Title: Distinct pre-stimulus and post-stimulus activation of VTA neurons correlates with stimulus detection

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Abstract

Dopamine neurons of the ventral tegmental area (VTA) signal the occurrence of a reward-predicting conditioned stimulus (CS) with a sub-second duration increase in post-CS firing rate. Important theories about reward-prediction error and reward expectancy have been informed by the substantial number of studies that have examined post-CS phasic VTA neuron activity. On the other hand, the role of VTA neurons in anticipation of a reward-predicting CS and analysis of pre-stimulus spike rate rarely has been studied. We recorded from the VTA in rats during the 3-choice reaction time task, which has a fixed duration pre-stimulus period and a difficult-to-detect stimulus. Using a stimulus that was difficult to detect led to behavioral errors, which allowed us to compare VTA activity between trials with correct and incorrect stimulus-guided choices. We found a sustained increase in firing rate of both putative dopamine and GABA neurons during the pre-CS period of correct and incorrect trials. The post-stimulus phasic response, however, was absent on incorrect trials, suggesting that the stimulus-evoked phasic response of dopamine neurons may relate to stimulus detection. The pre-stimulus activation of VTA neurons may modulate cortical systems that represent internal states of stimulus expectation and provide a mechanism for dopamine neurotransmission to influence preparatory attention to an expected stimulus.

Keywords: Attention, dopamine, reward, schizophrenia, ADHD, prefrontal cortex
Introduction

Dopamine neurons of the ventral tegmental area (VTA) have been ascribed a role in motivation and learning because they signal the expectancy and occurrence of reward and reward-predicting or salient stimuli (Cohen et al. 2012; Horvitz 2000; Pan et al. 2005; Schultz 1998; Schultz et al. 1997). These studies have demonstrated that dopamine neurons increase discharge after the onset of a reward-predicting conditioned stimulus (CS). The post-CS response may signal reward expectancy (Cohen et al. 2012). On the other hand, pre-CS VTA activity occurring as an organism expects the stimulus, has remained largely unexamined in the context of electrophysiological recordings (Bromberg-Martin et al. 2010; Schultz 2007).

Dopamine neurons likely contribute to cognitive states of stimulus expectancy, given that dopamine neurotransmission can modulate an organism’s ability to estimate the timing of stimuli (Coull et al. 2011), and that dopamine neurotransmission may be abnormal in psychiatric illnesses such as schizophrenia and ADHD which involve disrupted temporal organization of behavior (Allman and Meck 2012; Ward et al. 2012). These findings suggest that VTA neurons may control general stimulus expectation regardless of stimulus valence. One study that examined VTA activity during CS anticipation found a ramping decrease in firing rate that began when the presentation of a predictable stimulus was delayed or omitted, which is consistent with decreased dopamine activity when a CS is omitted (Bromberg-Martin et al. 2010). Therefore, previous studies on stimulus expectation have been limited to omission of an expected stimulus.

Our objective in the current experiments was to study the activity of VTA neurons during stimulus expectation. Given that dopamine may participate in the neural mechanisms underling stimulus expectation (Allman and Meck 2012; Coull et al. 2011; Ward et al. 2012), we hypothesized that dopamine neurons would change their firing rate during the time immediately preceding the CS ("pre-stimulus period") in expectation of the CS. Furthermore, given that temporal expectation of a stimulus improves stimulus detection (Nobre et al. 2007; Rohenkohl et al. 2012), we hypothesized that firing rate would correlate with behavioral performance in a task...
in which the stimulus was presented with predictable timing, but was difficult to detect. A
stimulus detection task (i.e., the 3-choice reaction time task, (Totah et al. 2009)), which used a
brief stimulus presented at randomized stimulus locations, was employed because it generated
incorrect and missed trials, which allowed us to assess VTA activity based on behavioral
performance. Notably, reduced cortical dopamine neurotransmission (Crofts et al. 2001; Granon
et al. 2000) results in impaired stimulus detection in the 3-choice reaction time task. These
findings suggest that in this task dopamine neuron spiking should correlate with behavior and
that increased spiking rate may correlate with accurate stimulus detection by contributing to the
neural mechanisms of stimulus expectancy.

To study stimulus expectancy, we analyzed single unit firing rate during an 8 second
pre-stimulus period. We also characterized the VTA neuronal firing rate immediately after
stimulus onset because VTA neurons have been observed to exhibit a short-duration “phasic”
response after the presentation of reward-predicting and salient stimuli (Dommett et al. 2005;
Schultz 2007). Consistent with involvement of the VTA in internal states of stimulus expectation
and temporal organization of behavior, we found that the putative GABA and dopamine neurons
had a sustained, elevated firing rate during the pre-stimulus period. The pre-stimulus activation
occurred during both trial types whereas post-stimulus VTA neuron activation was absent on
error trials supporting a role for the VTA in successful stimulus detection.

Materials and Methods

Subjects and behavioral methods
Male Sprague-Dawley rats (n=20) housed on a reverse light cycle and tested during their
active phase. All animal use procedures were approved by and carried out in compliance with
the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). The
behavioral task has been described in detail in our previous work (Totah et al. 2009). Briefly,
rats were trained and tested in operant chambers with a house light on the ceiling, 3 stimulus
ports with internal light-emitting diode (LED) lights on one wall, and an illuminated food magazine on the wall opposite from the stimulus ports. Nose pokes into the stimulus ports and the food magazine were registered by photosensors. A correct response, consisting of a nose poke into an illuminated stimulus port, was rewarded with sucrose. An incorrect response into an unlit stimulus port resulted in an extinguished house light. The rat was required to nose poke into a stimulus port within 5 sec after stimulus onset; otherwise, the house light was extinguished (i.e., an omission trial). The rat initiated each trial with a poke into the food magazine, which either contained sucrose pellets or was empty depending on whether the previous trial was correct or an error.

At the start of a trial, an 8 sec pre-stimulus period passed before the stimulus onset. Throughout this paper, we use the term “pre-stimulus” to refer to the time immediately preceding the CS, and not the unconditioned stimulus (US). On each trial, one of the 3 stimulus ports would illuminate. The location of the stimulus was selected at random from the three stimulus ports. There was a balanced distribution of the selection of the 3 ports, but the order of presentation was random. Each session lasted 30 minutes. Based on satisfying performance criteria (see previous work (Totah et al. 2009) for detailed training information), the stimulus duration was reduced gradually to 300 msec. Rats were deemed ready for electrode implantation when they met the performance criterion of > 70% accuracy [i.e., # of correct responses / (# of correct responses + # of incorrect responses)] and < 20% omissions (i.e., # of omitted responses / # of total trials) for six consecutive sessions using the 300 msec cue duration. The mean number of sessions needed to complete training was 42 sessions. During both correct and incorrect trials, rats oriented to the operant chamber wall that contained the stimulus ports and waited for the stimulus. Orientation to the wall of stimulus ports began approximately 2 sec before stimulus onset and was maintained throughout the pre-stimulus period. Review of video recordings showed that orienting behavior was similar for correct and incorrect trials; unfortunately, the video recordings did not allow us to quantify the exact
movement trajectory or head position. We were, however, able to confirm that, once they
oriented, rats faced the wall of stimulus ports for the remainder of the pre-stimulus period on
both correct and incorrect trials. During omission trials, the rats did not orient to the wall of
stimulus ports during the pre-stimulus period.

Electrophysiology procedure

Rats were implanted under isoflurane anesthesia with a microelectrode array of 8
Teflon-insulated stainless steel wires with an impedance of 300 – 700 kΩ (NB Labs, Denison,
TX). Twenty rats were implanted with one array in the VTA (-5.1 to -6.1 mm posterior to bregma,
0.4 to 0.6 mm lateral to bregma, and -8.0 mm ventral from the dura surface).

After 1 week of recovery, rats were acclimated to the recording cable in the operant box
for four 30-min sessions and re-trained to criterion performance. Once performance was stable
and above criterion, a 30 min session was recorded. Single units were recorded via a unity-gain
FET headstage and lightweight cabling, which passed through a commutator to allow freedom
of movement. Single unit activity was amplified using a 1,000X gain, band-pass filtered at 300 –
8,000 Hz, and digitized at a rate of 40 kHz using Recorder software (Plexon, Inc.). Single unit
activity was digitally high-pass filtered at 300 Hz. If voltage crossed an experimenter-defined
threshold, the single unit trace was recorded for 0.5 ms before and 2.5 ms after threshold
crossing, yielding a 3.0 ms duration waveform. Spike sorting was performed using Offline Sorter
(Plexon, Inc.) using manual sorting methods described previously (Totah et al. 2009). We used
standard criteria, which we have employed previously (Totah et al. 2009), to accept waveforms
as a single unit if the cluster in PCA space was >2 S.D. from the noise and the ISI > 1.1 ms.

Classification of single units into groups of putative neurons

Single units recorded in the VTA were separated into 3 groups: putative dopamine,
putative GABA, and “Other” units that could not be classified. Classification was according to
previously published methods (Fiorillo et al. 2003; Kim et al. 2012; Kim et al. 2010; Steffensen
et al. 1998; Ungless and Grace 2012), and based upon baseline firing rate and the waveform
Pre-stimulus VTA single unit activity

duration. Waveform duration was calculated from the average spike waveform across all spikes. Single units that had a firing rate $\leq 10.0$ Hz and a duration of $\geq 1.5$ msec were assigned to the putative dopamine neuron group. Single units that had a firing rate $> 10.0$ Hz and waveform duration of $< 1.5$ msec were assigned to the putative GABA neuron group. All other single units were assigned to a group designated as “Other” neurons. The use of these electrophysiological criteria for classifying VTA neurons recorded \textit{in vivo} in the awake rat may permit units that could use glutamate as a neurotransmitter to be classified as putative dopamine neurons (Ungless and Grace 2012); however, it is unlikely that they use GABA as a neurotransmitter (Steffensen et al., 1998). A recent study that used optogenetic targeting of VTA neuron types demonstrated that waveform duration is highly variable, but that firing rate separates neuronal types clearly (see the supplementary figures in (Cohen et al. 2012)). Our dopamine and GABA neuron groups are separated conservatively by firing rate, with the “Other” group likely containing members of both groups. Neural activity was analyzed both across all recorded VTA single units and according to these 3 groups of putative neurons.

\textit{Electrophysiological data analysis}

Electrophysiological data were analyzed with custom scripts written in MATLAB (Mathworks, Natick, MA). Single unit spiking was aligned to stimulus onset ($t = 0$ sec) and binned into 250 msec bins for the pre-stimulus period and 20 msec bins for the post-stimulus period. The pre-stimulus period was from -4 sec to 0 sec and the post-stimulus period was from 0 sec to 240 msec (but plotted until 500 msec). The shorter post-stimulus period was chosen to correspond with the characterized duration of the dopamine neuron response to stimuli. The longer pre-stimulus bin size and time window were chosen based on reviewing the data in different bin sizes and on our previous finding (Totah et al. 2009) that a 250 msec time bin was appropriate for plotting the data, but that a smaller or larger bin size did not change statistical significance. Firing rate was averaged across trials within trial type. For normalization (Z-score), we used the -5.75 sec to -4.0 sec as a baseline period to calculate a mean and standard
deviation of baseline firing rate. The baseline firing rate was calculated within trial type (i.e., separately for correct, incorrect, and omission trials). Single units were considered to be responsive (i.e., increase in firing rate from baseline) if there were at least 3 consecutive time bins with Z>2. All data are plotted as mean ± 1 standard error (shaded region). The chi-squared test was used to compare the proportion of single units responding on correct trials versus incorrect trials for only 1 neuron type (e.g., dopamine) or to compare the proportion dopamine versus GABA neurons responding during only 1 trial type (e.g., correct trials). Accordingly, all chi-squared tests had 1 degree of freedom. If any group of units was < 5, then we used a Fisher’s exact test.

**Perfusion and histology**

At the completion of recordings, rats were anesthetized with chloral hydrate and perfused with normal saline for 10 min and 10% buffered formalin for 10 min. After fixation, brains were sectioned (60 um thickness) and stained with cresyl violet. Electrode tracks and electrode tips were confirmed under light microscope and rats with incorrectly placed electrodes were excluded from analysis (yielding N = 20 rats after excluding inaccurate implantations).

**Results**

*Pre-stimulus VTA single unit activation correlates with subsequent stimulus-guided behavior*

We recorded neuronal activity in a 3 choice reaction time task (Figure 1). As described previously (Totah et al. 2009), during the pre-stimulus period (immediately preceding the CS), animals oriented to the wall of 3 stimulus ports in expectation of the upcoming stimulus. The stimulus was difficult to detect due to its short (300 msec) duration and its location being chosen randomly from one of the 3 stimulus ports on each trial. In animals used in the current study, incorrect stimulus location choice occurred in 24 ± 2% of trials confirming that the stimulus was difficult to detect. Accuracy (number correct / number correct + number incorrect) was similar for each stimulus port location (75 ± 18%, right port; 77 ± 13%, center port; 76 ± 15%, left port;
Pre-stimulus VTA single unit activity

mean ± s.d. from 20 rats. The reaction time (i.e., the latency between stimulus onset and the
nose poke into the illuminated stimulus port) also did not differ between stimulus port locations
(0.782 ± 1.072 sec and 1.902 ± 1.462 sec, right port; 0.704 ± 1.222 sec and 1.484 ± 1.271 sec,
center port; 0.766 ± 1.109 sec and 1.783 ± 1.748 sec, left port; mean ± s.d. for correct and
incorrect trials combined across 20 rats). Although there is some imprecision in measuring the
behavior during the pre-stimulus period, these data demonstrate that behavior was stereotypical
across the stimulus ports and may not have been heavily affected by trial-to-trial differences in
body or head position. Moreover, video analysis demonstrated that orienting behavior was
similar across animals and that, once rats oriented, they faced the wall of stimulus ports for the
remainder of the pre-stimulus period on both correct and incorrect trials. Overall, behavior
during the pre-stimulus period appeared to be similar between correct and incorrect trials in that
the rats oriented to the wall in a similar manner. Rats tended to stand in front of the middle
stimulus port. In some trials, the stimulus illuminated directly in front of the rat, but it still chose a
different location.

We analyzed changes in pre-stimulus VTA single unit firing rate during stimulus
expectation. We recorded 123 single units from 20 rats (Figure 2). Across the entire population
of 123 VTA units, we found that 29 (23.6%) increased their firing rate during the pre-stimulus
period on correct trials. Exemplar spike rasters and peri-stimulus time histograms of spike rate
for 2 single units are illustrated in Figure 3. The rasters are similar to previous observations of
changes (both increases and decreases) in tonic dopamine neuron firing over a period of
multiple seconds (Romo and Schultz, 1990; Fiorillo et al., 2003; Bromberg-Martin et al., 2010).
The proportion of significantly (using Z-score, see methods for details) responsive units was
reduced to 12 (9.8%) and 8 (6.5%) units on incorrect and omission trials, respectively (Figure
4A). The proportion of activated units was significantly different between correct and incorrect
trials ($\chi^2(1)=8.46$, p=0.004), as well as between correct and omission trials ($\chi^2(1)=14.03$,
p<0.0001). The mean firing rate (from the entire recording session) of the pre-stimulus
modulated single units was $17.26 \pm 19.88$ Hz (mean ± s.d.). Of the significantly activated units on correct trials, the mean increase in Z-score normalized pre-stimulus firing rate was largest on correct trials (Figure 4B). The mean increase in firing rate of activated neurons on correct trials was significantly larger than the increase during incorrect and omission trials [ANOVA (time as repeated measure); trial type (all 3 types) and time interaction, $F_{(12,688)}=10.27$, $p<0.0001$]. The difference was significant between each of the groups [ANOVA (time as repeated measure); trial type (correct versus incorrect) and time interaction, $F_{(23,644)}=7.05$, $p<0.0001$; trial type (incorrect versus omission) and time interaction, $F_{(23,644)}=4.34$, $p<0.0001$]. Note that the ANOVA tests compare data from the pre-stimulus period, which is baseline onset ($t = -5.75$ sec) until stimulus onset ($t = 0$ sec). The remaining units ($N = 94$) did not respond during the pre-stimulus period; however, in the non-responsive units, a large post-stimulus response is apparent (Figure 4C). A response at the start of the trial (~8 sec before stimulus onset) also occurred in both pre-stimulus responsive (Figure 4B) and non-responsive (Figure 4C) units. The trial start nose poke coincided with reward consumption (if the previous trial was correct, which was the majority of the trials). Thus, there was a non-specific modulation related to reward consumption (seen in both Figure 4B and 4C) and a specific modulation related to pre-stimulus period (seen in Figure 4B only). To further characterize the change in pre-stimulus firing rate, we plotted non-normalized (Hz) peri-event time histograms. The firing rate across pre-stimulus activated units was highly variable ($17.26 \pm 19.88$ Hz, mean ± s.d.) and is plotted as a histogram (inset, Figure 4B). Therefore, we divided units into 3 groups with pre-stimulus period mean firing rates of <1 Hz (Figure 4D), between 1 and 3 Hz (Figure 4E), and >3 Hz (Figure 4F). In the top panel of Figures 4D, 4E, and 4F, we show the SEM of firing rate and in the bottom panels we show firing rate for all units during correct trials only. The bottom panels clearly illustrate that the activation during the pre-stimulus period lasted for several seconds. Across units in all 3 ranges of firing rates, there was always an interaction between time (-6 sec to 0 sec) and all 3 trial types [ANOVA (time as repeated measure); trial type (all 3 types) and time interaction, Figure
The difference was due to an elevation of firing rate during correct trials compared to omission trials [ANOVA (time as repeated measure); trial type (correct and omission trials) and time interaction, Figure 4D, $F_{(23,184)}=4.63$, $p<0.0001$; Figure 4E, $F_{(23,138)}=4.20$, $p<0.0001$; Figure 4F, $F_{(23,276)}=2.58$, $p<0.0002$], whereas correct and incorrect trials were not significantly different [ANOVA (time as repeated measure); trial type (correct and incorrect trials) and time interaction, Figure 4D, $F_{(23,184)}=1.49$, $p=0.077$; Figure 4E, $F_{(23,138)}=0.95$, $p=0.528$; Figure 4F, $F_{(23,276)}=1.42$, $p=0.100$]. Although the Z-score normalization of firing rate was useful for visualizing data and controlling for firing rate variability across neurons (Figure 4B), it may have generated an artificial difference between correct and incorrect trials that could not be observed in the non-normalized data (Figure 4D, 4E, 4F). In summary, we observed sustained activation of VTA single units during the period immediately preceding the onset of a CS.

The next analysis examined the possibility that the pre-stimulus activation reflected motor planning and execution. The stimulus-guided behavior (i.e., a nose poke into a stimulus port) occurred $0.441 \pm 0.732$ sec after stimulus onset during correct trials (mean ± s.d. across all trials collected from $N = 20$ rats), whereas it occurred $1.100 \pm 1.221$ sec after stimulus onset during incorrect trials (Figure 5A, 5B). We tested the hypothesis that increases in firing rate were related to motor preparation and would align to nose poke events occurring sooner (as in correct trials) or later (as in incorrect trials). We aligned the activity of the pre-stimulus responsive units (shown in Figure 4) to the onset of the stimulus-guided nose poke (Figure 5C). We used Z-score normalization because it was clear from peri-event time histograms plotting firing rate (Figure 4D, 4E, 4F) that activation of these units occurred. The peri-event time histogram shows the mean change in normalized firing rate for pre-stimulus responsive neurons is aligned to the nose poke onset at $t = 0$ sec (black line). The orange and green vertical lines mark the average time of the stimulus onset before the nose poke (- 0.4 sec before correct nose poke, orange line and - 1.1 sec before incorrect nose poke, green line). Critically, the activation
began before stimulus onset, suggesting that motor preparation, alone, does not explain the increased firing rate during the pre-stimulus period. We further tested the hypothesis that these neurons were directly involved in motor preparation by plotting their firing rate aligned to the premature nose poke. Premature nose pokes were made during the pre-stimulus period and, therefore, reflected impulsive motor actions. On average, premature nose pokes (N = 35 ± 25 mean ± s.d. of premature response trials across N = 20 rats) occurred 7.200 ± 1.135 sec (mean ± s.d. across all trials collected from N = 20 rats) after the rat initiated the trial by poking into the food magazine. Premature nose pokes, therefore, usually occurred during the final 1 sec before stimulus onset would occur. Firing rate did not change in preparation for premature nose pokes (Figure 5D). These data demonstrate that VTA neurons increase firing rate before stimulus onset and that this change does not relate to motor preparation.

We assessed how this pre-stimulus sustained activation was represented in different groups of VTA units according to putative neuronal type. The VTA contains neurons that primarily use dopamine or GABA as neurotransmitter (Bayer and Pickel 1990; Carr and Sesack 2000a; Nair-Roberts et al. 2008; Swanson 1982). Single units were divided into groups using standard extracellular electrophysiology criteria, as described previously (Kim et al. 2012; Kim et al. 2010; Ungless and Grace 2012) (see Methods for details). We characterized the task-related activity of all recorded VTA single units and, when separating them into putative groups, we also showed data for “Other” units that could not be classified using these criteria. We recorded 72 putative dopamine neurons (mean ± s.d. of firing rate=3.40 ± 2.31 Hz), 14 putative GABA neurons (mean firing rate=33.38 ± 21.61 Hz), and 37 other neurons (mean firing rate=19.43 ± 20.48 Hz). Figure 6A shows representative example waveforms of a putative dopamine neuron and a putative GABA neuron. Figure 6B is a scatter plot of the firing rate and waveform duration for all recorded single units (N=123). Although the separation between high baseline firing rate, short-duration waveform single units (putative GABA neurons), and low baseline firing rate, long-duration waveform single units (putative dopamine neurons) is not substantial,
we note that the plot is consistent with the separation observed in other studies (Cohen et al. 2012; Matsumoto and Hikosaka 2009). Critically, a recent study that used optogenetic targeting of dopamine and GABA neurons in the VTA demonstrated that waveform duration is highly variable, but that firing rate separates neuronal types clearly (see the supplementary figures in (Cohen et al. 2012). Accordingly, given that the dopamine and GABA neuron groups are conservatively separated by firing rate in the present study, it is likely that they accurately reflect the phenotype of each neuronal population.

The pre-stimulus firing rate of dopamine, GABA, and other (unclassified) neurons were considered separately. The proportion of pre-stimulus activated neurons within each putative neuron group was not significantly different between these groups (Chi-squared test for correct trials: DA and GABA, $\chi^2(1)=1.16$, $p=0.28$; DA and Other, $\chi^2(1)=0.01$, $p=0.94$) (Figure 7A). The mean firing rate (from the entire recording session) of the pre-stimulus modulated units was $4.79 \pm 3.20$ Hz (mean ± s.d.) for putative dopamine neurons, $43.67 \pm 23.14$ Hz for putative GABA neurons, and $25.68 \pm 17.79$ Hz for other neurons. We plot the magnitude of pre-stimulus activation for each group of putative neurons that was activated on correct trials (Figure 7B). Therefore, all responsive VTA single units, regardless of putative neuronal type (dopamine or GABA), exhibited sustained activation during the pre-stimulus period.

Post-stimulus VTA single unit phasic response after stimulus onset

A short duration phasic increase in firing rate was observed within 250 msec after the stimulus onset in 21 out of 123 single units (17.1%) during correct trials. Although rats were oriented to the stimulus ports during both correct and incorrect trials, the proportion of responsive units was reduced significantly during incorrect trials ($N = 5$ units) and omission trials ($N = 1$ unit) compared to correct trials (Figure 8A, Fisher’s Exact Test; $p=0.0006$ for correct versus incorrect trials and $p<0.0001$, for correct versus omission trials). The single units that were activated phasically after stimulus onset were primarily putative dopamine neurons (Figure 8B). Twenty out of 72 putative dopamine neurons (27.8%) responded to stimulus onset
during correct trials, whereas only 1 out of 14 putative GABA neurons responded. The mean firing rate (from the entire recording session) of the putative dopamine neurons with a post-stimulus phasic response was 3.78 ± 2.10 Hz (mean ± s.d.). Of the putative dopamine neurons with a phasic response during correct trials, the magnitude of the Z-score normalized firing rate response was less during the incorrect and omission trials (Figure 8C) [ANOVA (time as repeated measure); trial type (all 3 types) and time interaction, $F_{(48,912)}=12.71$, $p<0.0001$]. The difference was significant between each of the trial types [ANOVA (time as repeated measure); trial type (correct versus incorrect) and time interaction, $F_{(24,456)}=12.61$, $p<0.0001$; trial type (incorrect versus omission) and time interaction, $F_{(24,456)}=2.24$, $p<0.001$]. Some of the excited putative dopamine neurons remained responsive for a longer duration after stimulus onset, while others were activated for only two or three 20 msec bins (Figure 8D). The single units (N = 20 putative dopamine neurons and N = 1 putative GABA neurons) that responded after stimulus onset did not have a sustained increase in firing rate during the pre-stimulus period (Figure 8D). Finally, we plotted the mean non-normalized firing rate of the putative dopamine neurons that exhibited a phasic response (Figure 8E, same neurons as in 8C and 8D). There was a significant interaction between time (0 sec to 300 msec) and all 3 trial types [ANOVA, $F_{(64,1216)}=5.89$, $p<0.0001$] as well as between correct and incorrect trials [$F_{(32,608)}=9.21$, $p<0.0001$] and correct and omission trials [$F_{(32,608)}=8.17$, $p<0.0001$]. However, there was no significant difference between incorrect and omission trials [$F_{(32,608)}=1.26$, $p=0.154$].

**Discussion**

We recorded from VTA single units during a 3-choice reaction time task. The task requires well-trained rats to orient toward a wall of three stimulus ports in anticipation of a brief visual stimulus, which changes between port locations randomly on each trial. The rat must make an instrumental response to the perceived location of the stimulus. We analyzed neural activity during two cognitively-relevant time periods: first, the pre-stimulus period during stimulus
expectation and second, during the period shortly after stimulus onset when VTA neurons would be expected to exhibit a phasic response to the onset of a salient stimulus (Horvitz 2000; Pan et al. 2005; Schultz 1998; Schultz et al. 1997). During the pre-stimulus period, we find that putative dopamine and GABA neurons increased their firing rate suggesting a role for VTA neurons in representation of internal states of stimulus expectation. We observed that the post-stimulus phasic response was absent on incorrect trials, suggesting that the stimulus-evoked phasic response of putative dopamine neurons may relate to stimulus detection.

Pre-stimulus activity in relation to current theories of reward and motivation

During the pre-stimulus period, putative dopamine neurons maintained an increased firing rate over a period of seconds. This effect did not differ between trial types. Previous work has demonstrated a similar sustained increase in firing rate before an unconditioned stimulus (reward) that was proportional to increased uncertainty about reward delivery (Fiorillo et al. 2003). A key distinction between our design and the previous work is that we examined activity related to expectancy of a CS as opposed to activity related to reward expectancy. In our task, it is unlikely that the difference in firing rate before the CS is related to reward expectancy because the rat was not provided with external information about trial outcome during the pre-stimulus period. Therefore, the change in pre-stimulus activity that we observe could be related to general stimulus expectation regardless of valence.

A previous study of VTA activity during expectancy of a CS has reported a tonic decrease in firing rate in the context of delaying or omitting an expected stimulus, which the authors note is consistent with reward prediction error theory (Bromberg-Martin et al. 2010). These authors did not find a change in activity before the CS, but only after the expected CS had been omitted. In contrast, our behavioral task had predictable stimulus timing paired with a stimulus that required cognitive effort to detect. As a control condition, Bromberg-Martin et al. (2010) examined pre-stimulus activity in a context similar to our task, in which the time of
stimulus onset was always predictable. In that case they found no change in VTA activity. It is possible that they would have found increased pre-stimulus activity before a predictable stimulus, had the task required higher cognitive demand to detect the stimulus.

The majority of studies about motivation have focused on VTA dopamine neurons, rather than VTA GABA neurons. We found that both putative dopamine and GABA neurons increased firing rate during the pre-stimulus period. One recent study also demonstrated increased VTA GABA neuron spiking over a multi-second period (Cohen et al. 2012). The authors used optogenetic targeting to differentiate VTA neuronal types and demonstrated that sustained increases in firing rate occurred in GABA neurons, but not dopamine neurons. The increase in GABA neuron activity occurred over a post-stimulus delay during reward expectancy. Our finding of sustained activation in both neuron types likely is due to the fact that our analysis was focused on stimulus expectancy during the pre-stimulus time period, rather than reward expectancy during the post-stimulus period.

Given that sustained increases in VTA dopamine neuron firing rate have been observed before self-initiated movement (Romo and Schultz 1990), we should emphasize that a limitation of our current task design prevents us from firmly concluding that the pre-stimulus activation is unrelated to motor planning and execution. Specifically, our video monitoring of body position and minor body movements was not adequate to completely dissociate stimulus expectancy from motor preparation. We, however, provide one important piece of evidence that the pre-stimulus activation is not related to nose poke execution because we do not observe increased firing rate before premature nose pokes. While this suggests that pre-stimulus activity is related to stimulus expectation, our interpretation should be taken with the caveat that the VTA activity could relate to body positioning, movements, and action preparation. Future experiments using detailed monitoring and control of orienting and motor behavior will address this limitation of the current study.
Finally, we recognize that the criteria we used to characterize single units into putative dopamine and GABA neurons may not provide enough accuracy. Although dopamine agonists can be used to test a dopamine neuron for auto-inhibition (Bunney et al. 1973; Grace and Bunney 1983; Groves et al. 1975), it is also the case that not all dopamine releasing neurons are sensitive to auto-inhibition (Margolis et al. 2006), including dopamine neurons projecting to the PFC (Lammel et al. 2008). We were interested primarily in PFC-projecting neurons because intra-PFC infusion of dopamine antagonists impairs performance of this task (Crofts et al. 2001; Granon et al. 2000). However, PFC-projecting dopamine neurons have a shorter waveform duration (Margolis et al. 2008) than that denoted by standard classification criteria (Ungless and Grace 2012). Recent experiments targeting dopamine (tyrosine hydroxylase positive) and GABA (glutamic acid decarboxylase positive) neurons and driving them to emit spikes using optogenetics have revealed that waveform duration is highly variable and that firing rate may be the best criteria with which to classify extracellularly recorded VTA units (Cohen et al. 2012). Notably, the characterization criteria may not be critical for interpreting VTA neuron activity during the pre-stimulus period given that all types of neurons were similarly activated. On the other hand, the post-stimulus phasic response that we observed was almost entirely putative dopamine neurons. The criteria that we employed may, therefore, affect the interpretation of those data.

Post-stimulus response of dopamine neurons in relation to current theories of reward and motivation

The brief, phasic increase in post-stimulus firing rate that we observed was similar to the widely reported dopamine neuron response to a CS or novel stimulus (Horvitz 2000; Pan et al. 2005; Schultz 1998; Schultz et al. 1997). We observed the post-stimulus phasic response, primarily, in putative dopamine neurons that were classified using standard firing rate and waveform shape criteria (Ungless and Grace 2012). Other work has demonstrated that both
dopamine and GABA neurons respond to a CS (Kim et al. 2010). This difference may be because the animals in that study were not over-trained or because the stimulus used in the present study was difficult to detect.

The phasic post-stimulus response was reduced significantly during incorrect and omission trials. The difference in phasic response does not reflect outcome because the stimulus did not provide any feedback about the outcome of the trial. Feedback was contingent on the instrumental response and was not received until approximately 750 - 1,500 msec after the stimulus onset. One potential explanation for the reduced phasic response during incorrect trials is that orientation to the stimulus on these trials could have reduced peripheral visual input, which is known to drive VTA neurons (Dommett et al. 2005). Although we did not record sensory cortex activity or control peripheral sensory input using head-fixation, we observed that the rats oriented to the stimuli in a similar manner in all trial types. Therefore, rather than reflecting afferent drive by peripheral sensory input (Dommett et al. 2005), the reduced phasic response during incorrect trials may be due to lack of stimulus detection. This is further supported by recent experiments in the monkey, which demonstrated that a difficult-to-detect stimulus that is not perceived by the animal still evokes a response in sensory cortex, but does not evoke a response from dopamine neurons or prefrontal cortex (PFC) neurons (de Lafuente and Romo 2011; 2012). Thus, in cognitively demanding tasks such as the one employed presently, dopamine neurons may not simply respond to peripheral sensory stimulation, but instead, along with PFC neurons, reflect an organism’s perception of the stimulus. Given this, the reduced VTA neuron responses during incorrect trials observed here may be caused, in part, by reduced top-down drive from the PFC to VTA dopamine neurons. PFC neurons provide top-down drive to dopamine neurons and cause them to spike more frequently (Lodge 2011). Recordings from PFC neurons during the same task (Totah et al. 2009) indicate that increased pre-stimulus firing rate occurs earlier in PFC neurons than in VTA neurons. It is noteworthy that the VTA neurons that exhibited a phasic response to the stimulus were, primarily, putative
dopamine neurons, given that PFC-projecting dopamine neurons, and not VTA GABA neurons, are preferentially innervated by PFC afferents (Carr and Sesack 2000b). Furthermore, microdialysis measurements of PFC dopamine release have suggested that the PFC can control its own dopamine release (Takahata and Moghaddam 1998). Therefore, VTA dopamine neurons could be driven selectively by PFC afferents in order to control cortical dopamine release during demanding cognitive tasks.

**VTA activity as a neuromodulator of cortical processing**

The sustained increase in dopamine neuron firing rate during the pre-stimulus period could have a tonic, modulatory effect on cortical neurons during periods of expectancy (Constantinople and Bruno 2011; Harris and Thiele 2011). This action may stabilize cortical ensembles by synchronizing up-states across neurons (Peters et al. 2004) and limiting PFC inhibitory interneurons to fire within focused time windows (Tierney et al. 2008). Increased cortical dopamine neurotransmission would enhance sustained activity in the PFC (Lapish et al. 2007), which is precisely the activity that we observe in the PFC during the pre-stimulus period of this task (Totah et al. 2009). Pre-stimulus activity before the onset of behaviorally-relevant events has been found not only in the PFC (Niki and Watanabe 1979; Pragay et al. 1987; Totah et al. 2009), but also in the striatum (Apicella et al. 1992). Both of these areas are modulated by dopamine (Fields et al. 2007); thus, pre-stimulus VTA activity may modulate target regions during periods of stimulus expectancy. As discussed in the previous section, this modulation may occur within a PFC – VTA – PFC loop, in which these interconnected regions (Carr and Sesack, 2000b) all have expectancy related activity (Niki and Watanabe 1979; Pragay et al. 1987; Totah et al. 2009).

In addition to dopamine neurons, the GABA neurons in the VTA also could provide long-range modulation of cortical processing. Our electrodes spanned the medial-lateral axis of the VTA but were primarily placed in the heavily PFC-projecting medial (as defined by (Lindvall et al.
Pre-stimulus VTA single unit activity

1978) and (Lammel et al. 2008)) sector of the VTA. While we cannot determine projection targets using our experimental paradigm, at least some of our electrodes were located in the parabrachial pigmented nucleus, which also contains tyrosine hydroxylase immuno-negative neurons that are presumably GABA producing neurons (Lammel et al. 2008). Furthermore, 60% of the VTA projection to the medial PFC in the rat is GABAergic (Carr and Sesack 2000a). Therefore, it is possible that at least a portion of the putative GABA neurons that we recorded were PFC projecting. Our data, therefore, indicate that the GABA projection from the VTA may play a role in regulating PFC activity during the pre-stimulus period. The activation of GABA neurons could produce pre-stimulus suppression of PFC neuron firing rate that we previously have demonstrated in this task (Totah et al. 2009).

Conclusion

We demonstrate that pre-stimulus VTA activity changes during a task that uses a predictable, but difficult-to-detect stimulus. Our results may be consistent with a preparatory increase in VTA neuron activity that underlies internal cognitive states of expectation, preparatory attention or timing of behavior. These cognitive states are fundamental to decision-making and behavior, but are not well understood (Driver and Frith 2000; Nobre et al. 2007; Totah et al. 2012). It is noteworthy that dopamine neurotransmission regulates both attention and time estimation (Coull et al. 2011; Swanson et al. 2011). Furthermore, individuals with schizophrenia, ADHD and Parkinson's disease who are thought to have an underlying pathology of dopamine neurotransmission also have attentional deficits, time estimation deficits and disrupted temporal organization of decision-making and behavior (Allman and Meck 2012; Arnsten 2011; Ward et al. 2012). Thus, a purpose of pre-stimulus VTA activation may be to modulate PFC neurons via both dopamine and long-range GABA projections during stimulus expectancy under cognitively demanding conditions. The sustained increase in dopamine neuron firing rate, and the resulting dopamine release, during the pre-stimulus period may
stabilize cortical ensembles by synchronizing up-states across neurons (Peters et al., 2004) and strengthen PFC sustained neural activity against degradation by task-irrelevant afferent neural activity (Totah et al., 2009; Totah et al., 2012). Finally, we found an unexpected reduction of phasic VTA neuron activation during the post-stimulus period of incorrect trials. These data suggest that dopamine neurons may function in a PFC-VTA-PFC loop that underlies stimulus detection.
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References


Carr DB, and Sesack SR. GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. Synapse 38: 114-123, 2000a.


Kim YB, Matthews M, and Moghaddam B. Putative gamma-aminobutyric acid neurons in the ventral tegmental area have a similar pattern of plasticity as dopamine neurons during appetitive and aversive learning. *Eur J Neurosci* 32: 1564-1572, 2010.


Figure Captions

Figure 1. A diagram of the timing of events during the behavioral task. The trial initiation, pre-stimulus period and CS were the same across trials. The only exception was that the CS presentation changed between 3 randomly selected locations on the operant chamber wall to which the rat oriented. After the CS, there were 3 possible behavioral choices: nose poke into the correct stimulus location, nose poke into the incorrect stimulus location, or response omission (not shown on the diagram). The timing of these nose poke events shown is approximate. Both correct and incorrect behavioral choices were followed 250 msec later by the trial outcome, either reward or extinguishment of the house light. Note that event times are shown to illustrate important points and are not drawn to scale. Analysis focused on firing rate changes during the pre-stimulus period immediately preceding the CS (-4000 msec to 0 msec) and during the post-stimulus period (0 msec to 240 msec). The behavioral response and the outcome occur well after (> 500 msec) the CS analysis window.

Figure 2. Extracellular recordings of single units were made in the VTA. The drawing indicates the location of recordings from each rat in relation to Bregma.

Figure 3. Waveforms, spike rasters and peri-event time histograms for firing rate (Hz) are presented for 2 single units. These units significantly increased their firing rate during the pre-stimulus period. Stimulus onset is at t = 0 sec.

Figure 4. Sustained activation of VTA single units before stimulus onset. (A) The largest proportion of units (N = 29 out of 123 total) was activated during correct trials, whereas the proportion of significantly activated units was reduced during incorrect and omission trials (**p<0.01). Significance was determined using Z-score (see methods for details). (B) The mean
Pre-stimulus VTA single unit activity normalized firing rate across units that were significantly activated before the stimulus is plotted (250 msec bins). The shading indicates standard error from the mean. Stimulus onset is at $t = 0$ sec. The magnitude of change was largest during correct trials and reduced during the other trial types ($p < 0.0001$). During omission trials, the firing rate did not change. The inset shows that non-normalized firing rate was highly variable across single units. The x-axis shows the mean rate from the pre-stimulus period (-6 sec to 0 sec) and the y-axis shows the number of units. (C) The mean normalized firing rate of the remaining non-responsive units ($N = 94$) is plotted with shading, indicating standard error from the mean. These units did not respond during the pre-stimulus period. However, the population responded after stimulus onset. (D, E, F) Units were split into groups with different firing rates to reduce variability in the population mean peri-event time histograms. Top panels show the mean and standard error of firing rate (Hz) in 250 msec bins. The lower plots show individual units on the y-axis and time around stimulus onset on the x-axis. Units were activated for a sustained period of multiple seconds before stimulus onset. Note that one unit with a mean firing rate of ~ 20 Hz was excluded from the plots to reduce variability but included in the statistical tests (ANOVA) reported in the results. VTA unit activation was observed during both correct and incorrect trials, but a change in firing rate did not occur during omission trials.

**Figure 5.** Pre-stimulus increases in firing rate were aligned to the subsequent stimulus-guided nose poke. (A, B) The latency to make a stimulus-guided nose poke differed between trial types. During correct trials (orange, A), rats responded with a mean latency of 0.441 sec after stimulus onset, whereas it occurred 1.100 sec after stimulus onset during incorrect trials (green, B). (C) The mean normalized firing rate of pre-stimulus responsive neurons aligned to stimulus-guided nose poke onset (at $t = 0$ sec, vertical black line). The shading indicates standard error from the mean. The orange and green vertical dotted lines mark the average time of stimulus onset preceding the nose poke for correct trials (orange) and incorrect trials (green). Units increased
firing rate before stimulus onset and continued to fire at an elevated rate until nose poke onset. The change in firing rate was greater during correct trials (orange line). (D) Pre-stimulus responsive neurons did not respond to nose poking events that were not stimulus-guided (i.e., premature nose pokes during the pre-stimulus period). These data suggest that VTA neurons respond during stimulus expectancy and continue until the stimulus-guided action, but do not respond to actions alone.

Figure 6. VTA single units were classified as putative dopamine and GABA neurons based upon firing rate and waveform duration. (A) Putative dopamine neurons were characterized by long waveform duration, whereas putative GABA neurons were characterized by short waveform duration. (B) Firing rate and waveform duration were used to classify single units into 2 groups.

Figure 7. All three groups of VTA neurons (DA, GABA and other) contained neurons that were activated during the pre-stimulus period. (A) Similar proportions of each putative neuron group significantly increased firing rate during the pre-stimulus period (correct trials). (B) Units from all three groups of putative neuronal types were activated. The mean normalized firing rate across all responsive units is plotted and the shading indicates standard error from the mean.

Figure 8. VTA units exhibited a phasic activation after stimulus onset that was significantly different between different types of stimulus-guided behavioral response. (A) The proportion of VTA single units that had a phasic response after stimulus onset was the largest on correct trials and reduced on the other trial types (**p<0.001). (B) Units from the group of putative dopamine neurons had a phasic response after stimulus onset, whereas other groups of neurons were not activated. (C) The normalized (Z-score) magnitude of phasic response of dopamine neurons was related to the subsequent stimulus-guided response. The response was
not related to the instrumental response or to the trial outcome, both of which occurred well after stimulus onset (> 500 msec). The mean normalized firing rate of units from the putative dopamine neuronal group that is activated after stimulus onset (t = 0 sec) is plotted in 20 msec bins. The shading indicates standard error from the mean. The largest activation was on correct trials and the magnitude was reduced during other trials (p<0.0001). (D) The phasic response each single unit (including 1 putative GABA neuron, plotted at y = 1) that is significantly activated after stimulus onset (t = 0 sec) is plotted across a large time window that illustrates firing rate during both the pre- and post-stimulus time periods. The normalized firing rate of all single units that were significantly activated after stimulus onset is displayed on the y-axis and time around stimulus onset (in 20 msec bins) is displayed on the x-axis. A Z-score >2 (yellow) signified a significant increase in firing rate from each single unit’s baseline firing rate. These neurons did not have a seconds-long activation before the stimulus onset; rather they had a phasic activation within 250 msec after the stimulus onset. (E) The firing rate (Hz) of neurons in the above panels is also shown non-normalized across a large time window including both pre- and post-stimulus periods (300 msec bins). The phasic response was significantly reduced during error trials.
Correct Response

Pre-Stimulus Period  CS  Poke  Reward delivery
-8000  0  300  ~500  ~750

Incorrect Response

Pre-Stimulus Period  CS  Poke Light off
-8000  0  300  ~1250  ~1500
Figure 2
Figure 3
Figure 5

(A) Correct trials

(B) Incorrect trials

(C) N = 29

(D) N = 29
Figure 6

(A) Dopamine-like neuron waveform
(B) GABA-like neuron waveform

B

Firing rate (Hz)

Waveform duration (ms)

- Dopamine
- GABA
- Other
Figure 7

Bar graph (A)

- DA: 15/72
- GABA: 5/14
- Other: 8/37

% of each neuron group:

- Correct

Z-score of firing rate vs. stimulus onset (sec) (B)

- Dopamine (N=16/72)
- GABA (N=5/14)
- Other (N=8/37)
Figure 8