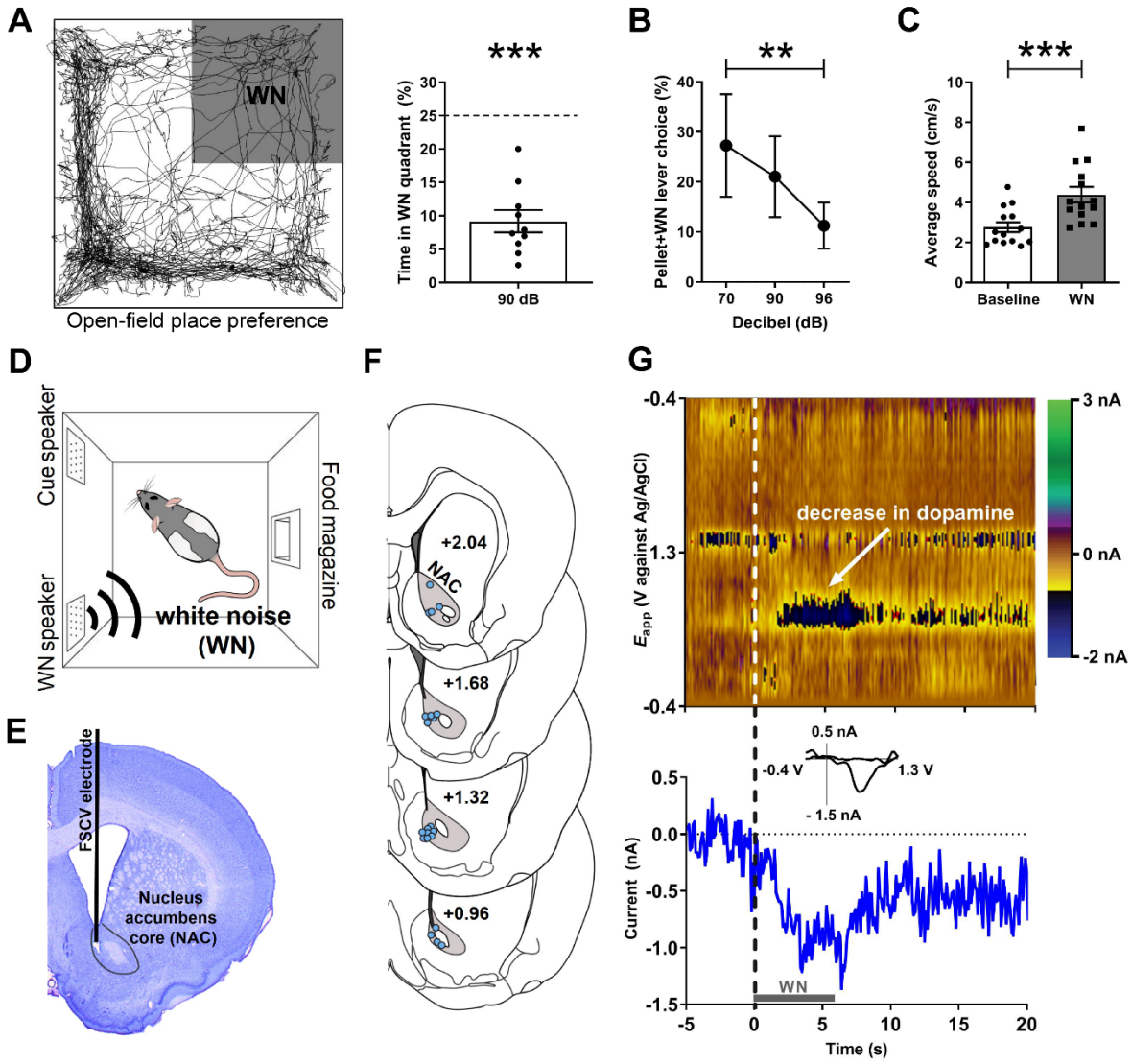

Figure 1 - White noise (WN) is an aversive stimulus that lowers dopamine concentration in the NAC. (A) Left: Example trajectory of a rat in the real-time place preference test (30 min), in which entry into the upper-right quadrant (shaded) led to 90-dB WN exposure. Right: Aversiveness of WN in the place preference test quantified as significantly decreased time spent in the WN quadrant (n = 10, 9.71 ± 1.65, $t(9) = 9.585$, $p < 0.0001$). **(B)** Rats (n = 6) discern between different WN magnitudes in an operant choice task, where they had to choose between pressing a lever that resulted in a food-pellet delivery and a lever that resulted in a food-pellet delivery plus simultaneous 5s of WN (Pellet+WN; Friedman test, $\chi^2(3) = 11.57$, $p = 0.0003$). 70 dB was less aversive than 96 dB ($p = 0.0027$). Data are mean±SEM. **(C)** Semi-random presentations of 6s-WN bouts increased the locomotion speed of rats in an operant box during the WN epoch compared to pre-WN baseline (post-hoc Dunn's test, $t(13) = 7.059$, $p < 0.0001$). **(D)** All fast-scan cyclic voltammetry (FSCV) recordings took place in operant boxes equipped with a food magazine, a multiple-tone generator (cue speaker), and a WN generator (WN speaker). **(E)** Example cresyl violet-stained brain slice depicting an electrolytic lesion in the NAC (outlined) at the tip of the FSCV electrode (vertical black line). **(F)** Schematic overview of FSCV recording locations (blue dots) in the NAC (gray) of all animals. **(G)** Single-trial pseudocolor plot (top panel), dopamine trace (bottom panel), and cyclic voltammograms (inset in bottom panel) for representative, dopamine-specific current fluctuations recorded in NAC, 5s before WN (dashed line), during 6s of WN (gray bar), and 14s after WN.

Different temporal NAC dopamine dynamics during aversive and appetitive Pavlovian conditioning

Dopamine release in the NAC is often consistent with a temporal-difference RPE, where an increase in dopamine activity, initially time-locked to the delivery of a reward, shifts backwards in time to its predicting cue. It is assumed that this phenomenon reflects the learned association between predictive cue and reward, where the reward becomes fully predicted by the cue and, therefore, no prediction error occurs at the time of reward delivery after sufficiently repeated cue-reward pairings (e.g., Schultz *et al.*, 1997; Flagel *et al*., 2011). Our first experiment investigated whether a similar phenomenon also applies to aversive stimuli and their predictors, and in what time frame such a shift may occur. Rats (n = 16) were exposed to 30 pairings of cue (5s) and 90-dB WN (6s) that were separated by a variable inter-trial-interval (Figure 2A). In order to visualize the rapid changes in dopamine response presumably reflecting learning, the first five trials are depicted individually, and based on their stable visual appearance, trials 6-10 and 11-30 were binned together. Using one-way repeated measures ANOVAs, in which we compared the average dopamine concentration during baseline, cue, and WN epochs, we found significant main effects in all trials (Trial 1: $F(1.403, 19.65) = 6.853$, $p = 0.0102$; Trial 2 : $F(1.324, 19.86) = 8.205$, $p = 0.0059$; Trial 3: $F(1.265, 18.98) = 6.737$, $p = 0.013$; Trial 4: $F(1.100, 16.50) = 5.016$, $p = 0.0363$; Trial 5: $F(1.548, 23.23) = 21.90$, $p < 0.0001$; Trials 6-10: $F(1.025, 15.38) = 15.94$, $p = 0.0011$; Trials 11-30: $F(1.093, 16.39) = 38.42$, $p < 0.0001$). Post-hoc analyses using Wilcoxon signed-rank tests with a Holm-Bonferroni multiple-comparison correction revealed significantly lower dopamine concentrations during the WN epoch compared to pre-cue baseline during trial 1 ($Z = -1.988$, $p = 0.0235$), trial 2 ($Z = -2.430$, $p = 0.0075$), trial 3 ($Z = -2.327$, $p = 0.010$), trial 5 ($Z = -3.464$, $p < 0.0005$), trials 6-10 ($Z = 3.103$, $p = 0.001$) and trials 11-30 ($Z = -3.516$, $p < 0.0001$), indicating that the decrease in dopamine during WN is an unconditioned response, since it is observed already during the first trial. In contrast, we only observed significantly lower dopamine in the cue epoch compared to pre-cue baseline during trial 5 ($Z = -1.965$, $p = 0.0245$), trials 6-10 ($Z = -2.999$, $p = 0.0015$), and trials 11-30 ($Z = -3.516$, $p < 0.0001$), indicating that this decrease develops over time and, therefore, is a conditioned response. In these first 30 trials, the decrease in extracellular dopamine during the WN epoch did not disappear or decrease. Thus, WN does not provoke a substantial temporal shift of NAC dopamine signaling from unconditioned to conditioned (predictive) cue within the first session of training.

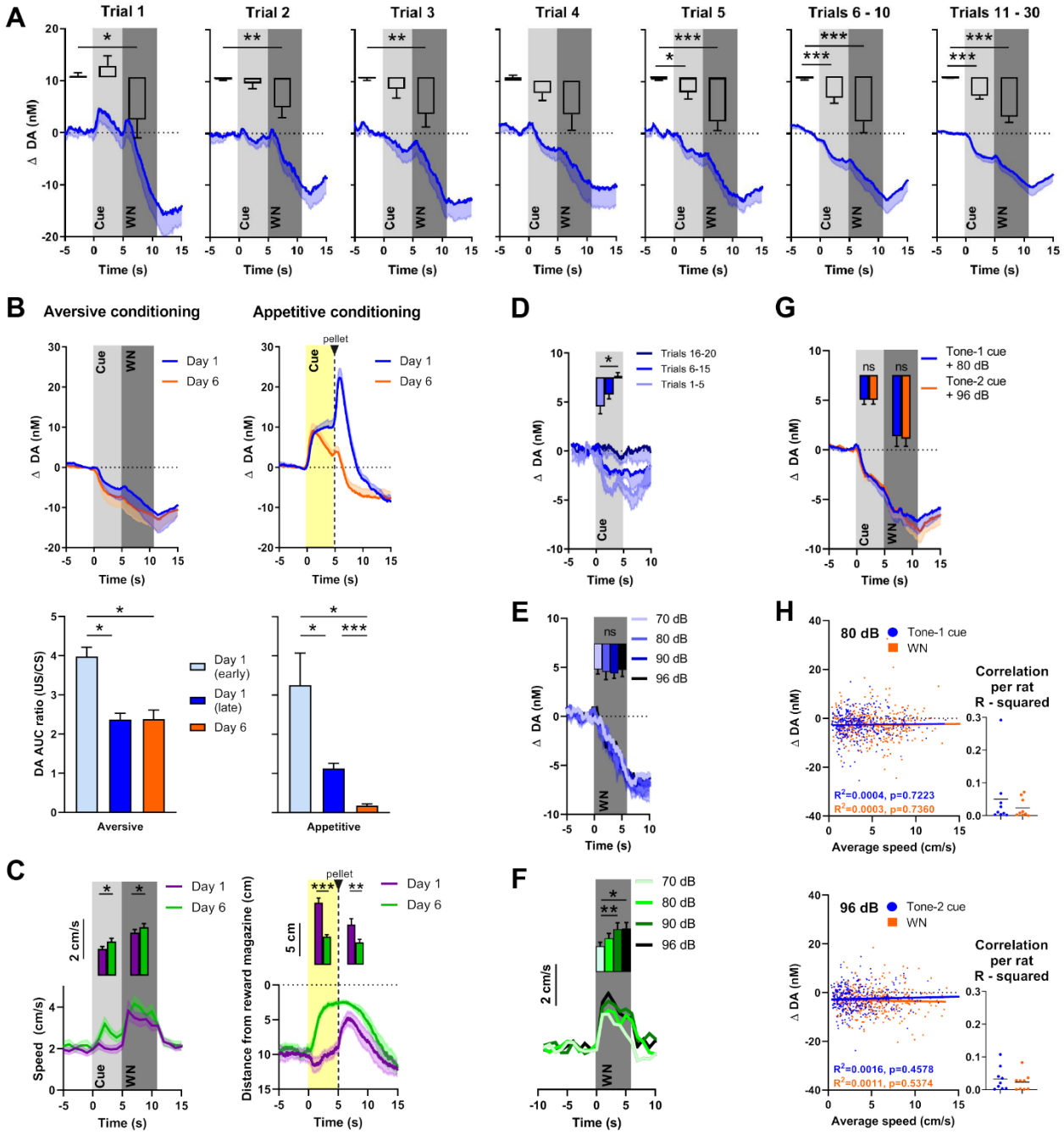

Figure 2 - NAC dopamine signaling and rat behavior during Pavlovian WN-cue conditioning and varying WN intensities. **(A)** Average extracellular concentrations of dopamine (DA; in nM) in the NAC (dark-blue line; SEM is shaded light-blue) during the first 30 pairings of cue (5 s tone) and WN (6 s, 90 dB) (16 rats). To illustrate the immediate, unconditioned effects of WN, the first five trials are displayed individually. The bar-graph insets depict dopamine release (+SEM) averaged for baseline, cue, and WN epochs. WN decreased dopamine significantly in all trials, except trial 4. The WN-paired cue began to decrease dopamine significantly starting at trial 5. **(B)** Comparison of dopamine release during aversive (left, n = 4) and appetitive (right, n = 10) Pavlovian conditioning. Top: Subsecond changes in dopamine concentration (nM) on day 1 (blue) and day 6 (orange). Bottom: Ratio (+SEM) of AUCs between the CS and US (US/CS) on day 1 (early and late trials) and day 6 during aversive (left) and appetitive (right) conditioning. For aversive conditioning, dopamine differed between day 1 (early) and day 1 (late) ($p = 0.0138$), and between day 1 (early) and day 6 ($p = 0.0318$). For appetitive conditioning, a significant difference was found between early and late conditioning on day 1 ($p=0.0441$) and dopamine differed between day 1 (early) ($p = 0.0102$) and day 1

(late) ($p < 0.0001$) compared to day 6. **(C)** Conditioned behavioral response corresponding to (B). Left: During aversive conditioning, locomotion speed during cue presentation increased from day 1 to day 6 ($Z = , p = 0.0491$), and also during WN ($Z = -2.343$, $p = 0.0.019$) ($n = 17$). Right: During appetitive conditioning, time spent in proximity of the reward magazine increased between day 1 and 6 both during cue presentation ($t(9) = 6.962$, $p < 0.0001$) and after pellet delivery ($t(9) = 2.572$, $p = 0.0301$). **(D)** In an extinction session, for 20 consecutive trials, WN was withheld after cue presentation ($n = 6$), and dopamine differed significantly between trials 1-5 and 16-20 ($p = 0.0133$). **(E)** In contrast, we detected no differences in dopamine release between exposure to varying WN intensities (70, 80, 90, or 96 dB; ($F(2.380, 11.90) = 0.1655$, $p = 0.8813$), $n = 6$). **(F)** While we do find a main effect of WN intensity on locomotion speed ($\chi^2(3) = 13.80$, $p = 0.0032$) and significant differences between 70 and 90 dB ($p = 0.005$) and 70 and 96 dB ($p = 0.0143$)($n = 13$). **(G)** We observed no significant differences in dopamine during cue ($Z = -0.059$, $p = 0.953$) or WN ($Z = -0.178$, $p = 0.859$) when two separate tones were used as predictors for 80-dB (blue) or 96-dB (orange) WN ($n = 9$). **(H)** Trial-by-trial correlation between locomotion speed and dopamine concentration during cue (blue) and WN (orange) for either 80-dB (top) or 96-dB WN (bottom) were not significant. Each dot represents one trial. Trials from all animals ($n = 9$) were pooled. Top left: No correlation during 80-dB WN ($R^2 = 0.0016$, $p = 0.457$) or its cue ($R^2 = 0.0004$, $p = 0.7223$). Bottom left: No correlation during 96-dB WN ($R^2 = 0.0011$, $p = 5374$) or its cue ($R^2 = 0.0003$, $p = 0.7360$). Right: R^2 values calculated separately for each individual rat confirms there is no significant correlation between locomotion speed and dopamine.

One possible explanation for an incomplete shift of dopamine signaling from WN to cue is that 30 pairings are insufficient to fully acquire the association. Therefore, we conditioned a subset of rats ($n = 4$) for five additional days. Another group of rats ($n = 10$) received food-pellet rewards paired with a predictive cue to compare the temporal dynamics of aversive (90-dB WN; Figure 2B, left) and appetitive (reward) conditioning (Figure 2B, right). Changes in dopamine are illustrated across time (Figure 2B, top), and in order to quantify the shift of the dopamine response from the US to the CS, we calculated the ratio between the areas under the curve (AUC) of the US and the CS during the very first trials of conditioning ('early day 1'), the rest of the trials of day 1 ('late day 1') and on the sixth day of conditioning ('day 6') (see bottom figure 2B). During aversive conditioning we find, using a mixed-effects analysis, a significant main effect of the amount of conditioning ($F(0.9586, 2.396)=117.3$, $p =0.0043$), and post hoc testing using Tukey's multiple comparisons test reveals significant differences between the ratios of day 1 early trials and day 1 later trials ($p = 0.0138$), as well as between day 1 early trials and day 6 ($p = 0.0318$). However, no difference was observed between day 1 later trials and day 6 ($p = 0.9852$). During appetitive conditioning we also find a main effect of the amount of conditioning on the ratio between the US and the CS ($F(1.034,8.788)=13.88$, $p=0.0047$) and in contrast to aversive conditioning the ratio on day 6 is significantly different from both day 1 early trials ($p=0.0102$) and day 1 later trials ($p<0.0001$). In addition, we find a significant difference between day 1 early trials and day 1 later trials ($p=0.0441$). The comparison of conditioned behavioral responses to 90-dB WN between day 1 and day 6 using Wilcoxon-signed rank tests and a Holm-bonferonni correction for multiple comparisons reveals an increase in locomotion speed (Figure 2C, compared to baseline which, on day 1, was restricted to the WN epoch alone (Cue: $Z = -0.876$, $p=0.381$. WN: $Z = -3.621$, $p <0.0001$). On day 6 we observe an increase in locomotion speed during both the cue ($Z = -3.053$, $p = 0.002$) and WN ($Z = -3.621$, $p < 0.0001$) epoch compared to baseline which are both also significantly higher compared to day 1 (cue: $Z = - 2.485$, $p = 0.013$. WN: $Z = -$

2.343, p = 0.019). During appetitive conditioning we see the same temporal evolution in the conditioned response, where rats approach the reward magazine more during the cue epoch on day 6 compared to day 1 ($t(9) = 6.962$, $p < 0.0001$). However, during appetitive conditioning, but not during aversive conditioning, an almost complete shift of the dopamine response from the CS to the US occurred. Together, these results demonstrate distinct differences in the temporal dynamics of dopamine signaling during aversive and appetitive conditioning.

Extinction of cue-induced dopamine signaling

In addition to having monitored the quick acquisition of the cue's dopamine-decreasing properties (Figure 2A), to further verify that these properties were a learned response, we tested how fast the association between cue and WN could be extinguished. Rats with well-established cue-WN associations (that were conditioned for more than 6 days) were exposed to 20 consecutive trials in which WN was omitted. Using a Friedman test, we found a significant effect of extinction ($\chi^2(2) = 8.4$, $p = 0.005$). Post-hoc analysis using a Dunn's multiple comparison test revealed a significant difference between the decrease in extracellular dopamine concentration of trials 1 - 5 and trials 16 - 20 ($p = 0.0133$, Figure 2D), with the latter no longer showing a decrease in dopamine. Thus, over the course of 15 extinction trials, the cue lost its conditioned dopamine response.

NAC dopamine does not reflect WN intensity and WN-induced behavior

For the previous experiments, we used WN with an intensity of 90 dB. We asked whether WN of different intensities would differentially influence extracellular dopamine in the NAC, since we observed increased avoidance of higher intensities of WN (Figure 1B). First, we tested whether there is a dose-response relationship between different WN intensities and dopamine. We exposed rats ($n = 6$) to four different intensities of WN (70, 80, 90 and 96 dB), which were delivered in a semi-random order (Figure 2E). Although all WN intensities decreased dopamine release, we found no significant effect of intensity on extracellular dopamine ($F(2.380, 11.90) = 0.1655$, $p = 0.8813$). In order to confirm the rats' capability to discriminate between the different WN intensities in this experimental setting, we exposed additional rats to the same intensities and quantified their locomotion speed (Figure 2F; $n = 13$). In contrast to dopamine release, we do find a main effect of WN intensities on baseline-subtracted locomotion speed ($\chi^2(3) = 13.80$, $p = 0.0032$) and significant differences between 70 and 90 dB ($p = 0.005$) and 70 and 96 dB ($p = 0.0143$), which demonstrates that rats were able to discriminate between the different WN intensities. In a different experiment, we trained rats ($n = 9$) on an aversive conditioning paradigm in which two cues (2 kHz or 8 kHz tones) predicted exposure to 6s of either 80-dB or 96-dB WN, respectively (Figure 2G). Again, no significant differences were found in extracellular dopamine release between WN intensities ($Z = -0.178$, $p = 0.859$), nor between the effects of their respective predicting cues ($Z = -0.059$, $p = 0.953$). Both of these experiments indicate that extracellular dopamine in the NAC does not encode WN intensity, and, therefore, the relative aversiveness or aversive value of WN is not encoded by NAC dopamine in the NAC.

Many studies have demonstrated the involvement of dopamine in movement (e.g., Syed *et al*., 2015; Alves da Silva *et al*., 2018; Cuddington & Dudman, 2019). Therefore, we tested if a correlation between locomotion speed and extracellular dopamine concentration existed during cue and WN epochs. We performed a trial-by-trial analysis for both 80-dB (Figure 2H, top) and 96-dB (figure 2H, bottom) WN exposures and found no correlation during the cue (80 dB: $R^2 = 0.0004$, $p = 0.7223$; 96 dB: $R^2 = 0.0003$, $p = 0.7360$) nor during WN (80 dB: $R^2 = 0.0016$, $p = 0.4578$; 96 dB: $R^2 = 0.0011$, $p = 0.5374$). The dots in the inset graphs represent the R^2 values of the average locomotion speed and dopamine concentrations during the cue and WN period in the recording session for each individual animal.

NAC dopamine signals contain little prediction error

Although the WN-predictive, conditioned cue acquired the ability to reliably suppress NAC dopamine release, no substantial transfer of this effect from US to CS occurred (a prerequisite for a prediction error signal). To further evaluate whether NAC dopamine might function as an APE, we introduced several deviations from the expected outcomes. First, we exposed rats to two different cues (2 kHz or 8 kHz tones) that were associated with different probabilities followed by either 80-dB or 96-dB WN (see Figure 3A). Even though dopamine did not encode WN intensity, we hypothesized that dopamine may nonetheless convey an error component to be reflected as diminished dopamine decrease during the better-than-expected condition (occurence of low-probability (25%) 80-dB WN), and augmented decrease in dopamine during the worse-than-expected condition (occurence of low-probability (25%) 96-dB WN). As expected, we did not observe differences in dopamine release during the cue epoch due to the uncertainty of which intensity would follow (Tone 1: $t(8) = 0.5983$, $p = 0.5662$, Figure 3B; Tone 2: $t(8) = 1.432$, $p = 0.1901$, Figure 3C). However, during the WN epoch, we also did not find significant differences between the two intensities, neither for tone 1 ($t(8) = 0.6698$, $p = 0.5218$), nor for tone 2 ($t(8) = 0.4452$, $p = 0.6680$). Together, these results indicate that the prediction error of outcomes deviating from expected probability is not encoded by NAC dopamine concentrations.

Since WN intensity had little influence on dopamine release, we investigated whether an APE was detectable when deviations from the expected outcome occurred in the temporal domain (i.e., duration of WN). We randomly exposed rats to a small number of probe trials with a) a longer-than-predicted 90-dB WN (12s instead of 6s), in other words a worse-than-predicted outcome (Figure 3D), b) omitted WN, in other words a better-than-predicted outcome (Figure 3E), or c) unpredicted WN, in other words another version of a worse-than-predicted outcome, but in this case lacking the prediction completely (Figure 3F). These three types of probe trials were implemented in a session in which the first 30 trials consisted of exclusive, deterministic pairings of cue (5s) and 90-dB WN (6s), after which these regular predicted WN trials were intermixed with the above-mentioned probe trials. During the worse-than-predicted WN trials where the WN was extended by 6s, we observed an extended suppression of dopamine, which decreased with the same rate as during the initial 6s, and which ceased immediately upon termination of WN, resulting in a overall lower dopamine concentration in the 11-25 s epoch (Figure 3D; $t(10) = 1.863$, $p = 0.046$, after Holm-Bonferroni correction for multiple

comparisons). During better-than-predicted trials (omitted WN), we found overall higher concentrations of dopamine in the 5-20 s epoch (Figure 3E; t(10) = 3.751, p = 0.0019, after Holm-Bonferroni correction). For the unpredicted WN, which constitutes a prediction error since the predictive cue is lacking, we aligned dopamine concentrations for predicted and unpredicted WN at its onset, in order to compare the impact of WN per se. We observed significantly lower dopamine concentrations exclusively in the epoch after the termination of the WN (Figure 3F; 11-25 s, t(10) = 2.453, p = 0.0170, after Holm-Bonferroni correction), but not during the WN epoch itself.

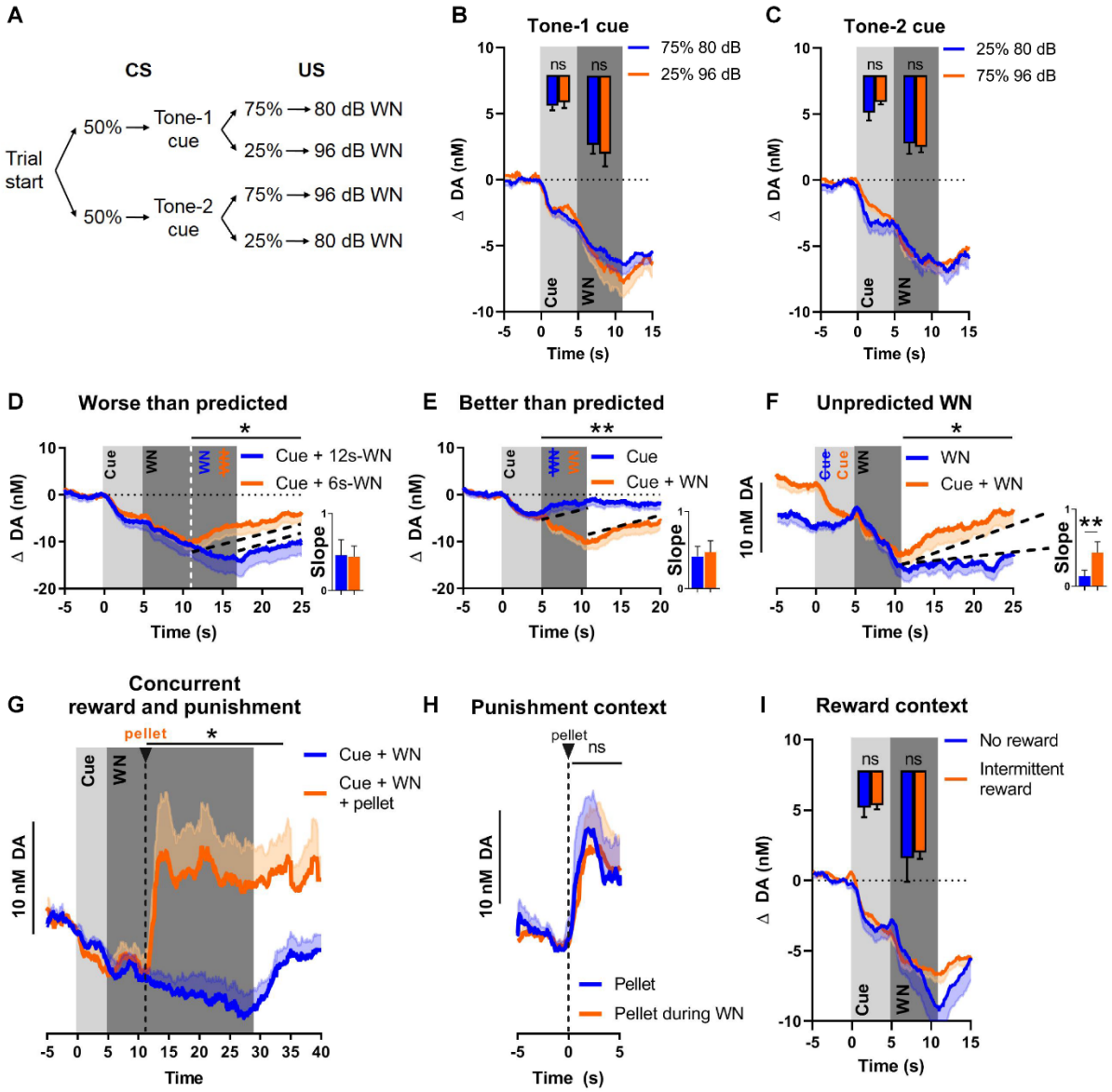

Figure 3 - NAC dopamine consistently tracks prediction and duration of WN with little aversive prediction-error function. (A) Trial structure of the probabilistic Pavlovian WN task. **(B)** Dopamine concentration in the probabilistic task during the presentation of tone-1 cue, which was followed by 80-dB WN (blue) in 75% of trials and by 96-dB WN (orange) in the remaining 25% of trials. Bar-graph inset: No significant differences in average dopamine concentration (n = 9) during cue (t(8) = 0.5983, p = 0.5662) and WN (t(8) = 0.6698, p = 0.5218). **(C)** Dopamine concentration in the probabilistic task during the presentation of tone-2 cue, which was followed by 80-dB WN (blue) in 25% of trials and by 96-dB WN (orange) in the remaining 75% of trials. Bar-graph inset: No significant differences in

average dopamine concentration (n = 9) during cue (t(8) = 1.432, p = 0.1901) and WN (t(8) = 0.4452, p = 0.6680). **(D)** Comparison of dopamine between predicted 6s-WN (orange) and worse-than-predicted 12s-WN (blue) (n = 11) demonstrates significantly lower average dopamine in the epoch between 11-25 s during worse-than-predicted 12s-WN (t(10) = 1.863, p = 0.046). Bar graph (right): Slopes of dopamine-concentration trajectories (black dotted lines) show no significant difference between worse-than-predicted and predicted WN (Z = -1.432, p = 0.0775). **(E)** Comparison of dopamine between predicted 6s-WN (orange) and better-than-predicted, omitted WN (blue) (n = 11) demonstrates significantly higher average dopamine in the epoch between 5-20 s during better-than-predicted, omitted WN (t(10) = 3.751, p = 0.0019). Bar graph (right): Slopes of dopamine-concentration trajectories (black dotted lines) show no significant difference between better-than-predicted and predicted WN (Z = 1.334, p = 0.091). **(F)** Comparison of dopamine between predicted 6s-WN (orange) and unpredicted 6s-WN (blue) (n = 11) demonstrates significantly lower average dopamine in the epoch between 11-25 s during unpredicted WN (t(10) = 2.453, p = 0.0170). Bar graph (right): Slopes of dopamine-concentration trajectories (black dotted lines) show a significantly flatter slope during unpredicted WN compared to predicted WN (Z = -2.490, p = 0.0065). **(G)** Dopamine release during prolonged 24s-WN exposure (blue) continues to incrementally decrease over time. Unexpected reward delivery (n = 6) during such 24s-WN (orange) induces an increase in dopamine in the epoch between 11-35 s (t(5) = 3.108, p = 0.0266). **(H)** Comparison of dopamine after unexpected pellet delivery (blue) and after unexpected pellet delivery during WN exposure (orange) (n = 6) show no significant difference in average dopamine in the epoch between 0-5 s (t(5) = 0.08753, p = 0.9336). **(I)** Comparison of dopamine during WN exposure in a testing context without rewards (blue) and a testing context with intermittent rewards (orange) (n = 6) shows no significant difference in average dopamine during the cue (t(5) = 0.2841, p = 0.7877) and WN (t(5) = 0.3151, p = 0.7654).

Although we did not detect a dopamine error-signal during exposure to WN (i.e., deviations from expected WN), we hypothesized that such unexpected events may alter dopamine after WN-offset. Thus, we compared the slope (or rate) of change of dopamine concentration during the recovery epoch (after WN cessation), since using slope allows for integration of the change in dopamine concentration over time, when the animals were presented with deviations from the predicted aversive event. Specifically, we found no significant slope difference between fully predicted WN trials and "worse-than-expected" trials (t(10) = 0.1511, p = 0.4415, after Holm-Bonferroni correction, Figure 3D, bar graph) or "better-than-expected" trials (t(10) = 0.4809, p = 0.3205, after Holm-Bonferroni correction, Figure 3E, bar graph). Thus, when our rats were exposed to unexpectedly extended WN or to the unexpected omission of WN, dopamine concentration reflected only the duration of WN exposure, but not a prediction error. In contrast, we found a significant difference in recovery slope between unexpected and expected WN (t(10) = 2.895, p = 0.0080, after Holm-Bonferroni correction, Figure 3F, bar graph), which indicates that, in the case of an unexpected aversive stimulus, dopamine does not only track the duration of this aversive stimulus, but displays a differential response and, thus, may serve as a qualitative teaching signal.

Dopamine integrates information about appetitive and aversive stimuli

We then investigated whether dopamine could still encode rewards during ongoing WN exposure, i.e. while extracellular dopamine concentrations are continuously decreasing. To test this, we delivered food pellets unexpectedly during a prolonged WN epoch. We observed a significant increase in dopamine release upon pellet delivery (t(5) = 3.108, p = 0.0266, Figure 3H), which was comparable to the increase in dopamine release we observed upon pellet delivery in the absence of WN (t(5) =

0.08753, p = 0.9336, Figure 3I). These results indicate that dopamine is still responsive to rewarding events during an aversive event and, thus, integrates information about appetitive and aversive events.

A previous study reported that dopamine is more prone to encode an APE in an experimental context with a low probability of intermittent reward delivery (Matsumoto *et al.,* 2016). Thus, we compared dopamine release during cue and WN exposure embedded in two task contexts with different reward probabilities (i.e., different "reward contexts"). In the first task context, no rewards were delivered during the entire session, whereas in the second context a low chance of reward delivery existed (reward-trial probability = 0.1). We did not observe a significant difference in dopamine concentration between these reward contexts during the cue epoch ($t(5) = 0.2841$, $p = 0.7877$), nor during the WN epoch ($t(5) = 0.3151$, $p = 0.7654$) (Figure 3J). Consistently, in another experiment, we did not observe a significant difference in dopamine concentration (during cue and WN epochs) when comparing a no-reward context with a high-reward context (reward-trial probability= 0.5; cue: $t(17)=1.448$, $p=0.0829$, WN: $t(17)=1.428$, $p=0.0857$; data not shown).

DISCUSSION

In this study, we set out to delineate the role of the dopamine system in processing aversive stimuli, by systematically investigating subsecond fluctuations in rat NAC dopamine concentration in response to an aversive auditory stimulus (WN), as well as its prediction by auditory tone cues. First, we validated the aversiveness of WN in a real-time place-preference task and in an operant task, where we found that WN aversiveness scales with loudness. Trial-by-trial analysis of the first WN exposures revealed that WN as an unconditioned stimulus diminishes the concentration of extracellular dopamine in the NAC, and that a predicting cue rapidly takes on the roll of conditioned stimulus (reversible by extinction), eliciting WN-like behavioral activation and dopamine depression. Dopamine during cue and WN was not correlated with locomotion speed. In contrast to appetitive conditioning, only a very limited temporal shift of the dopamine response from WN to the cue occurred. Dopamine responses to WN and its predictive cue were not affected by aversive value (WN intensity), context valence (introduction of intermittent rewards), or probabilistic contingencies. Instead, prediction and duration of the aversive WN were accompanied by a relatively slow and steady decrease in NAC dopamine concentration (a declining ramp that continued without plateauing for at least 24 seconds), which was followed by an equally slow recovery of dopamine upon cessation of WN. The slope of this rebounding dopamine ramp was altered only by unpredicted presentation of WN (not by better-than-predicted or worse-than-predicted outcomes), revealing a function of dopamine that sometimes goes beyond simple real-time tracking the presence of conditioned and unconditioned aversive stimuli. Finally, we find the integration of rewarding and aversive stimuli is of parallel nature, as WN-associated dopamine depression did not modify the rapid surge of dopamine triggered by unexpected

reward delivery. Together, our findings indicate that negative dopamine signals in the NAC mostly track the prediction and duration of aversive events, with few aspects that are consistent with an APE.

WN is a versatile aversive stimulus that suppresses dopamine release and increases locomotion

We chose WN as an aversive stimulus to probe the limbic dopamine system's role in aversive conditioning, as it has several advantages compared to more commonly-used aversive stimuli. First, WN is mildly to moderately aversive (Campbell & Bloom, 1965; Hughes & Bardo, 1981), and as such does not induce freezing, but instead provokes mild behavioral activation. This is particularly relevant with regard to studies relating dopamine function to behavioral read-outs, since lack of movement is often associated with diminished activity of the dopamine system and, thus, may confound the interpretation of negative dopamine signals in the context of aversive events. Second, WN is reliably effective and tolerated across many trials and sessions, supporting the detection of neuronal signals by providing sufficient data for averaging across trials and enabling complex experiments with varying valence and contingencies. Third, WN is distinct, well-controllable, and easy to produce, where intensity and duration can be titrated effortlessly. Fourth, WN does not require attention to be detected (i.e., the animal will hear it anywhere in an experimental environment). Fifth, animals cannot interfere with WN delivery, as opposed to air puffs or electric foot shocks, which can be influenced by the animal's actions and position (i.e., closing its eyelids or decreasing contact surface with the charged grid floor). Sixth, WN does not interfere with data recording in FSCV, electrophysiology, or fluorescence imaging. Together, the above-mentioned merits make WN an experimentally valuable stimulus with great potential to uncover aversion-relevant brain mechanisms.

In the presented work, we report that WN diminishes extracellular dopamine concentration in the NAC upon first exposure, characteristic of an unconditioned stimulus or primary reinforcer. A predicting cue quickly adopted this property upon subsequent exposures, which was reversible by extinction. Such dopamine responses were stable across trials and sessions. Interestingly, the use of WN revealed a rare relationship between dopamine and behavior: Increased locomotion speed was associated with a decrease in dopamine release. Behavioral activation is usually associated with increased dopamine signaling (Boureau & Dayan, 2011; Berridge & Robinson, 1998; Alves da Silva *et al*., 2018; Cuddington & Dudman, 2019), whereas a lack of movement or even freezing, depending on stimulus intensity, is often associated with decreased dopamine (e.g., Oleson *et al*., 2012; Badrinarayan *et al*., 2012). These frequently-observed association patterns have prompted the hypothesis that the directionality of changes in dopamine concentration reflects the chosen strategy: An active or passive behavioral reaction to aversive events (Badrinarayan *et al*., 2012). Our results, however, prove that this hypothesis is not universally applicable. The behavioral activation we observe in response to WN might reflect an increased motivation to escape, which we cannot ascertain as our task was Pavlovian (thus, without an active avoidance component: the WN was inescapable). Notably, the observed decline in dopamine was not at all correlated with movement on

a trial-by-trial basis; thus, it is conceivable that during mild WN exposure, NAC dopamine was uncoupled from its usual, more direct behavioral impact.

What is and what is not encoded by NAC dopamine?

Many studies have investigated the role of dopamine in aversion by testing the system's reaction to the exposure to aversive stimuli (see above), but only a few scrutinize dopamine's precise function therein, or whether dopamine encodes a "true" APE. Their conclusions range from "dopamine is insensitive to aversiveness" (Fiorillo, 2013), to the other extreme of "dopamine serves as an APE" (Matsumoto *et al.,* 2016). Fiorillo (2013) ruled out the existence of a dopamine APE because 1) dopamine-neuron firing did not differ between presentation of aversive and neutral stimuli, 2) prediction of an aversive event did not affect firing, and 3) no integration of rewarding and aversive values was observed. In contrast, Matsumoto *et al.* (2016) found evidence for all three of these requirements and, therefore, concluded that dopamine neurons are capable of encoding a value prediction error (equally for both rewards and aversive stimuli). This discrepancy could partially be explained by the fact that Matsumoto *et al.* (2016) recorded from dopaminergic neurons in the VTA (of mice), whereas the majority of the neurons that Fiorillo (2013) recorded were in the substantia nigra (of monkeys). Since we measured extracellular concentrations of NAC dopamine, which is released from terminals that originate from neurons in the VTA (Ikemoto, 2007), we expected to find an APE in our data.

Indeed, our results meet Fiorillo's (2013) three requirements for an APE stated above: 1) During the first pairing of cue and WN, when the predictive auditory cue was still neutral, dopamine concentration during the WN epoch differed significantly from that during baseline and cue presentation, but the latter (cue and baseline dopamine) did not differ from each other. 2) After only four cue-WN pairings, cue presentation diminished dopamine concentration, thus prediction of the aversive event did alter dopamine activity. Although we did not find a significant difference in overall dopamine concentration between predicted and unpredicted WN, we did observe a difference in their post-WN recovery slopes. 3) Finally, although we did not detect an "interactive" integration (modulated signal) of aversive and reward values during concurrent presentation of WN and a food pellet (as the absolute magnitude of released dopamine was equal to that of a pellet delivered outside of WN exposure), both the rewarding and aversive stimuli were encoded in parallel. The signals are thus integrated in the sense that both are processed at the same time, in an additive manner; as opposed to an exclusive organization, where the dopamine system may be "turned off" or unresponsive towards rewards during the presence of an aversive stimulus. Taken together, up to this point, our results fit best with the conclusion of Matsumoto *et al.* (2016); although a noteworthy contrast is that in our data, context was irrelevant to the magnitude of dopamine response to the aversive event and reward: It made no difference for the acute dopamine response magnitude whether the aversive stimulus was delivered in rewarding contexts or not.

Next, however, we took inspiration from Hart *et al.* (2014), who used a mathematical approach developed by Caplin and Dean (2007) to confirm the encoding of RPE signals by NAC

dopamine. They used a deterministic and a probabilistic choice task in order to determine whether dopamine signals fulfilled three axioms that were considered necessary for a RPE signal: “consistent prize ordering”, “consistent lottery ordering”, and “no surprise equivalence”. We employed the above-mentioned deterministic and probabilistic Pavlovian conditioning tasks to identify an APE, instead of an RPE, by exposing rats to low- and high-dB WN, predicted by two different tones, with either a 100% probability (deterministic task) or with different probabilities (probabilistic task). We did not observe differences in dopamine concentration during the WN epoch in the deterministic task, which fulfills the third axiom (no surprise equivalence), since the prediction error is zero for both of these conditions. But the first two axioms were not fulfilled, since we did not detect differences in dopamine during the WN epoch, when different WN intensities were presented with different probabilities. Rats avoided higher-dB WN more than lower-dB WN (Figure 1B) and exhibited WN dB-dependent locomotor activation (Figure 2F), indicating that WN aversiveness scales with WN intensity and that rats are able to discriminate between different WN intensities. Thus, we conclude that the NAC dopamine signals we observed did not fulfill the axiomatic criteria of an APE, when aversive-stimulus intensity or value was varied.

Finally, we performed experiments to probe the dopamine signal in conditions where the aversive stimulus deviated from the expected duration, in other words, when trials were worse or better than predicted based on WN duration, but with a stable intensity of 90 dB. First, we extended WN duration or omitted WN in occasional trials. Extended WN elicited a continuation of the same declining dopamine-concentration slope, which ceased promptly at WN cessation, after which dopamine slowly ramped back up towards baseline with a reversed, inclining slope. Thus, although the signal reflected the duration of extended WN, no discernable error component was evident. When WN was omitted, we did not observe an error signal either: Instead, again, the signal slowly returned to baseline levels. Second, in another version of the “worse-than-expected” condition, we occasionally delivered WN unexpectedly, without a preceding cue (after animals had learned the cue-WN association well), and observed a difference in the recovery slope after WN-offset as compared to predicted WN. This flattened recovery slope indicates that NAC dopamine signals more than simply track the presence of aversive stimuli; in addition, it may relate to the failed anticipation of an aversive event (based on reliance on the predictive cue), and, thus, indicate altered cue-WN contingencies. In summary, we conclude that dopamine precisely tracks aversive-stimulus duration, and the only evidence of an APE-like signal in our data was found after unpredicted WN, whereas several of our other experimental accounts are incompatible with an APE function of NAC dopamine. This places our results firmly in the middle ground between the no-APE (Fiorillo, 2013) and the full-APE conclusions (Matsumoto *et al.* 2016) described above.

Aversion versus reward

Consistent with most literature (Badrinarayan *et al.,* 2012; Oleson *et al.,* 2012; DeJong *et al.,* 2019; Stelly *et al.,* 2019), we find that NAC dopamine encodes rewarding and aversive events with opposite directionality. Furthermore, we report that a cue predicting an aversive stimulus can adopt the ability

to prompt a decrease in dopamine the way the aversive stimulus itself would. Taken together, this suggests that NAC dopamine encodes both reward and aversive prediction. However, decreases in dopamine concentration did not scale with WN intensity, unlike what is well-established for reward processing, where reward size or probability is encoded both for the reward itself and for predictive stimuli (Gan *et al.,* 2010; Tobler *et al.,* 2015; Watabe-Uchida *et al*., 2017). Furthermore, encoding of a prediction error, which is one of the best characterized features of reward-related dopamine signaling, did not occur for aversive events. Thus, NAC dopamine does not encode aversive and appetitive stimuli (and their prediction) in the same way. Moreover, the basic nature of aversion-related dopamine signals in our data was different from that of rewards. For example, the temporal signal shift towards the earliest predictor of the respective reinforcing stimulus, as described for rewards, is incomplete for aversive conditioning. Another example is that reward-related changes in extracellular dopamine concentration are substantially larger and faster compared to aversive events. These discrepancies may be partially attributable to general differences between dopamine release into and removal from the extracellular space. More specifically, the dopamine system presumably has a bigger dynamic range for increasing activity; it can do so, for example, by increasing the number of cells firing and their firing frequency (and thereby the total number of dopamine-containing vesicles being released). In contrast, dopamine-signaling reduction cannot drop below a certain point, since the cells' maximum response is to cease firing altogether and extracellular dopamine can only be removed relatively slowly or must diffuse away. This disparity could translate into a structurally-limiting factor on what can be encoded by a reduction in dopamine concentration and explain some of the above-mentioned differences in function. However, the slow-ramping declining and recovery slopes we observed do not reflect the system limits, since the very first exposure to WN resulted in a steeper decline and rewards given during WN resulted in steeper increases. Furthermore, disparate qualitative differences were also found in NAC dopamine responses to the presentation of ultrasonic vocalizations that are associated with rewarding and aversive events (Willuhn *et al.*, 2014b). Taken together, our results indicate there are a few similarities between dopamine encoding of rewards and aversive stimuli, but overall find more differences between them - hinting at aversive events being encoded by NAC dopamine more rudimentarily, in a qualitative instead of quantitative fashion.

In summary, our findings demonstrate that WN is a valuable and versatile aversive stimulus that is well-suited to probe how the brain processes aversive stimuli. Overall, we conclude that dopamine tracks the anticipation and duration of an aversive event. This tracking materializes as a perpetually declining dopamine ramp that progresses without altering its slope until offset of the aversive stimulus (even WN lasting for 24 s did not reach a plateau of minimal dopamine concentration). Such aversion tracking may play an anticipatory role for certain defensive behaviors, since the animals were behaviorally activated during the aversive event. Furthermore, we speculate that these slowly ramping aversion signals may contribute to a qualitative learning signal (other than a quantitative or scalar APE signal), since the unexpected aversive stimulus elicited a response beyond simply tracking the stimulus. Thus, we conclude that dopamine tracks both positive and negative valence in their temporal aspects and prediction, but that quantitatively speaking, the exact value and error is only

encoded for rewards, in the upward direction of NAC dopamine concentration. This implies that aversive value and APEs are encoded in other brain regions.

MATERIALS & METHODS

Animals

Adult male Long-Evans rats (300-400 g) were housed individually and kept on a reversed light-dark cycle (light on from 20:00 till 8:00) with controlled temperature and humidity. All animal procedures were in accordance with the Dutch and European laws and approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences. 37 rats underwent surgery, 21 of which exhibited a functional FSCV electrode with a histologically verified location in the NAC, and were therefore included in the study. An additional 19 rats, which did not undergo surgery, were used for behavioral tasks. All rats were food-restricted to 90% of their free-feeding bodyweight, and water was provided *ad libitum*.

Stereotaxic surgery

Rats were induced under isoflurane anesthesia and placed into the stereotaxic frame, on an isothermal pad maintaining body temperature. The analgesic Metacam (0.2mg meloxicam/100 g) was injected subcutaneously and the shaved scalp was disinfected using 70% ethanol. Upon incision of the scalp, it was treated with lidocaine (100 mg/ml). Holes were drilled in the cranium and the dura mater was cleared for targeting the NAC (1.2 mm AP, 1.5 mm ML and -7.1 DV). Chronic carbon-fiber electrodes (Clark *et al.,* 2010), made in-house, were positioned in the NAC, and an Ag/AgCl reference electrode was placed in a separate part of the forebrain. The electrodes were secured to screws in the skull using cranioplastic cement. Following surgery, rats received subcutaneous injection of 2 ml saline, and were placed in a temperature-controlled cabinet to be monitored for an hour. Rats were given 1-2 weeks post-surgery to recover before food restriction, behavioral training, and recording.

Behavioral procedures

All behavioral experiments, except the real-time place preference task, were conducted in modified operant boxes (32 x 30 x 29 cm, Med Associates Inc.), equipped with a food magazine (connected to an automated food-pellet dispenser) flanked by two retractable levers (with cue lights), a house light,

multiple tone generators, two WN generators, and metal grid floors (Med Associates Inc.). Each operant box was surveilled by a video camera. The boxes were housed in metal Faraday cages, that were insulated with sound-absorbing polyurethane foam. An overview of experiments, animal numbers, and read-outs is provided in Supplementary Table 1.

Real-time place preference

Rats (n = 10) were placed for 30 minutes in a light-shielded, square, Perspex open field (60x60x60 cm), made in-house (Netherlands Institute for Neuroscience (NIN) mechanical workshop). A camera mounted in the center above the open field recorded the position of the rat, which was tracked in real-time by the open-source software Bonsai (Lopes *et al.,* 2015). One quadrant (randomly assigned for each rat) of the open field was paired with exposure to 90-dB WN, which was produced by a WN generator from Med-Associates Inc., mounted on top of one of the open-field walls. WN was automatically switched on as long as the head of the rat was present in the chosen quadrant, and switched off as soon as the rat exited the quadrant. The WN-quadrant position was fixed throughout the session. The percentage of time rats spent in the WN-paired quadrant was compared to chance level (25%).

WN and reward choice task

Rats (n = 6) were trained to press one of the two levers in the operant box to receive food-pellet rewards (Dustless precision pellets, 45 mg, Bio-Serv). During the first training days, a single lever was inserted at variable inter-trial intervals. Pressing this lever prompted delivery of a single food pellet and immediate retraction of the lever. Omissions (no lever press for 10s) resulted in 10s house-light illumination. After reaching a 90% success rate, the other lever was introduced in training sessions that consisted of 20 “forced” trials, in which one of the two levers was presented, followed by 80 choice trials where both levers were presented. Any lever press resulted in delivery of a single food pellet and retraction of extended levers (marking the end of the trial). After 5 consecutive sessions with over 90% success rate, we paired reward delivery of one of the levers with simultaneous 5s of 90-dB WN exposure. Rats were trained under these contingencies for 5 days (n = 6), after which half of the animals (n = 3) were switched to 96-dB and the other half to 70-dB WN (n = 3). After five sessions, WN intensities were transposed between the two groups of animals for an additional 5 sessions, so that every animal received each WN intensity. In each of these WN sessions, animals could earn a maximum of 100 pellets (in 100 trials). Both levers were presented simultaneously at a variable inter-trial-interval averaging 25s (range: 15-35s). Just as at the start of training, one lever press induced immediate retraction of both levers and prompted reward delivery (end of trial), whereas omissions (no press within 10s upon lever insertion) ended the trial and resulted in 10s house-light illumination. We compared the relative number of WN-paired lever presses on the fifth day of training across different WN-intensities.

FSCV during aversive Pavlovian conditioning with 90-dB WN

On the first day of aversive Pavlovian conditioning, a new group of 16 rats was tethered to the FSCV recording equipment and placed in the operant box. In this and all paradigms described below, prior to behavioral session start, two unexpected deliveries of a single food pellet (spaced apart by two minutes), confirmed electrode viability to detect dopamine. The session started with the illumination of the house light. The first 30 trials consisted of the presentation of a 5 s cue (1.5 kHz, 75 dB tone) followed by 6 s of WN (90 dB). Trials were separated by a variable inter-trial interval of 60s (range: 30-90 s). For a subset of the rats (n = 11), these initial 30 trials were followed by 55 trials, in which 5s-cue/6s-WN pairings were randomly mixed with four trials with unpredicted WN (6s of 90-dB WN without cue), four trials with WN omission (5s-cue without WN), and four trials with 5s-cue followed by 12s of 90-dB WN (longer-than-expected condition).

A subset of the initial 16 rats (n = 4) was conditioned for an additional 5 days (days 2-6), of which the first 4 days consisted of sessions with 30 trials of pairings of 5s-cue/6s with 90-dB WN, and on the fifth day (sixth day of conditioning in total) another FSCV recording session took place (as described for day 1). An additional group of animals (n=13), without implanted FSCV electrodes were conditioned for 6 days, in order to characterize behavioral responses to different WN intensities.

For the analysis of the first 30 conditioning trials, we compared the average dopamine concentration during the cue (5 s) and the WN (6 s) epoch to baseline (-5 to 0 s before cue onset). To analyze trials with different contingencies (unpredicted, omitted, or longer WN), we compared average dopamine during the relevant epochs (unpredicted WN: 11-25 s after cue onset; better than predicted (omitted WN): 5-20 s after cue onset; worse than predicted (longer WN): 11-25 s after cue onset) with average dopamine in the respective epochs in immediately preceding trials 5s-cue/6s-WN pairings (trials 25-30), during which dopamine decreases had stabilized and were unaffected by different contingencies. Slopes of dopamine traces were compared between trials with different contingencies and predicted WN trials since using the slope allows for integration of the change in dopamine concentration over time, as opposed to averaging concentrations over an epoch (in which there is no integration over time). All traces were aligned before WN onset.

To compare dopamine concentration during cue and WN between days 1 and 6 and to quantify the shift of dopamine release from the US to the CS, we subdivided the results of day 1 into “day 1 (early)” (trials 2-4; trial 1 was excluded to remove the saliency response to the first cue exposure) and “day 1 (late)” (trials 5-30). We calculated the ratio between US and CS dopamine signals as a deviation from baseline (in the respective up or down direction). For aversive conditioning, this ratio was determined by (area above the curve of the WN epoch)/(area above the curve of the cue epoch). For appetitive conditioning, this ratio was determined by (area under the curve of the pellet epoch)/(area under the curve of the cue epoch).

Appetitive Pavlovian conditioning

Rats (n = 10) were placed in the operant box, and on days 1 and 6, they were tethered to the FSCV recording equipment. Illumination of the house light signaled the beginning of the session. Sessions

consisted of 40 pairings of cue-light illumination (5 s) with a pellet delivery (delivered immediately after cue offset), which were separated by variable inter-trial-intervals averaging 60 s (range: 30-90 s).

WN dose response

Rats (n = 6) were tethered to the FSCV recording equipment and placed into the operant box. The two WN generators with custom-made volume control dials (NIN mechanical workshop) were used to switch between different WN intensities. The FSCV recording session consisted of 6 blocks in which two different WN intensities (70, 80, 90 or 96 dB) were presented for 6 s in random order, 4 times each, with a variable inter-trial-interval averaging 30 s (range: 25-35 s). Between blocks, the volume dial was used to change WN intensities. During the different blocks, all WN intensities were presented in pairs of two and, therefore, each intensity was presented in 3 of the 6 blocks and played 12 times in total. We compared the average dopamine concentration during the WN exposures between the different intensities.

An additional group of rats (n=13), without implanted FSCV electrodes, were placed into an operant box and underwent WN exposure in order to characterize behavioral responses to the four different WN intensities (70, 80, 90 or 96dB; randomly ordered in blocks of 15 trials) presented for a duration of 6s per trial, followed by an average variable inter-trial interval of 60s (range: 30-90 s). Before the start of each block, 3 food pellets were delivered with a variable inter-trial interval averaging 30 s (range 20-40). We compared the average baseline-subtracted locomotion speed of the animals during the WN exposures between the different intensities.

Aversive Pavlovian conditioning with 80-dB and 96-dB WN

Rats (n = 9) underwent 4 aversive conditioning sessions in the operant box in which a 2 kHz and 8 kHz tone (5 s cue) predicted the exposure to 80-dB or 96-dB WN (6 s), respectively. Sessions consisted of 88 trials, of which 40 trials with 80-dB WN and 40 trials with 96-dB WN, predicted by their respective cues, were presented in random order. In the remaining 8 trials, an unpredicted food pellet was delivered. These deliveries were distributed across the session so that in every block of 10 WN exposures, one pellet was delivered at a random trial number. Trials were separated by variable inter-trial-intervals averaging 60 s (range: 30-90 s). On the fourth conditioning day, a recording session took place, for which the rats were connected to the FSCV recording equipment. We compared average dopamine concentrations during the cue (5 s) and during the WN (6 s) epochs.

During the subsequent 4 aversive conditioning sessions, we changed the probability of exposure to 80-dB and 96-dB WN following their associated cues. The total number of presentations of tone 1, tone 2, WN, and pellet deliveries remained the same. However, tone 1 was now followed by 80-dB WN (6 s) in 75% of the trials and by 96-dB WN (6 s) during the remaining 25% of the trials. Tone 2 was followed by 96-dB WN (6 s) in 75% of the trials and 80-dB WN (6 s) during 25% of the trials. A recording session took place on the fourth conditioning day. We compared the average dopamine concentrations during the cue (5 s) and WN (6 s) epochs.

Concurrent reward and WN, and cue extinction

Rats (n = 6) were connected to the recording set up and placed in the operant box. The conditioning session began with 10 pairings of the 5-s cue (1.5 kHz tone) and 6 s WN (90dB). Next, followed a block of 20 trials pairing the 5-s cue and 24 s of WN; during half of these trials (randomized) a pellet was delivered 6 s into the WN exposure. The recording session was concluded with a block of 20 extinction trials, in which only the 5s cue was delivered. This recording session was the last to take place, the rats had experienced 9-11 conditioning sessions prior to this recording.

FSCV measurements and analysis

As described previously (Willuhn *et al.,* 2014a), fast-scan cyclic voltammetry (FSCV) was used to detect subsecond changes in extracellular concentration of dopamine using chronically implanted carbon-fiber microsensors that were connected to a head-mounted voltammetric amplifier, interfaced with a PC-driven data-acquisition and analysis system (National Instruments) through an electrical commutator (Crist), which was mounted above the test chamber. Every 100 ms, voltammetric scans were repeated to achieve a sampling rate of 10 Hz. The electrical potential of the carbon-fiber electrode was linearly ramped from -0.4 V versus Ag/AgCl to +1.3 V (anodic sweep) and back (cathodic sweep) at 400V/s (8.5 ms total scan time) during each voltammetric scan, and held at -0.4 V between scans. Dopamine is oxidized during the anodic sweep, if present at the surface of the electrode, forming dopamine-o-quinone (peak reaction detected around +0.7 V), which is reduced back to dopamine in the cathodic sweep (peak reaction detected around -0.3 V). The ensuing flux of electrons is measured as current and is directly proportional to the number of molecules that undergo electrolysis. The background-subtracted, time-resolved current obtained from each scan provides a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances (Phillips & Wightman, 2003). Chemometric analysis with a standard training set was used to isolate dopamine from the voltammetric signal (Clark *et al.,* 2010). All data was smoothed with a moving 10-point median filter and baseline (set at 1 s before cue onset or in case of an absent cue 1s before WN onset) subtraction was performed on a trial-by-trial basis prior to analysis of average concentration. Analyses were performed on dopamine concentration during cue (5 s) and WN (6 s) epochs and were compared to baseline dopamine concentrations or to the same epoch in a different experimental condition. Prior to each FSCV recording session, two unexpected deliveries of a single food pellet (spaced apart by two minutes) confirmed electrode viability to detect dopamine. Animals were excluded from analysis when: 1) A lack of dopamine release in response to unexpected pellets before start of the behavioral session, 2) FSCV recording amplitude background noise that was larger than 1nA in amplitude.

Analysis of operant-box behavior

DeepLabCut software (Mathis *et al.,* 2018) was used to track rat movement in the operant box using video data recorded during FSCV measurements. This tracking data was analyzed in MATLAB (The Mathworks, Inc. Version 2019a) to determine distance to the reward magazine and speed of

742 movement (cm / s). Analyses were performed using the average distance or locomotion speed during
743 the cue (5 s) or WN (6 s) epochs. During the WN- and reward-choice task, the number of presses on
744 each lever was registered via an automated procedure.

745

746 **Histological verification of recording sites**

747 After completion of the experiments, rats were deeply anesthetized using a lethal dose of
748 pentobarbital. Recording sites were marked with an electrolytic lesion before transcardial perfusion
749 with saline, followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed in PFA for
750 24 hours after which they were placed in 30% sucrose for cryoprotection. The brains were rapidly
751 frozen using an isopentane bath, sliced on a cryostat (50 µm coronal sections, -20°C), and stained
752 with cresyl violet.

753 **Statistical analysis**

754 FSCV and behavioral data were analyzed using one- or two-tailed paired or unpaired t-tests, repeated
755 measures ANOVAs, regression analysis, or their nonparametric equivalents when appropriate. Post-
756 hoc analyses were conducted when necessary and p-values were adjusted when multiple
757 comparisons were made. Statistical analyses were performed using Prism (Graphpad software) and
758 SPSS statistics version 25.0 (IBM); graphical representations were made using Prism. Statistical
759 significance was set to $p < 0.05$. Sample size was not explicitly determined by a power analysis when
760 the study was being designed, but was, instead, based on the lab's experience with this type of data.

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764 **COMPETING INTERESTS**

765 The authors declare that no competing interests exist.

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958 Supplementary Table 1

Experiment	N	FSCV readout	Behavioral readout
Real time place preference (Fig 1A)	10		X
WN and reward choice task (Fig 1B)	6		X
Aversive Pavlovian conditioning day 1 - First 30 trials (Fig 2A, B and C) - Mix trials (Fig 3D, E and F)	 16 11	 X X	 X
Appetitive Pavlovian conditioning (Fig 2B and C)	10	X	X
Aversive Pavlovian conditioning days 2 – 6 (Fig 2B and C)	4 13	X	X X
Dose response (Fig 2E and F)	6 13	X	X X
Deterministic experiment (Fig 2G)	9	X	
Probabilistic experiment (Fig 3A, B and C)	9	X	
Concurrent reward & WN and extinction (Fig 2D, 3G and H)	6	X	