Dopaminergic neurons of the substantia nigra pars compacta (SNC) and ventral tegmental area (VTA) exhibit spontaneous firing activity. The dopaminergic neurons in these regions have been shown to exhibit differential sensitivity to neuronal loss and psychostimulants targeting dopamine transporter. However, it remains unclear whether these regional differences scale beyond individual neuronal activity to regional neuronal networks. Here, we used live-cell calcium imaging to show that network connectivity greatly differs between SNC and VTA regions with higher incidence of hub-like neurons in the VTA. Specifically, the frequency of hub-like neurons was significantly lower in SNC than in the adjacent VTA, consistent with the interpretation of a lower network resilience to SNC neuronal loss. We tested this hypothesis, in DAT-cre/loxP-GCaMP6f mice of either sex, when activity of an individual dopaminergic neuron is suppressed, through whole-cell patch clamp electrophysiology, in either SNC or VTA networks. Neuronal loss in the SNC increased network clustering, whereas the larger number of hub-neurons in the VTA overcompensated by decreasing network clustering in the VTA. We further show that network properties are regulatable via a dopamine transporter but not a D2 receptor dependent mechanism. Our results demonstrate novel regulatory mechanisms of functional network topology in dopaminergic brain regions.

Key words: complex network analysis; dopamine; dopaminergic neurons; dopamine transporter; substantia nigra; ventral tegmental area

Significance Statement

In this work, we begin to untangle the differences in complex network properties between the substantia nigra pars compacta (SNC) and VTA, that may underlie differential sensitivity between regions. The methods and analysis employed provide a springboard for investigations of network topology in multiple deep brain structures and disorders.
et al., 2014; Tiklová et al., 2019). While these differences have been hypothesized to underlie differential sensitivity to neurotoxins (Mattamal et al., 1995; Mosharov et al., 2009; Martin et al., 2016; Knowlton et al., 2018), drugs of abuse (Margolis et al., 2006; Hnasko et al., 2010; Lüscher, 2016; Morales and Margolis, 2017), and neurodegeneration (Braak and Braak, 2000; St Martin et al., 2007; Branch et al., 2014; Howell et al., 2020; Lin et al., 2020), it remains unclear how these differences manifest within the VTA and SN regional networks.

Furthermore, dopaminergic neurons vary in their intrinsic electrophysiological properties (Grace and Bunney, 1983; Rapport et al., 1992; Morales and Margolis, 2017), such as spontaneous firing rate, excitability, and spike duration, suggesting that individual neurons may differentially contribute to the synchronization of activity in dopaminergic networks. However, it remains unknown how networks arise from local interactions of dopaminergic neurons, what properties these regional neuronal networks exhibit, whether they differ by specific midbrain regional [VTA vs SN pars compacta (SNC)] identity, how they are regulated or fluctuate in time.

Recently, network neuroscience has emerged as an important tool to answer integral questions about brain network organization (Bassett and Sporns, 2017). Previous work using network theory found that densely connected neurons (hubs) not only exist but orchestrate synchrony (Sporns et al., 2007; Yu et al., 2008; Bonifazi et al., 2009; Sporns, 2013). Recent works have suggested that dopamine network synchrony drives interregional connectivity and whole brain synchronization (Beeler and Dreyer, 2019; Palacios et al., 2019). However, dopaminergic networks have yet to be investigated for the relative impact of the activity of individual neurons to network topology. Hubs, or highly connected neurons, are a common feature of complex networks (Sporns et al., 2007; Sporns, 2013; van den Heuvel and Sporns, 2013; Gutierrez and Marder, 2014; Schroeter et al., 2015), but dopaminergic networks have yet to be tested empirically for the existence of hub neurons. Consistently, many investigations describe the SNC dopaminergic network as more vulnerable to neuronal loss, as often observed in Parkinson’s disease, than the adjacent VTA, albeit with a less understood mechanism (Sulzer, 2007; Boger et al., 2008; Mosharov et al., 2009; Vaillancourt et al., 2009; Evans et al., 2017; Lieberman et al., 2017; Tarfa et al., 2017; Fernandes et al., 2020b; Gigüere et al., 2019). While the origin of these regional differences has remained nebulous, network analysis can provide clues to how functional network organization of these regions may contribute to resilience or failure within the network that ultimately lead to neurocognitive and behavioral dysfunction.

The dopamine transporter, the spatial and temporal regulator of dopamine signaling (Prasad and Amara, 2001; Ingram et al., 2002; Beckstead et al., 2004; Kahlig et al., 2005; Fog et al., 2006; Schmitt and Reith, 2010; Lin et al., 2016), which is expressed in nearly every dopamine neuron in both VTA and SN, providing a unique modicum of communication that is specific to monoamine systems. It has been shown previously that stimulation of dopamine transporter induces alteration in local network properties in reduced systems (Miller et al., 2019), and repeated exposure to modifiers of dopaminergic signaling, such as psychostimulants, regulates dopaminergic signaling machinery and brain activity (Sandrova et al., 2000; Volkow et al., 2001a,b, 2002; Wang et al., 2004; Kahlig et al., 2006; Chang et al., 2008; Krasnova et al., 2011; Volkow and Morales, 2015; Miller et al., 2019). Dopaminergic signaling relies heavily on volume transmission (Rice, 2000; Cragg et al., 2001; Beckstead et al., 2004; Cragg and Rice, 2004; O’Neill et al., 2017), enabling communication between both adjacent and distant neurons (Pucak and Grace, 1994; Cragg et al., 2001; Gentet and Williams, 2007; Gantz et al., 2013; Rice and Patel, 2015). Termination of the dopaminergic signal is thereby achieved by reuptake through dopamine transporter. However, it remains unexplored how dopamine transporter activity regulates interactions between dopaminergic neurons, leading to overall regulation of functional connectivity and ultimately dopaminergic network properties within the SNC and VTA. Previous models of dopaminergic networks suggest that information is encoded through either firing rate or synchronized activity (Beeler and Dreyer, 2019; Kremer et al., 2020; Starkweather and Uchida, 2020). However, these models do not explain whether dopamine transporter function is critical to the functional structure of dopaminergic networks, or whether perturbations of dopamine transporter activity result in restructuring of the network.

In this work, we used high speed live-cell calcium imaging to investigate dynamic functional connectivity and network properties of the SNC and VTA. Moreover, we determined regional-specific contributions of individual neurons to dopaminergic networks through electrophysiological manipulations and identified the role of dopamine transporter to the regulation of dopaminergic networks. We found that dopaminergic neurons in both VTA and SNC exhibit dynamic activity with similar magnitudes and variance of functional connectivity, but the incidence of hub-like neurons was significantly lower in SN dopamine neurons than in the adjacent VTA. Consequently, suppression of activity of an individual dopaminergic neuron within the SNC network increased network clustering, which measures triplet interactions important for functional integration, whereas it decreased network clustering in the VTA. Furthermore, we found that methamphetamine stimulation of dopamine transporter alters network assortativity and small-world properties, which can contribute to network vulnerability to insults like aberrant protein aggregation or neurotoxins. These results suggest that the regional heterogeneities of dopaminergic networks influence functional network topology. Furthermore, differences in functional network topology contribute or ameliorate network dysfunction in the presence of neuronal loss, and whether networks can be controlled through dopamine transporter regulation. This work begins to untangle the differences in complex network properties between the SNC and VTA, that may underlie differential sensitivity between regions and provides a springboard to investigations of multiple deep brain structures and disorders.

Materials and Methods

Animals

DAT<sup>RES</sup> and Ai95(RCL-GCaMP6f)-D knock-in mice were obtained from The Jackson Laboratory [stock numbers: 006660 (DAT<sup>RES</sup>), 024105 (Ai95D)]. Only animals exhibiting bright EGFP fluorescence were used for experiments. Approximately equal numbers of males and females were used for each experimental condition. All animals were maintained in the University of Florida animal facilities. All experiments were approved by the Institutional Animal Care and Use Committee at University of Florida. Animals were housed under standard conditions of 22–24°C, 50–60% relative humidity, and a 12:12 h light/dark cycle.
Preparation of acute midbrain slices in imaging
Postnatal days 28–42 DAT-cre/loxP-GrCaMP6f mice of either sex were used. Mice were decapitated and brain extracted immediately into ice-cold oxygenated artificial CSF (ACSF) consisting of 126 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 26 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM MgSO4 and 10 mM dextrose, equilibrated with 95% O2 – 5% CO2, 300–310 mOsm, and pH adjusted to 7.35–7.4. Brain was cut coronally to PEC on the coronal plane to create a flat surface to glue to the cutting stage. Brain was then submerged in fresh ice-cold ACSF as previously described; 200–300-μm sections containing either the SN or VTA were cut using a DSK MicroSlicer Zero 1N and transferred to a recovery chamber at 32–34°C and allowed to recover for 45–60 min and maintained at room temperature (22–24°C) until used.

Electrophysiological recordings and analysis
To facilitate electrophysiological recordings, the slicing preparation was modified with slicing performed in a sucrose-sodium-replacement solution containing 250 mM sucrose, 26 mM NaHCO3, 2 mM KCl, 1.25 mM NaH2PO4, 11 mM dextrose, 7 mM MgCl2, and 0.5 mM CaCl2, and recovery (ACSF) was supplemented with 10 μM MK-801 to reduce glutamate toxicity. Slices were transferred to a low-flow open diamond bath imaging chamber (RC-26GLP, Warner Instruments) under constant perfusion of ACSF (described previously) at 36–37°C at a rate of 2 ml/min. Dopamine neuron cell bodies were identified first by GrCaMP6f fluorescence through a 40× water immersion objective on an Eclipse FN1 (Nikon Instruments) upright microscope using a Spectra X light engine (Lumencor) equipped with a 470/24-nm solid-state illumination source and recorded using a 12-bit Zyla 4.2 sCMOS camera (Andor Technology, Ltd., Belfast, Northern Ireland). Neuronal morphology was then visualized using infrared differential interference contrast (IR-DIC). Borosilicate glass capillaries (1.5-mm O.D., Sutter Instrument Company) were pulled on a P-2000 laser puller (Sutter Instrument Company) to an open tip resistance of 4–10 MΩ and filled with a potassium gluconate-based internal solution comprised 120 mM K-gluc, 20 mM KCl, 2 mM MgCl2, 10 mM HEPES, 0.1 mM EGTA, 2 mM Na2ATP, 0.25 mM NaGTP, and 5 mM biocytin, 290–295 mOsm, pH adjusted to 7.25–7.3. Recordings were made using an Axon Axopatch 200-B microelectrode amplifier and digitized with a Digidata 1440a through ClampEx 10.2 software (all Molecular Devices, LLC). Analysis of electrophysiological recordings was performed with custom code written for this project using Python 3.7 and the pyABF module (https://pyppi.org/project/pyabf).

Calcium imaging
Acute coronal slices were illuminated as described previously (see above, Electrophysiological recordings and analysis). Images captured at 20–25 frames per second with no delay between frames. Videos were acquired either simultaneously or independent of electrophysiological recordings, depending on the experiment performed. Drug concentrations were selected after a careful review of the literature to model in vivo drug exposure in slice application (Ingram et al., 2002; Goodwin et al., 2009; Branch and Beckstead, 2012; Lin et al., 2016; Sambo et al., 2017; Miller et al., 2019). The literature suggests that 10 μM methamphetamine evokes its maximum responses (Goodwin et al., 2009; Lin et al., 2016), which is blocked by 10 μM nomifensine (Sambo et al., 2017).

Image correction, segmentation, and signal preprocessing
Nikon nd2 files were imported into ImageJ2 via the Bio-Formats importer. Motion correction was performed as necessary with the moco plugin (Dubs et al., 2016). A rolling ball algorithm was applied to each image frame where a pixel value is determined by averaging a 50-pixel ball around the present pixel and then subtracted from the original image to reduce spatial variations. Cell bodies of spontaneously active dopamine neurons were manually segmented within clear borders of interest (ROI), ensuring that ROIs did not overlap and exported to CSV. CSV files were imported into MATLAB and signals were smoothed using the loess method (span = 0.5% of data points). Signals were normalized using relative fluorescence by the following calculation:

$$\Delta F/F_0 = \frac{F_t - F_{t-250}}{F_{t-250}}$$

Where $F$ at time $t$ is normalized to the baseline measurement. The baseline is calculated as the median value of the first 25 s of the recording on a per neuron basis.

Dynamic functional connectivity and network analysis
Dynamic functional connectivity was computed using pairwise Spearman’s correlation coefficients (MATLAB function: corr, type: Spearman) calculated over a sliding 100 frame window with a one frame step size between neurons. Since no threshold is privileged and arbitrary thresholds affect network measurements (Langer et al., 2013; Garrison et al., 2015; van den Heuvel et al., 2017), multiple proportional thresholds were applied to each correlation matrix from 5% to 100% at 1% intervals. Thresholds were excluded if all connections remaining were to one neuron or if less than four neurons remained in the network. For modularity and path length measurements, negative correlations were set to 0 to enable proper computation. All network analysis used the weighted function versions from the Brain Connectivity Toolbox (Rubinov and Sporns, 2010). Control weighted networks were created by randomizing the connection topology while maintaining the size, density, and degree distribution (Maslov and Sneppen, 2002) or latticed (Sporns and Kötter, 2004) where appropriate. Network metrics were calculated at each threshold and then averaged to give a time-resolved network metric that is balanced for specificity and sensitivity before moving to the next matrix.

For all networks, usage of node refers to a neuron. Normalized network strength was defined as the sum of all weights per neuron divided by the number of neurons in the observed network (Rubinov and Sporns, 2010) as defined by:

$$S_i = \frac{\sum w_{ij}}{N-1}$$

Importantly, normalized network strength was calculated using the full correlation matrix without thresholding.

Network assortativity, an evaluative measurement of preferential connectivity to neurons with similar or dissimilar connection strength (Newman, 2002; Foster et al., 2010; Teller et al., 2014; Miller et al., 2019). Assortativity ($r$) was calculated as defined by:

$$r(\alpha, \beta) = \frac{\sum \left( j_i^\alpha - \bar{F} \right) \left( k_j^\beta - \bar{F} \right)}{\sigma^\alpha \sigma^\beta}$$

Where the nodal strength of neuron $i$ (source) and nodal strength of neuron $j$ (target) at connection $i$ of all connections ($E$). With $\sigma$ defined similarly for $\alpha$ and $\beta$:

$$\sigma^\alpha = \sqrt{\sum E^{-1} \left( j_i^\alpha - \bar{F} \right)^2}$$

Network clustering, an extension on pairwise-correlation to triplet correlations (Chambers and MacLean, 2016; Miller et al., 2019), was determined by calculating clustering coefficients (Onnela et al., 2005). Clustering coefficients were calculated as defined by the following:

$$C_i = \frac{2}{k_i(k_i-1)} \sum_{j,k} (w_{ij}w_{jk}w_{ki})$$

Where $k_i$ is the degree of node $i$ and the weight $w$ between nodes $i$ and $j$, computed across the triangle inclusion of nodes $i,j,k$. 
Characteristic path lengths, the average minimum number of connections between any two neurons in the network that must be traversed to connect them (Rubinov and Sporns, 2010), infinite path lengths were omitted from the calculations. Following the original definition by Watts and Strogatz (1998; Telesford et al., 2011), the small-world parameter ($\omega$) was calculated as defined by the following:

$$\omega = \frac{L_r}{L} - \frac{C}{C_l}.$$  

Where $L$ and $C$ refer to path length and clustering coefficient and $L_r$ is the path length of a weighted random lattice and $C_l$ is clustering of a weighted random network.

Network modularity, the existence of communities around neurons is quantified by the $Q$ value index (Newman, 2006; Reichardt and Bornholdt, 2006).

Statistical analysis and network graph construction
All statistical analysis was performed in MATLAB (version 2020a, MathWorks). Two-sample $t$ tests were used where appropriate. Effects were determined as significant at the $\alpha = 0.05$ level. Undirected network connections were imported with corresponding XY positions from the image plane into Gephi v0.9.2 (Bastian et al., 2009). Data and code availability
All raw ROI fluorescence data, and processed functional connectivity and network metrics are provided in MAT files (MATLAB). Custom MATLAB code is intended to run as compiled code on a high-performance computing cluster. MATLAB livescript versions of the code, with raw and processed data, are available on request.

Results
Establishing a temporally and spatially-resolved model system to investigate the functional connectivity and network properties of dopaminergic neurons in the VTA and SNC
To investigate the intrinsic modulation of dynamic functional connectivity and properties of dopaminergic networks of the VTA and SNC, we used highspeed calcium imaging of neural activity (Fig. 1). Dopaminergic neurons signal through their canonical neurotransmitter, dopamine, at multiple release sites,
notably through somatodendritic release via a calcium-dependent manner (Beckstead et al., 2004; Borisovska et al., 2013). Furthermore, dopaminergic neurons signal through multiple timescales (Beeler and Mourra, 2018; Berke, 2018), via presynaptic (Pucak and Grace, 1994; Benoit-Marand et al., 2001; Lindgren et al., 2001; Ingram et al., 2002; Branch and Beckstead, 2012; Dreyer and Houngsgaard, 2013; Rice and Patel, 2015) and postsynaptic mechanisms (Ingram et al., 2002; Lin et al., 2016; Aversa et al., 2018). While dopaminergic neurons receive diverse inputs into their respective regions, little is known about the self-organized intrinsic dopaminergic networks of the VTA and SNC. For these reasons, we used live-cell calcium imaging in acute midbrain slices spanning the VTA and SNC of mice, which provides a readout of neural activity that is intrinsically related to dopamine neuronal activity (Engelhard et al., 2019). We achieved high-specificity expression of GCaMP6f in the dopamine neurons, within these regions, by crossing a GCaMP6f reporter line with DAT-cre. The recordings were performed at 20–25 Hz with an average of 13 individually resolvable neurons per experiment. Thereby, our model system provides a rigorous approach to resolve dopamine neuronal activity with both high spatial and temporal resolution afforded by GCaMP6f kinetics, as well as recording from both SNC and VTA within the same slice.

Calcium influx and GCaMP6f responses exhibit a nonlinear relationship (Chen et al., 2013). Additionally, dopaminergic signaling occurs at multiple timescales, and synchronized neuronal activity is pivotal in dopamine communication (Beeler and Mourra, 2018; Berke, 2018; Beeler and Dreyer, 2019; Palacios et al., 2019). For these reasons, we used Spearman’s \( \rho \) on the calcium signal between every neuron within the field of view over a 100-frame sliding window to construct a stack of adjacency matrices. Each matrix of correlation coefficients is then averaged to give the instantaneous functional connectivity of the network.

The time resolved adjacency matrices were then used to construct dynamic networks. Network measurements have previously been shown to be particularly sensitive to threshold values (Garrison et al., 2015; Zalesky et al., 2016), e.g., correlations that are believed to be meaningful. Importantly, there is no consensus on a meaningful correlation (Zalesky et al., 2016; van den Heuvel et al., 2017), i.e., a correlation of 0.2 could be as important as a value of 0.9; therefore, we strategized with a sliding threshold function where relative thresholds were applied starting at a retainer of 5% of the strongest correlations, to a fully connected network with all correlations retained, at 1% intervals. Network metrics were calculated at each threshold and then averaged to give a time-resolved network metric that is balanced for specificity and sensitivity before moving to the next matrix. This procedure enabled detailed and comprehensive investigation of the dynamic properties of dopaminergic networks of the VTA and SNC, and how they are mechanistically regulated.

**Benchmarking temporal and spatial isolation of activity in individual neurons**

To better understand the accuracy of the manual segmentation on the spatial correlations between neurons, we generated a representative dataset with a laser scanned confocal reference volume of the region imaged in the calcium imaging dataset. In the laser scanning confocal image (Fig. 2A, left), neurons can easily be matched to the segmentation image from the calcium imaging dataset (Fig. 2A, right), with ROI boundaries non-overlapping of proximal neurons. Yellow arrows show neurons 7, 8, 9 that were selected for their high degree of spatial proximity and thus testing the accuracy of the manual segmentation on the spatial correlations between neurons. We considered these three neurons, because neuron 9 is situated between neuron 7 and neuron 8. ROI centroid distances between neuron 9 and neuron 7 is 43 pixels and for neuron 9 and neuron 8 the distance is 54 pixels. One would predict, by spatial proximity for the correlation between neuron 7 and neuron 9 to be significantly higher than neuron 8 and neuron 9. Therefore, we looked at the raw signals found from each neuron (Fig. 2B). Contrary to our prediction, we find that the correlation between neurons 7 and 9 to be 0.3696, while the correlation between neurons 8 and 9 is 0.6202 despite neuron 7’s ROI being closer to neuron 9’s than neuron 8’s (Fig. 2C). Thus, the analyses and the conclusions made here are not significantly affected by spatial correlation overlap.

We also noticed that the correlation between neuron 7 and neuron 8 are lower than other correlations. For instance, these two neurons are farther apart from each other than either from neuron 9, suggesting distance and correlation of activity might be related. Therefore, we examined the relationship between distance and correlation by considering the average correlation between neuronal pairs as a function of distance. Investigating analyses of spatial correlation between these neurons, did not reveal a strong correlation between distance and activity correlation \((r = -0.18, p = 0.06; \text{Fig. 2D})\). Therefore, in acute slice preparation, distance does not dictate degree of correlation as a regionally extrinsic process.

**SNC and VTA exhibit similar patterns of dynamic functional connectivity**

Dynamic functional connectivity quantifies the coupling between the time-series activity of a pair of neurons within a network as a function of time. Traditionally, these are applied to time-locked behavior events (Lurie et al., 2020). However, dopaminergic neurons exhibit tonic spontaneous firing activity that perseveres when excitatory neurotransmission is blocked (Bean, 2007; Puopolo et al., 2007; Lin et al., 2016), suggesting a basal pattern of activity occurs in each region. Here, we use correlated calcium signals to determine dynamic functional connectivity of SNC and VTA dopaminergic networks. First, we assayed the average functional connectivity (average of correlations between all neuronal pairs within the observed network) over the entire duration of the experiment within each region followed by analysis of the variance of this measurement across time to determine whether SNC and VTA exhibit similar functional connectivity over time. Surprisingly, the SNC and VTA exhibited similar average functional connectivity (two-tailed unpaired \( t \) test, \( p = 0.7139, n = 5–8 \) sections from 5 animals; Fig. 3B), suggesting that functional connectivity alone does not sufficiently provide an understanding of differential vulnerability between VTA and SNC dopaminergic neurons.

We next asked whether these two regions would exhibit similar patterns in functional connectivity over time. To do so, we measured the coefficient of variation in the functional connectivity of each region and found similar patterns of functional connectivity within the SNC and VTA dopaminergic networks (two-tailed unpaired \( t \) test, \( p = 0.6887; \text{Fig. 3C} \)). Similar average and dynamic functional connectivity patterns in the VTA and SNC dopamine neurons suggest that each region has similar basal oscillatory processes, which have been theorized to underlie how dopaminergic networks encode information (Beeler and Dreyer, 2019). The limitation of average functional connectivity is that it does not provide information about the structure of the functional network, and specific properties these may confer.
In contrast to functional connectivity, complex network theory provides a well-accepted approach to investigate patterns of connectivity, which in turn facilitate or limit information transfer between dopamine neurons and across the network, that can reveal predisposition to failure, loss of information transfer within functional networks (Rubinov and Sporns, 2010; Bassett and Sporns, 2017). Therefore, we used the complex network analysis to examine the functional network properties of the SNC and VTA.

Emergent variance of network hubs in the SNC and VTA
To investigate patterns of functional network connectivity in each region, we examined the strength of connections (magnitude of the correlated activity between neurons) in the SNC and the VTA. Within the observable neurons of the field of view in each region, we found a wide distribution of connection strengths between dopamine neurons, where some neurons would exhibit low connectivity to each neuron within the network, while others would be strongly interconnected, suggesting that hub neurons may exist within the dopaminergic neuronal network in the VTA and SNC (Fig. 4A,B). Therefore, we calculated the nodal strength of each neuron by integrating the connection values to every other neuron per matrix and over time. Since more connections can arbitrarily inflate nodal strength values, temporal nodal strength means were normalized to the relative size of the network (Fig. 4C). Similar to the pattern of network connectivity of the network visualizations in Figure 4A, B, we found differential patterns of connectivity between dopaminergic neurons of the SNC and VTA. In the SNC, we found skewing of the connection strength distribution to lower strength values. Whereas, VTA neurons exhibited stronger connections across the network compared with the SNC [mean normalized node strength (n) = 3.97 (89 neurons), SNC, 5.24 (70 neurons), VTA, two-tailed unpaired t test, p = 1.89 × 10⁻⁹]. Because of the insurmountable technical restraints using brain slices, one of the limitations of our approach is that we have computed node strength in relatively small neuronal networks, in each brain structure, comprising 10–20 nodes. Nevertheless, even in this limited scale our analyses revealed the SNC to be less densely interconnected than the VTA, suggesting a potentially increased vulnerability of the region to insults.

The variances in nodal strength distributions between regions suggest that some neurons behave as network hubs, or neurons that significantly contribute more to the network than others. As nodal strength belongs to a group of network measurements known as nodal centrality, nodal strength provides a simplistic evaluation for hub-like properties of neurons within the region. We combined the weighted connection distributions (magnitude

Figure 2. Representative benchmark of temporal and spatial isolation of calcium activity. A, 3D laser-scanning confocal image (left) of a VTA slice from a live-cell calcium imaging dataset (right). B, Raw calcium activity from neurons 7, 8, and 9 (shown in A) were selected for their high degree of spatial proximity. C, Correlation matrix of calcium activity between neurons 7, 8, and 9. D, No significant relationship between neuronal soma distance and calcium activity is observed in the representative dataset.
of Spearman’s ρ between each neuron) of both regions to determine an average nodal strength value regardless of region, and calculated the relative percentages above and below the value for the SNC and VTA. Only a relatively small fraction of SNC neurons met this criterion (32.58%) while the vast majority of neurons in the VTA (78.57%) exceeded it (Fig. 4). These results suggest that loss of hub neurons in the SNC may have more dramatic consequences to network properties and its potential lower resilience to failure than a similar loss in the VTA, potentially underlying the differential sensitivity ascribed to the two regions (Achard, 2006; Gao et al., 2016).

Network properties of the SNC and VTA

Similar to other brain networks (Sporns, 2011), it is likely that dopaminergic network connectivity is non-random in functional structure. However, functional network topology can emerge from randomly interacting neurons (Pernice et al., 2011; Orlandi et al., 2013). Therefore, we sought to determine first whether real network properties of the SNC and VTA are distinct from chance events. To determine whether the dynamic properties of each region deviate from a random network of equivalent size, each property was evaluated against a random weighted network (or random lattice, which is appropriate for small-world network measurements; Telesford et al., 2011) of equivalent size and functional connectivity magnitude. Importantly, these random networks were generated through either a rewiring parameter performed for 1000 iterations randomly or with latticization (Maslov and Sneppen, 2002; Sporns and Kötter, 2004). Dopaminergic networks of the SNC and VTA were then assessed for network assortativity (preferential connectivity), modularity (delineation of clear non-overlapping groups), path length (average network distance between nodes), and clustering coefficient (higher order interactions and functional segregation).

We first assayed network assortativity, which ascertains preferential attachment of a neuron to other neurons within the network. Specifically, if a neuron with many connections is primarily connected to other neurons with many connections (preferential connectivity to neurons with similar connectivity), that neuron will have a positive assortativity value, whereas if a neuron with many connections primarily connects to neurons with only one connection (preferred connectivity to neurons with dissimilar connectivity), that neuron will have a negativity assortativity value. Importantly, positively assortativity has previously been shown to provide robustness and resilience to networks (Teller et al., 2014; Lim et al., 2019), while negativity assortativity increases speed of information transfer (Gallos et al., 2008; Murakami et al., 2017). Therefore, examination of

![Figure 3](https://example.com/figure3)

**Figure 3.** SNC and VTA exhibit similar patterns of dynamic functional connectivity. Intrinsic dynamic functional connectivity of dopamine neurons of the SN (SNC) and VTA exhibit similar patterns of functional connectivity. Functional connectivity is defined as the average pairwise Spearman’s correlation across all neuronal pairs. **A**, Representative dynamic connectivity in the SN and VTA. **B**, Mean functional connectivity is similar in the SN and VTA (mean FC = 0.386, 0.407 SNC, VTA, respectively, two-tailed unpaired t test, p = 0.7193). **C**, Variability of functional connectivity (coefficient of variation, CV) is similar across the two regions (mean CV = 0.694, 0.673 SNC, VTA, respectively, two-tailed unpaired t test, p = 0.6887); n = 5–8 sections from 5 animals.
network assortativity provides critical information about resilience and speed of information transfer in the VTA and SNC dopaminergic neurons/network. We found that the SNC and VTA dopaminergic networks both exhibit weakly positive assortativity (random networks generally exhibited negative assortativity), suggesting that neurons preferentially connect to neurons with similar connectivity properties that oscillate between positive and negative values over time (Fig. 5A–C).

We next quantified modularity of the network, or the degree to which neurons form specific groups that are more

**Figure 4.** VTA dopaminergic networks are more strongly interconnected than the SNC. A, B, XY-projection network graph of the functional connectivity matrices overlaid on SNC and VTA suggest differences in connection strength distributions. Connection strength, a neuron’s relative contribution to the network, is displayed through coloration (blue = minimum, white = mean, red = high). Neurons are overlaid with circles representing their weighted degree (node strength, blue = minimum, white = mean, red = high). Scales reflect distribution within the investigated network. C, Normalized node strength is determined through integrating the magnitude of the correlation values of each neuron to all other neurons within the network, and then normalized by dividing by $N-1$, where $N$ = total number of neurons within the network. D, VTA neurons tend to exhibit larger normalized node strength values with a right skewed distribution while SNC exhibit a more Gaussian-like distribution centered on a lower value (mean normalized node strength ($\mu$) = 3.97 (89 neurons), SNC, 5.24 (70 neurons), VTA, two-tailed unpaired $t$ test, $p = 1.89 \times 10^{-11}$). Node strengths are pooled for each region from five to eight slices from five animals. E, Normalized node strength values were pooled for midbrain average node strength and then neurons were classified whether they exceeded or fell below this value. The VTA exhibits a significantly greater proportion of neurons exceeding this average midbrain node strength than SNC (78.57%, VTA; 32.58%, SNC; two-tailed unpaired $t$ test, $p = 3.80 \times 10^{-11}$).
interconnected with each other than neurons outside the group, ranging from 0 to 1, where 0 represents no modular separation and 1 indicates a strong modular structure. Highly modular networks enable subspecialization of the neurons within a network (Sporns and Betzel, 2016; Paldino et al., 2017). Similarly, both regions exhibited equivalent non-random consistent modular functional structures (Fig. 5D–F), suggesting that modular segregation, and thereby subspecialization of neuronal groups, occurs consistently in the SNC and VTA. To investigate the relationship of robustness and efficiency of information transfer, we evaluated the network beyond pairwise comparisons to triplets, determined by clustering coefficients, which assays the connectivity between three neurons simultaneously (MacLean et al., 2005; Chambers and MacLean, 2016; Miller et al., 2019). High clustering coefficients are associated with network robustness (Bullmore and Sporns, 2009). We additionally quantified global efficiency using path length, where a low path length suggests that information can quickly move between clusters facilitating temporally efficient transfer (Bullmore and Sporns, 2009). Path length and clustering coefficients in the SNC and VTA dopaminergic networks were significantly different from random networks and random lattices of equivalent size and connection distribution. Although significantly different, these followed patterns found in random networks, suggesting

Figure 5. Complex network analysis reveals non-random ordered topology of SNC and VTA dopaminergic networks. We investigated regional dynamic network properties to determine whether they differ from those of a random network of equivalent size and connection distribution. Networks were assayed for (A) assortativity, preferential connectivity of a neuron to other neurons with similar (positive) or dissimilar (negative) number of connections; (B) modularity, the degree to which neurons form subspecialized groups; (G) path length, the average number of connections separating any two neurons within the network; and (J) clustering coefficient, a triplet interaction between three neurons. Across each measurement, SNC and VTA networks exhibited similar network assortativity (B), modularity (E), characteristic path length (H), and clustering coefficient (K) that were significant from their randomized counterparts and exhibited similar dynamics (C, F, I, L); n = 5–8 sections.
that more complex topology may be present, such as small-worldness (Watts and Strogatz, 1998; Bassett and Bullmore, 2006).

We next sought to investigate SNC and VTA dopaminergic networks for small-world topology, which is characterized by high clustering, similar to random lattice clustering, and low path length, similar to random path length (Watts and Strogatz, 1998; Telesford et al., 2011). Importantly, small-world networks can confer processing efficiency, resiliency and synchronization (Watts and Strogatz, 1998; Albert et al., 2000; Lago-Fernández et al., 2000; Achard, 2006; Yu et al., 2008). SNC and VTA networks met the standard criteria of a small-world coefficient \( \omega \) between \(-0.5\) and 0.5 (Telesford et al., 2011), suggesting that both regions exhibit this property robustly across time (Fig. 6A–C).

**SNC fragility and VTA resiliency in neuronal loss**

Although the SNC and VTA exhibit surprisingly similar and robust dynamic network properties, the literature suggests these regions to be differentially sensitive to neuronal loss (Sulzer, 2007; Mosharov et al., 2009; Lieberman et al., 2017). Furthermore, it remains unexplored whether loss of a single neuron significantly alters network properties. Therefore, we sought to investigate to what degree the functional network in each region is affected by the loss of a single neuron as determined by restructuring of network properties. To do so, we used simultaneous whole-cell patch clamp electrophysiology and calcium imaging to selectively hyperpolarize individual dopamine neurons to remove their contribution to clamped electrophysiology and calcium imaging to selectively hyperpolarize individual dopamine neurons to remove their contribution to the functional network activity (Fig. 7A). Network measurements were performed at two stages: (1) at baseline firing activity, while the neuron is patched, but no current is injected (Fig. 7A, note there is no change in the spontaneous firing of the patched neuron) and (2) during hyperpolarization, where a hyperpolarizing negative current is applied to suppress spontaneous firing activity (Fig. 7A, note the absence of firing activity during the hyperpolarization step).

Intriguingly, the networks retained many of their properties such as assortativity, which ascertainment whether neurons preferentially connect to neurons with similar connection distributions, and modularity, which measures the degree to which the network forms subgroups (assortativity, \( p = 0.8127 \) SNC, \( p = 0.2418 \) VTA, modularity, \( p = 0.6604 \) SNC, \( p = 0.1792 \) VTA). Strikingly, we found that loss of an individual neuron in SNC increases clustering, suggesting that SNC neurons become more coordinated following neuronal loss to potentially overcome the loss and likely increases metabolic burden. Conversely, VTA networks exhibited significantly decreased clustering coefficients, which may provide resiliency to the region by preventing failures from cascading across the network.

**Dopamine transporter modulation regulates dopaminergic network properties**

Dopaminergic signaling is tightly regulated through dopamine transporter mediated reuptake. Previously we and others have shown that methamphetamine increases the DAT-dependent sodium current, intracellular calcium levels leading to increased firing frequency of dopamine neurons (Jones et al., 1999; Gnegy et al., 2004; Khoshbouei et al., 2004; Kahlig et al., 2005; Lin et al., 2016; Sambo et al., 2017). These data led us to the hypothesis that regulation of network properties may be under direct control of dopamine transporter activity. In order to test this hypothesis, we used methamphetamine to stimulate dopamine transporter activity. To determine specificity, these experiments were performed in the presence or absence of an antagonist cocktail that blocks the synaptic receptors D1, D2, GABA\(_A\), GABA\(_B\), AMPA, and NMDA (5 \( \mu \)M SCH-23390, 5 \( \mu \)M sulpiride, 10 \( \mu \)M SR-95531, 100 \( nM \) CGP-35348, 10 \( \mu \)M CNQX, and 20 \( \mu \)M MK-801, respectively) as well as a cocktail supplemented with a dopamine transporter inhibitor (nomifensine, 10 \( \mu \)M). Drug concentrations and duration of exposure (6 min) were chosen based on previous efficacious work (Lin et al., 2016; Sambo et al., 2017). This experimental paradigm identifies network properties that are specifically dependent on the activity of dopamine transporter, are dopamine receptor mediated, or occur independent of dopamine transporter or dopamine receptor activity. We found methamphetamine increased assortativity in both methamphetamine alone (drug vs baseline, \( p = 0.0152 \), paired samples two-tailed \( t \) test; Fig. 8A) and with antagonists (drug vs baseline, \( p = 0.0361 \), paired samples two-tailed \( t \) test), but not in the presence of nomifensine blockade of dopamine transporter (drug vs baseline, \( p = 0.0503 \), paired samples two-tailed \( t \) test), although a similar trend is observed. A similar effect was observed for small-world coefficients (drug vs baseline, methamphetamine alone \( p = 0.0125 \),...
Figure 7. Suppression of individual neuronal activity increases network clustering in SNC but decreases network clustering in the VTA. A, top, Individual neurons within the field of view are patched sequentially with simultaneous calcium imaging. Middle, During the imaging paradigm, neurons are hyperpolarized to suppress their activity and eliminate contribution to the network. Bottom, Networks are analyzed with the neuron in the network (baseline) and while activity is suppressed (hyperpolarized). The hyperpolarized neurons were excluded from network measurements. Network assortativity (B), modularity (C), path length (D), and small-world coefficient (F) remained stable across the SNC and VTA when individual neurons were hyperpolarized (hyperpolarized vs baseline, assortativity, p = 0.8127 SNC, p = 0.2418 VTA, modularity, p = 0.6604 SNC, p = 0.1792 VTA, path length, p = 0.2947 SNC, p = 0.1249 VTA, small-world coefficient, p = 0.3264 SNC, p = 0.5243 VTA, paired samples two tailed t test). Clustering coefficient (E) significantly differed in both regions (hyperpolarized vs baseline, p = 0.0271 SNC, 0.0353 VTA, two-tailed paired samples t test), but were opposite in valence; n = 4–5 networks per group; *p < 0.05.
Figure 8. Dopamine transporter modulation regulates dopaminergic network properties. A, Dopaminergic networks stimulated with methamphetamine exhibit increased assortativity whether methamphetamine alone or with antagonist cocktail blocking: D1, D2, GABA_A, GABA_B, NMDA, AMPA receptors is applied (methamphetamine alone $p = 0.0152$, methamphetamine with antagonists $p = 0.0361$, $p = 0.0503$ methamphetamine with nomifensine and antagonists, two-tailed paired samples $t$ test). B, Network modularity increased across all conditions.
methamphetamine with antagonist cocktail \( p = 0.0046 \), methamphetamine, nomifensine, and antagonists \( p = 0.0713 \), paired samples two-tailed \( t \) test; Fig. 8E). Since methamphetamine decreases dopamine uptake (Bennett et al., 1998; Sulzer et al., 2005), increases dopamine efflux (Stephans and Yamamoto, 1995; Kahlig et al., 2005; Fog et al., 2006), and increases firing activity of dopamine neurons (Branch and Beckstead, 2012; Saha et al., 2014; Lin et al., 2016), it is not surprising that methamphetamine exposure increased modularity and reduced network clustering across all conditions (drug vs baseline, modularity methamphetamine alone \( p = 0.0104 \), methamphetamine with antagonist cocktail \( p = 0.0012 \), methamphetamine, nomifensine, and antagonists \( p = 0.0323 \), clustering coefficient, methamphetamine alone \( p = 0.0159 \), methamphetamine with antagonist cocktail \( p = 0.0068 \), methamphetamine, nomifensine, and antagonists \( p = 0.0488 \), paired samples two-tailed \( t \) test; Fig. 8B,C), while path length remained consistent throughout (methamphetamine alone \( p = 0.9238 \), methamphetamine with antagonist cocktail \( p = 0.3215 \), methamphetamine, nomifensine, and antagonists \( p = 0.1489 \), paired samples two-tailed \( t \) test; Fig. 8D). These results collectively suggest that modulation of dopamine transporter activity can regulate dopaminergic network properties.

**Technical and conceptual limitations of this study**

In the current study, we leveraged genetic restriction of GCaMP and complex network analysis to determine the intrinsic properties of dopaminergic networks of the SNC and VTA. Although this study is the first to our knowledge to examine dopaminergic networks in this manner, the methods employed also impose technical and conceptual limitations in the study. Specifically, the utilization of slices in the coronal plane undoubtedly severed many physical connections and projections between neurons. Furthermore, the 40× objective and 1-photon imaging used limits the observable region, and correspondingly the number of neurons, than can be achieved with a 20× objective (Cossart et al., 2003; Bonifazi et al., 2009; Pachitariu et al., 2017). We have formed our interpretations with these concerns (van Wijk et al., 2010; Miele et al., 2019) in mind, and future studies will overcome these limitations as dopaminergic networks are further examined in multiple planes, as well as the potential for in vivo implementations that have recently begun to be explored in these regions (da Silva et al., 2018; Fernandes et al., 2020a).

**Discussion**

Dopaminergic neurons and by proxy their regional networks exhibit a rich diversity of neurochemical and electrophysiological properties (Margolis et al., 2006; Fu et al., 2012). The focus of the field often relies on results from individually recorded neurons (Ingram et al., 2002; Margolis et al., 2006; Puopolo et al., 2007; Lin et al., 2016, 2020), or distinctions derived from immunohistochemistry (Fu et al., 2012) or transcriptomics (Poulin et al., 2014; Tiklová et al., 2019) that cannot capture whether the activity in one neuron influence the activity of local neurons and ultimately the output of dopaminergic network. To address these limitations, we used simultaneous highspeed calcium imaging and electrophysiology to isolate interregional differences in interactions of spontaneously active dopaminergic neurons of neighboring dopaminergic nuclei, the SNC and VTA at baseline and following exposure to methamphetamine known to increase dopamine transmission via multiple mechanisms.

Our results show for the first time that the dopaminergic networks of the SN (SNC) and VTA exhibit stable and rich dynamic network properties. We identified increased prevalence of network hubs within the VTA compared with the SNC, differential responses to loss of a single neuron, and modulation through dopamine transporter dependent and independent mechanisms.

Functional networks emerge from local interactions between neurons (Gutierrez and Marder, 2014; Schroeter et al., 2015; Palacios et al., 2019). In dopaminergic networks, dopamine neurons have the machinery to directly interact through both synaptic and non-synaptic signaling (Beckstead and Williams, 2007; Aversa et al., 2018; Miller et al., 2019). However, these studies do not account for dopaminergic network properties and dynamics, and how they differ by brain region. Identification of regional differences offer greater understanding of differential sensitivity of dopaminergic regions, such as loss of SNC dopamine neurons in Parkinson’s disease (Sulzer, 2007; Mosharov et al., 2009; Vaillancourt et al., 2009). Within the subsets of each regional area sampled, we found an overrepresentation of hub-like neurons in VTA dopaminergic networks than in the adjacent SNC. Therefore, the event of loss of a hub neuron in the SNC would have a greater detriment to behavioral outputs that are circumscribed to the SNC. Finally, although the observed regions represent a small fraction of the total area of the region, which is the general limitation of live cell imaging in the randomly selected regions in the SN and VTA, our computational analyses still revealed non-trivial differences in network topology between regions. Future work will determine the degree to which this is consistent across the three-dimensional axes (rostral-caudal/medial-lateral) of the SNC and VTA.

The observed differences in basal network properties led us to test the hypothesis that neuronal loss should differentially affect these two regions. Selective removal of neurons from functional networks through electrophysiological suppression resulted in surprisingly increased clustering of the SNC with decreased clustering in the VTA (Fig. 7E). While high clustering, a higher order measure of connectivity (MacLean et al., 2005; Chambers and MacLean, 2016), is associated with network robustness (Bullmore and Sporns, 2009), increases in clustering suggest that SNC networks respond to neuronal loss by increasing functional segregation (Rubinov and Sporns, 2010) to potentially maintain network output, while the VTA responds by becoming more dispersive. Higher clustering in the SNC suggests an increase of neuronal resources that would maintain network output despite the reported lower average firing rates which have been observed previously (Janezic et al., 2013). Increasing resources may create metabolic stressors that may predispose further neuronal dysfunction and consequentially network failure (Thompson, 2004; Ekstrand and Galfet, 2009; Lieberman et al., 2017). Thus, the network attempting to maintain synchrony by temporally aligning may then contribute to dysfunction of other nuclei within the basal ganglia (Brown, ...
2003; Burkhart et al., 2009). Conversely, the response found within the VTA would suggest robustness resilience to cascades of energy usage or pathogens (Rubinov and Sporns, 2010; Cai et al., 2020). However, loss or dysfunction of VTA dopamine neurons also occurs in Parkinson’s disease and experience cognitive deficits associated with VTA dopamine neurons (Alberico et al., 2015). A VTA lesion study found unrefined executive functioning (Pioli et al., 2008), as was also found in a case report of a patient exhibiting VTA damage (Adair et al., 1996). Furthermore, cortical areas innervated by the VTA exhibit hypodopaminergic states in schizophrenia (da Silva Alves et al., 2008; Pogarell et al., 2012) and lower clustering coefficients (Lynall et al., 2010). Therefore, given enough dispersion of VTA dopamine networks, the outcome of loss of higher order synchrony and functional specialization may underlie these symptoms.

Beyond single neurons, dopaminergic networks feature volume transmission and therefore can interact with both proximal and distal targets (Rice, 2000; Rice and Patel, 2015). Dopamine transporter regulates the degree to which dopamine diffusion can occur through the reuptake of dopamine and ending dopamine signaling (Jones et al., 1999; Cragg et al., 2001). Furthermore, influx of dopamine or methamphetamine through the transporter produces depolarizing currents that alter the firing properties of these neurons (Ingram et al., 2002; Lin et al., 2016; Aversa et al., 2018), which both transporter activity and firing rate are critical to dopaminergic synchronization (Dreyer and Hounsgaard, 2013; Beeler and Dreyer, 2019). Disruption of dopamine transporter function would therefore likely cascade across the network, disrupting network topology. Our data support the interpretation that network structure is under tight control by dopamine transporter, as all but one metric (path length) significantly altered through manipulation of dopamine transporter, and these effects persisted under blockade of many of the receptor types found within the ventral midbrain, but not when the transporter was blocked (Fig. 8). Therefore, dopamine transporter can act as a master regulator of dopaminergic networks through regulation of dopaminergic signaling. Additionally, this provides a direct pharmacological target that may be able to revert dopaminergic networks with dysfunctional properties, such as those found in Parkinson’s disease (Fischer et al., 2019) and addiction (Tomasi et al., 2009; Ding et al., 2013; Orsini et al., 2018). However, as the experiments were performed in a cocktail of antagonists, future experiments will be necessary to provide more nuanced interpretations to the regulation of dopaminergic networks through simultaneous polypharmacy approaches of multiple targets that have been shown to alter network synchrony (Li et al., 2011). Importantly, while acute midbrain slices provide regional investigations into the regulatory mechanisms within each region, they do not account for active afferent input during behavioral state-dependent changes observed in freely behaving animals (Arbuthnott and Wickens, 2007; Kim et al., 2012; Lohani et al., 2019). Therefore, future experiments will require in vivo monitoring of network activity to determine whether and how dopaminergic networks of the SNC and VTA shift their topology to produce or respond to state-dependent changes.

These results expand and complement the current body of literature from the firing rates of individual neurons and synchrony to networks of interacting dopaminergic neurons whose remarkable heterogeneity give rise to exquisite regional differences, both in sensitivity and resiliency. Therefore, our approach provides a foundation for future experiments into the functional structure of dopaminergic networks and how changes in functional structure underlie behavior and disease.

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