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Acute cocaine exposure elicits rises in calcium in arousal-related laterodorsal tegmental neurons

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Abstract

Cocaine has strong reinforcing properties, which underlie its high addiction potential. Reinforcement of use of addictive drugs is associated with rises in dopamine (DA) in mesoaccumbal circuitry. Excitatory afferent input to mesoaccumbal circuitry sources from the laterodorsal tegmental nucleus (LDT). Chronic, systemic cocaine exposure has been shown to have cellular effects on LDT cells, but acute actions of local application have never been demonstrated. Using calcium imaging, we show that acute application of cocaine to mouse brain slices induces calcium spiking in cells of the LDT. Spiking was attenuated by tetrodotoxin (TTX) and low calcium solutions, and abolished by prior exhaustion of intracellular calcium stores. Further, DA receptor antagonists reduced these transients, whereas DA induced rises with similar spiking kinetics. Amphetamine, which also results in elevated levels of synaptic DA, but via a different pharmacological action than cocaine, induced calcium spiking with similar profiles. Although large differences in spiking were not noted in an animal model associated with a heightened proclivity of acquiring addiction-related behavior, the prenatal nicotine exposed mouse (PNE), subtle differences in cocaine's effect on calcium spiking were noted, indicative of a reduction in action of cocaine in the LDT associated with exposure to nicotine during gestation. When taken together, our data indicate that acute actions of cocaine do include effects on LDT cells. Considering the role of intracellular calcium in cellular excitability, and of the LDT in addiction circuitry, our data suggest that cocaine effects in this nucleus may contribute to the high addiction potential of this drug.

Abbreviations

ACh, acetylcholine; ACSF, artificial cerebral spinal fluid; bNOS, brain nitric oxide synthase; CPA, cyclopiazonic acid; CPP, conditioned place preference; DA, dopamine; DTN, dorsal tegmental nucleus; IP_3, inositol 1,4,5-trisphosphate; LDT, laterodorsal tegmental nucleus; PFA, paraformaldehyde; PND, postnatal day; PNE, prenatal nicotine exposure; PSE, prenatal saccharine exposure; ROI, region of interest; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; TTX, tetrodotoxin; VTA, ventral tegmental area.

Introduction

Cocaine blocks the reuptake of dopamine (DA) by the DA transporter (Koe 1976a,b; Ritz and Kuhar 1987; Ritz et al. 1987). The pharmacological effect of the prolonged DA rise due to block of DA reuptake, rather than a stimulation of release, is believed to underlie the reinforcing properties of cocaine. Cocaine, similar to other drugs of abuse, induces sustained levels of DA within the mesoaccumbal circuitry innervated by DA-rich projections sourcing from the ventral tegmental area (VTA). Rises in DA in mesoaccumbal circuitry signals saliency of

stimuli and reinforces the triggering behavior. Cocaine's profile of actions which does not extend to release of DA per se, but rather reductions in DA uptake resulting in prolongment of duration of DA within the synapse (Koe 1976a), would be expected to include actions of DA in the cleft at autoinhibitory synapses known to be present on DA VTA cells, resulting in reductions in DA levels. However, autoinhibition of DA VTA neurons was found to be reduced from expectation (Einhorn et al. 1988; White et al. 1990), suggesting that other cocaine-stimulated mechanisms were counteracting expected autoinhibition and maintaining DA VTA cell activation in the face of this inhibition. One proposed mechanism that could contribute along with others in countering inhibitory actions of DA VTA cell activation was cocaine-induced excitation of afferents directed to DA VTA neurons. Consistent with this interpretation, cFOS expression was heightened in afferents directed to the VTA, including those from the pontine tegmentum, following six consecutive days of self-administration of cocaine (Geisler et al. 2008).

One brain stem source of the cocaine-induced cFOS-positive afferents labeled following retrograde tracer injections in the VTA was the laterodorsal tegmental nucleus (LDT) (Geisler et al. 2008)), which was not surprising as evidence has emerged in the last decade that strongly supports the interpretation that the LDT is an important player in the cellular processes involved in drug-dependent behaviors (Grace et al. 2007; Maskos 2008; Lammel et al. 2012; Kohlmeier 2013; Xiao et al. 2016). The behaviorally-relevant firing pattern of DA neurons resulting in release of DA in the nucleus accumbens (nAc) sufficient to signal stimulus saliency depends on an intact and functioning LDT (Lodge and Grace 2006; Lammel et al. 2012; Chen and Lodge 2013). The LDT sends cholinergic, glutamatergic, and likely GABAergic projections to DA VTA neurons comprising the mesoaccumbal circuit and the majority of these projections are excitatory, based on synaptic anatomical profiles (Omelchenko and Sesack 2005, 2006; Lammel et al. 2012; Dautan et al. 2014). This pathway is functional in vivo, as electrical and pharmacological stimulation of the LDT results in rises of acetylcholine (ACh) and glutamate in the VTA (Forster and Blaha 2000; Forster et al. 2002a,b) and stimulation of afferents directed from the LDT to the VTA results in excitatory membrane currents in VTA neurons (Lammel et al. 2012), including stimulation selectively of ACh-containing afferents (Xiao et al. 2016). This brain stem to mid brain pathway is involved in drug-addiction behaviors as optogenetic stimulation of LDT neurons sending projections to the VTA induced conditioned place preference (CPP), a standard, preclinical model of reward, even in the absence of drug (Lammel et al. 2012). Further, reinforcement of operant responding was detected in lever pressing rats who received optogenetic activation of LDT axons providing afferent innervation of the VTA (Steidl and Veverka 2015).

The LDT has been shown to be involved in cocaine-induced behaviors, suggesting that cocaine either directly or indirectly influences LDT cellular activity. Pharmacological suppression of cholinergic neurons of the LDT attenuated the acquisition of cocaine-induced CPP (Shinohara et al. 2014), leading to the interpretation that cholinergic afferents from the LDT to the VTA were critically involved in acquiring and retrieving, cocaine-related cues (however, see, (Steidl et al. 2015)). Glutamatergic mechanisms within the LDT were found to be involved in the expression of CPP, although, inhibition of NMDA and AMPA receptors did not fully block cocaine-mediated induction of the conditioned behavior, suggesting that nonionotropic, glutamate-dependent mechanisms were involved in activation of LDT cells. (Shinohara et al. 2014). Finally, the ability of blockade of muscarinic and nicotinic receptors in the VTA to reduce expression of cocaine-induced CPP suggested a prominent role for the cholinergic LDT cells in this behavior (Shinohara et al. 2014).

Results from cocaine behavioral studies, which have targeted the LDT, provide strong indications that cocaine has cellular actions on neurons of the LDT. Supporting this interpretation, examination of effects of cocaine within the LDT revealed that chronic cocaine exposure induced synaptic and cellular plasticity in the LDT (Kurosawa et al. 2013; Kamii et al. 2015). In brain slices from rats treated for 5 days with cocaine, presynaptic release of glutamate from terminals directed to identified cholinergic LDT cells was heightened, suggesting cocaine-induced synaptic plasticity (Kurosawa et al. 2013). Further, chronic cocaine exposure increased the activity of a persistent sodium conductance resulting in heighted cellular excitability suggesting an influence of chronic cocaine on postsynaptic plasticity (Kamii et al. 2015).

Although acute effects of cocaine on naive LDT cells have never been reported, when taken together, the cellular studies that have been conducted in the LDT show that chronic effects of this drug are associated with alterations in synaptic strength within this nucleus (Kurosawa et al. 2013; Kamii et al. 2015), suggesting that acute actions, perhaps involving calcium, are induced by this drug. Accordingly, by using calcium imaging in mouse brain slices, we addressed the hypothesis that first-time exposures of cocaine induce cellular actions involving alterations in calcium in LDT cells. Further, we and others have shown changes in cellular functioning in addiction-related neurons, including the LDT, in the prenatal nicotine-exposed mouse (PNE), which could underlie the

higher addiction liability in this animal model of drug-dependent-related behaviors (Slotkin et al. 1991, 2006, 2011; Pauly et al. 2004; Christensen et al. 2015). Therefore, we hypothesized that LDT cellular responses to acute cocaine exposures were altered in this model.

Using calcium imaging in mouse brain slices containing the LDT, we show for the first time that single exposures of cocaine have actions on activity of cells of the LDT. Given the role of the LDT in VTA DA neuronal excitability, our findings of acute effects of cocaine in this nucleus suggest that these actions could participate in maintaining DA VTA neuronal activation, and facilitate signaling of stimuli saliency and thereby contribute to conferring positive incentive for its use. Further, subtle alterations in cocaine effects on calcium in the LDT of the PNE could be involved in alteration in neuronal processes involved in coding relevancy to environmental stimuli and these data shed further light on the neuronal alterations induced by gestational exposures to drugs of abuse, which could participate in heightening addiction vulnerability.

Materials and Methods

Animals

All animal studies complied with the regulations by the Danish Ministry of Justice and the European Communities Council Directive (86/609/EEC), and all animal protocols were approved by the Animal Experiments Inspectorate (2009/561-1707). Naval Medical Research Institute (NMRI) mice were purchased from Harlan Laboratories (Horst, The Netherlands). Mice were kept in the stables of the institute of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, where they were housed in open cages in a parameter-controlled room (temperature 20–23°C, humidity 45–65% and 12 h light-cycle from 6 AM to 6 PM). The animals had free access to food and water at all times.

For studies with naive mice, a lactating female arrived with pups. The pups arrived at postnatal day (PND) 7 in a 1:1 gender mix, allowing an equal gender distribution among the mice used in the conducted experiments. The pups were given at least 1 day of acclimatization before being included in the study. Pups from PND 8 to no older than PND 21 were included, due to a well-documented decreased uptake of the calcium imaging dye in slices obtained from animals beyond this age (MacLean and Yuste 2009; Yuste et al. 2011).

Prenatal nicotine exposure model

Adult NMRI mice (>8 weeks of age) were utilized for in-house breeding of two treatment groups. Two groups of mice were exposed during gestation to either; (1) nicotine and saccharin or (2) saccharin only through maternal ingestion via the drinking water during the pregnancy. Treatment and control groups are termed PNE and prenatal saccharin exposure (PSE), respectively. Breeding of the different treatment groups was effectuated at the same time to ensure similar environmental conditions, and experiments were conducted across several litters to avoid the data being driven by litter effects.

Drinking solutions were changed twice per week. The PSE group received 2% w/v of the artificial sweetener, saccharin sodium dihydrate salt (Merck KGaA, Darmstadt, Germany) in ddH_2O (double-distilled water). The treatment group (PNE) received 50, 100, 200, and 300 μg/mL nicotine (Sigma Aldrich, St. Louis, MO, USA) in 2% w/v saccharin at week 1, 2, 3, and 4, respectively. The slow increase in nicotine exposure was done to reduce possible adverse effects of treatment and saccharin was used as vehicle to mask the bitter taste of nicotine. Breeding was initiated once the final nicotine concentration had been reached. The final nicotine concentration was derived from several previous studies indicating that the resulting plasma concentration of nicotine via delivery in this manner results in tolerance and dependence (Sparks and Pauly 1999; Pauly et al. 2004), as well as alterations in cellular functioning in the LDT (Christensen et al. 2015; McNair and Kohlmeier 2015).

Tissue preparation

Mice were deeply anesthetized by inhalation of isofluorane (Baxter A/S, Allerød, Denmark), which was assessed by loss of righting reflex and failure to respond to a deep pinch of the paw. After decapitation, the brain was rapidly removed and submerged into ice-cold artificial cerebral spinal fluid (ACSF). The standard ACSF solution contained NaCl (124.03 mmol/L), KCl (4.99 mmol/L), Na_2HPO_4-$2H_2O$ (1.20 mmol/L), $CaCl_2$-$2H_2O$ (2.70 mmol/L), $MgSO_4$ anhydrous (1.20 mmol/L), dextrose anhydrous (9.99 mmol/L) and $NaHCO_3$ (25.95 mmol/L) at pH 7.4 and saturated with carbogen (95% O_2/5% CO_2). A block of the brain containing the LDT was coronally sectioned at 250 μm using a VT1200S vibrating blade microtome (Leica, Wetzlar, Germany) in which vertical deflection had been minimized (Vibrocheck, Leica). The LDT was located by known landmarks visible to the unaided eye; the dorsal tegmental nucleus (DTN) and the fourth ventricle, and typically, only one brain slice could be obtained from each animal which contained the LDT. Slices containing the LDT were incubated in carbogenated ACSF for 15 min at 37°C to facilitate removal of fat from cut myelin, and to hasten

termination of injured cells, followed by 1 h of submersion in ACSF held at room temperature for equilibration.

Calcium imaging

To prepare for single-photon, calcium imaging, cells of the LDT were loaded with the membrane-permeable derivative of the ratiometric fluorescent calcium indicator dye, Fura-2 (Fura-2 AM, Life Technologies, OR, USA), which following the crossing of cell membranes, is cleaved by endogenous cellular esterases, leaving the calcium indicator trapped in the cell. To this end, slices containing the LDT were incubated in 1 mL ACSF containing 15 μmol/L Fura-2 AM freshly prepared from a 3.3 μmol/L stock solution of the dye dissolved in the solvent dimethyl sulfoxide (DMSO; 1:1000 final dilution) and equilibrated with carbogen. Incubation was performed at 32°C for 10 min + 1 min for every PND of the animal (e.g., 20 min for an animal at PND 20), as recommended in other studies (MacLean and Yuste 2009; Yuste et al. 2011). Loading with Fura-2 AM has previously been demonstrated to be efficient for loading of neurons within the LDT (Kohlmeier et al. 2004). During and after incubation, the slice was exposed to a minimum of light due to a risk of photobleaching.

Following incubation, slices were transferred to a submersion recording chamber attached to a BX51WI or a BX50WI upright microscope (Olympus, Hamberg, Germany) and were continuously flushed with carbogen-saturated ACSF by perfusion with a RP-1 peristaltic four-head pump (Rainin Instrument Co., Emeryville, CA, USA) at a flow rate of 1.3 mL/min. The slice was rinsed in the chamber for 10–15 min before recordings began in order to ensure temperature equilibration, fura-2 AM de-esterification and removal of free dye debris.

Localization of the LDT was determined under bright-field illumination using a 4× objective (Olympus) guided by the DTN and the fourth ventricle as anatomical landmarks. Individual cells from which fluorescence would be monitored were localized under fluorescent light using a LUMPlan FL N 40× or LUMPlan FL 40× water immersion lens (Olympus). The focus of the microscope was adjusted to distinguish fluorescent dye debris from out of focus cells. Excitation of the fluorescent dye was conducted by a xenon light bulb at wavelengths of 340 nm and 380 nm. At these two wavelengths, fura-2 is excited and emits light at 510 nm, which was transmitted by a Fura-2 cube filter (Chroma, VT, USA) and detected via a 12-bit cooled CCD camera Sensicam 672LD3687 or 382KL1090 (PCO AG, Kelheim, Germany). Wavelength switching was effectuated by ICU control of a Polychrome V or Polychrome VI monochromator (FEI Munich GMbH, Munich, Germany).

The cooled CCD camera was controlled by, and data were collected with, the software Live Acquisition v. 2.5.0.20 or TILLvisiON v. 4.0.1 (FEI Munich GmbH). Pixels in images were binned at 2 × 2, which was the best balance between spatial and temporal resolution so as to maintain visibility of cells during quite long recording sessions (30–80 min). Regions of interests (ROIs) were delineated just outside the perimeter of fluorescent-filled cells based on size and presence of clearly defined processes indicative of dendrites. A background ROI was selected from a region devoid of labeled cells.

Exposure time for images was adjusted such that the intensity of fluorescence at the brightest point was within the range of 300 cd/m_2 (candela per square meter), with the value of 0 as black. This range ensured that less than 10% of the total levels of the 12-bit camera was utilized, allowing a large dynamic range unlikely to alias evoked fluorescent signals. Exposure and cycle times were a compromise between two parameters: (1) higher image frequency to avoid aliasing of cell responses, and (2) limit of light exposure to avoid phototoxicity of cells and photobleaching. Exposure times varied from 30 to 700 ms and the cycle time (i.e., the interval between consecutive 340 nm/380 nm image-pairs) also varied with a range of 1000–3000 msec.

Drugs and solutions

Drugs were "bath applied" via the ACSF. The standard ACSF was used with the exception that in recordings designed to examine the role of flux of calcium across the membrane, a low calcium external solution was utilized which contained in mmol/L: NaCl (127), KCl (5), Na_2HPO_4-H_2O (1,2), $CaCl_2$-$2H_2O$ (1,2), $MgSO_4$ (1,2), dextrose (10), $NaHCO_3$ (26). Stock solutions of drugs were made from solid drugs dissolved in ddH_2O and frozen in appropriate aliquots. Drug to be applied to brain slices were prepared from aliquots of stock dissolved in carbogenated ACSF with a final dilution of at least 1:1000. A total volume of 3 mL of compounds which were expected to increase DA levels in the synapse, was perfused to the recording chamber via the bath. This volume was chosen for agonists as in preliminary experiments robust responses could be elicited, and as we wished to attempt wash-out of drug for repeatability studies, applying an additional volume seemed counter-productive. Cocaine (Cocaine chloride, Nordisk Droge- & Kemikalieforretning, Copenhagen, Denmark), was applied at a concentration of 5 μmol/L. Effects of 10 μmol/L D-amphetamine sulfate (Sigma Aldrich) and 30 μmol/L dopamine hydrochloride (Tocris, Bristol, United Kingdom) were also examined. In some of these studies, SCH-23390, the selective D_1-like receptor antagonist (Tocris),

and 3,5-Dichloro-N-[[(2S)-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide (raclopride, Tocris), the selective D_2-like receptor antagonist were applied at concentrations of 10–20 μmol/L. In some recordings 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 15 μmol/L, Sigma) and D(−)-2-amino-5-phosphonopentanoic acid (AP5, 50 μmol/L, Sigma), were used to block AMPA and kainate, and NMDA, receptors, respectively. Tetrodotoxin (TTX, Alomone), which blocks voltage-gated sodium channels, was applied at a final concentration of 500 nmol/L. Cyclopiazonic acid (CPA, Tocris) was applied at a concentration of 30 μmol/L and success of this compound in "dumping" IP_3-sensitive intracellular calcium stores was monitored during its wash-in by noting changes in fluorescence, indicative of rises in calcium. Concentrations of agonists and DA receptor antagonists were chosen based on available literature conducted in brain slices or cultured cells which showed that these concentrations resulted in DA presence in the synapse and/or elicited DA-mediated cellular effects (Smith et al. 1995; Zhang et al. 1996; Ferris et al. 2012; Calipari et al. 2013; Siciliano et al. 2014). This allowed comparisons to be made between our data and that acquired by other groups.

Recordings were initiated 2.5–3 min before drug arrival at the chamber, to ensure for stable baseline data, and continued for a total of 30–80 min. In recordings conducted with DA and glutamate receptor blockers, control recordings in ACSF were collected to ensure whether the antagonist had effects on fluorescence, although, effects of these compounds on fluorescence were not noted in preliminary studies. In all studies with these antagonists, these drugs were applied for 5–15 min prior to 3 mL application of cocaine.

Data processing and analysis

In this study, we complied with all journal design and statistical requirements. We have utilized cell groups for statistical analysis which exceeded a size of 5. Inclusion and exclusion criteria have been noted. Blinding during the experiment was not used, however, where possible, analysis was done blinded to treatment. Fluorescent data are presented as normalized data, which is standard, and explained in the Data Presentation section below. All groups contained variance, which is reported as SEM.

After recordings were complete, offline analysis was conducted with the software Offline Analysis v. 2.5.0.20 (FEI Munich GmbH, Germany) or TILLvisiON v. 4.0.1. Recordings which exhibited movement of tissue, responses obscured by floating debris, or a major change in focus were excluded. Responses were categorized into several different response profiles based on kinetics. Responses exhibiting a change in fluorescence <2% were excluded as not being easily distinguishable from noise.

Kinetics exhibiting a spiking profile was quantified using Igor Pro v. 6.3.6.4 (WaveMetrics, Portland, OR, USA) with built-in tools and third party macros to quantify the response amplitude, which was calculated as an average of the height measured from trough to peak of the highest spike and the two neighboring spikes. Graphpad Prism v. 6.05 (Graphpad Software Inc., La Jolla, CA, USA) and Igor Pro v. 6.3.6.4 were used for statistical analysis of the data. All data sets were tested for normality, using D'Agostino & Pearson omnibus or Shapiro–Wilk normality tests. In cases where data were not normally distributed, nonparametric tests were utilized. The Fisher's exact test or Chi Square test was used to examine the significance of the proportion of response types. A two-tailed Mann–Whitney test was used to examine the significance between response amplitudes between treatment groups. Furthermore, the Kolmogorov–Smirnov (K–S) test was performed for comparison of response amplitude distributions between treatment groups. Results were considered significant at $P < 0.05$, and asterisks were used to define the level of significance; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Data presentation

All graphs and plots were generated using Igor Pro v. 6.3.6.4 and Graphpad Prism v. 6.05. Absolute calcium values were not calculated due to several different, well-known uncertainties present in conducting imaging with calcium-binding dyes in brain slices (Connor and Cormier, 2000). Instead, all conducted ratiometric measurements were converted to relative fluorescence which allowed comparison of data in which absolute fluorescence likely varied due to unequal loading or due to differences across the two different recording set ups. Accordingly, data are presented as relative changes in percentage of calcium designated %deltaF/F. DeltaF is the change in the fluorescence emission ratio (F340/F380 at peak -F340/F380 baseline) recorded during the run, which is then divided by the average baseline fluorescence intensity (F), measured before the drug was applied. Background fluorescence was subtracted from all images. Upgoing traces in figures of %delta F/F0 ratios represent a rise in intracellular calcium. In some rare cases, bleaching across the run was removed by a subtraction of an equation of the best-fit line using a custom macro in Igor Pro v. 6.3.6.4.

Immunohistochemistry

While the LDT is heterogenous and comprised of several distinct cellular phenotypes, presence of the cholinergic

cells defines the perimeter of this nucleus. Cholinergic neurons of the LDT selectively co-localize the enzyme brain nitric oxide synthase (bNOS), which can successfully be detected in mouse brain slices subjected to hours of recordings (Veleanu et al. 2016). Therefore, post hoc identification of the LDT was performed using immunohistochemical labeling for bNOS. To this end, following recordings, brain slices were submerged in 4% paraformaldehyde and stored overnight. Slices were then cryoprotected by saturation in a 30% sucrose solution. Thereafter, brain slices were resectioned on a Leica cryostat CM 3050S (Leica) at a thickness of 40 μm for subsequent histological procedures. Following a 24 h incubation with the bNOS primary (anti-bNOS, rabbit polyclonal, Sigma-Aldrich, Denmark A/S), bNOS-positive cells were visualized by labeling with an appropriate FITC-conjugated secondary antibody (Alexa Fluor 488, goat anti-rabbit IgG, Molecular Probes/Invitrogen, Denmark). Mounted sections were imaged using a FITC filter set with a monochrome digital camera (Axiocam MRM, Zeiss, Germany) mounted on an epi-fluorescence microscope (Axioskop 2, Zeiss, Germany). Images were collected using Axiovision 4.6 (Zeiss) software and post hoc image processing, which consisted of contrast optimization applied equally across the entire image field, was conducted by use of Adobe Photoshop CS3 (v. 10.0.1).

Results

All data from this report source from recordings of cells in brain slices containing the LDT which were conducted from naive mice (male $n = 34$, female $n = 27$, over 1200 cells), PSE mice (male $n = 5$, female $n = 4$, 299 cells), and PNE mice (male $n = 7$, female $n = 5$, 270 cells). In all investigations, the total number of cells derived from at least three animals with only one slice being used from each animal, however, data were not analyzed for differences across genders. Preliminary analysis revealed no differences across the range of ages examined, so data from animals between P8 to P21 were pooled.

As glial cells and their potential role in cocaine cellular actions playing a role in addiction were not within the scope of this study, neurons were distinguished from glia based on anatomical features presented in other studies (Kohlmeier et al. 2004; Boucetta and Jones 2009; Boucetta et al. 2014). Average soma sizes of 22.7 ± 1.0 μm, 18.9 ± 4.7 μm, and 17.2 ± 1.9 μm have been reported for cholinergic, glutamatergic, and GABAergic neurons, respectively, which distinguishes them from the smaller glial cells (Boucetta and Jones 2009; Boucetta et al. 2014). In addition, neurons were identified in this study by visibility and size of processes, which has been shown to be a reliable way of detecting indicator-filled neurons in this nucleus ((Kohlmeier et al. 2004), Fig. 1A). Although cholinergic neurons do not represent the major cellular phenotype of the LDT, they are considered the principle cell type and their presence defines this nucleus. To verify that calcium imaging was conducted in the LDT, immunohistochemistry was performed to detect neurons containing brain nitric oxide synthase (bNOS), which has been shown to be a marker of cholinergic neurons in this nucleus (Vincent et al. 1983, 1986; Hope and Vincent 1989; Hope et al. 1991). Recordings were found to source from cells contained within the boundary of bNOS-positive cells, indicative that data derived from cells of the LDT ((Veleanu et al. 2016), Fig. 1B).

Cocaine induced robust calcium rises in a substantial proportion of LDT cells

Acute exposure of cocaine (5 μmol/L, 3 mL) to Fura-2AM loaded LDT cells induced large changes in fluorescence (dF/F) in a substantial portion of cells in this nucleus, which is a novel finding.

In 47% of the imaged Fura 2-filled LDT cells (178/375; 12 animals), changes in dF/F were elicited by cocaine, suggestive that acute cocaine evokes elevations of somatic calcium in these cells. Although chronic cocaine exposure has been shown to induce changes in cellular functioning within the cellular network of the LDT, ours is the first study to show that cocaine has acute actions on individual cells as well.

Responses were characterized based on kinetic profile. The most abundant response profile was designated "spiker" (55%, $n = 98/178$), based on presence of at least four transients during the response. It is worth noting that in other Fura-2 AM calcium imaging studies conducted in the LDT, using non-DA-related compounds, similar spiking did not consistently result from tested compounds (Kohlmeier et al. 2004, 2013; McNair and Kohlmeier 2015). Further, in preliminary experiments, drug-free ACSF failed to consistently induce similar spiking ($n = 50$; 5 animals). Spiking behavior often arose on a gradually rising change in dF/F, designated as a plateau. As three distinct onset latencies of the spiker kinetic were noted in preliminary studies, spikers were further categorized as early, middle, or late spikers based on the onset of spiking from the time cocaine hit the bath (Fig. 1C). Early spikers arose with a latency of 2–4 min (Fig. 1D), middle spikers with a latency of 6–8 min (Fig. 1E), and late spikers were apparent at 12–15 min after cocaine reached the bath (Fig. 1F). Of the three spiker profiles, the middle latency spiker was the most common spiker response, as 58% of the spiker types exhibited this profile ($n = 57/98$, Fig. 1C). One-quarter (25%, ($n = 24/98$) and

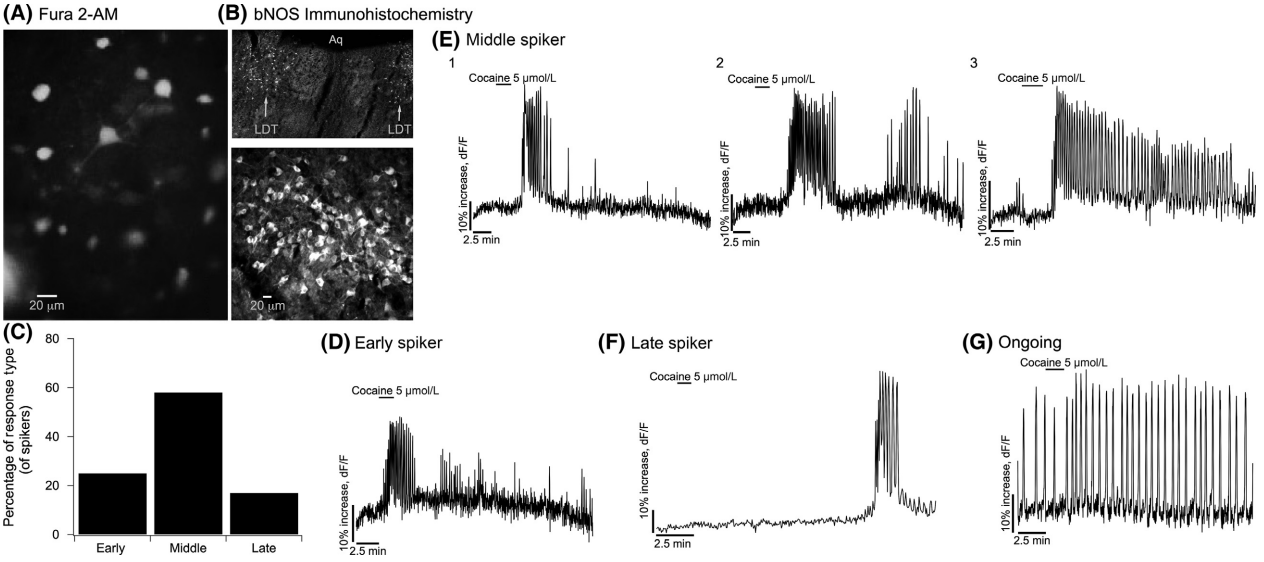

Figure 1. Cocaine (5 μmol/L, 3 mL) induced changes in fluorescence (dF/F) indicative of rises in intracellular calcium in nearly half of the Fura-2AM loaded cells examined in the laterodorsal tegmental nucleus (LDT). (A) LDT cells loaded with the calcium indicator dye, Fura-2, are visible under fluorescent illumination with an excitation wavelength of 380 nm. Changes in fluorescence induced by applied drugs were measured within regions of interest drawn around each calcium indicator-loaded cell. (B) Although the LDT was chosen by anatomical landmarks during recordings, bNOS immunohistochemistry was used post hoc to confirm that recordings sourced from brain slices containing the LDT. The perimeter of bNOS-positive cells has been reported as a reliable method by which to define the boundaries of the LDT in mouse (Veleanu et al. 2016), and as shown in the top image in (B), which is a slice used in this study, at 4× magnification under fluorescent optics (wavelength excitation: 488 nm), bilateral clusters of bNOS-positive cells can be seen ventral to the aqueduct (Aq). Recordings were conducted in these clusters. The bottom fluorescent image is from the same slice, at a higher magnification (20X), which allows for heightened visualization of the cellular details of the bNOS-positive cells. (C) The most common response type induced by cocaine was categorized as a spiking response (Spikers), which exhibited a rapid rise and fall of fluorescence. Spikers could be further divided into three subtypes based on latency of occurrence of spiking (early, middle and late). The middle latency spiking response type was the most commonly-occurring of the three types of spiking behavior, which is reflected in the histogram in C, that illustrates the proportion of each of the three types of spiking response occurring in a population of cells (n = 178 spikers/375 tested LDT cells). (E1, 2, 3) Examples of the most common spiking response are shown from three different representative cells responding to cocaine with changes in fluorescence categorized as a middle latency type of response. The two other types of spiker responses are shown in D and F, and designated as early and late, respectively. (G) In some cases, cells were exhibiting ongoing changes in fluorescence prior to drug application indicating spontaneous fluctuations in calcium activity. This ongoing behavior could be silenced by cocaine, but was often enhanced in amplitude and frequency by cocaine, as shown in this representative LDT cell. LDT, laterodorsal tegmental nucleus.

17% (n = 17/98) of the spiking responses were classified as early and late spikers, respectively (Fig. 1C). In a minority of cells, spiking behavior was ongoing before cocaine exposure, which is a cellular behavior we have noted in other studies conducted in the LDT using multi-cell loading with Fura-2AM (Kohlmeier et al. 2004). In some of those instances, cocaine was found to silence this activity (n = 20/375; data not shown), whereas in a few cases, the frequency of spiking was enhanced by cocaine beyond the baseline level (n = 15/375, Fig. 1G). As the most common response was to elicit spiking behavior from a quiescent baseline, subsequent focus was placed on this response type and cells exhibiting ongoing behavior before drug application, or the minority of cells that exhibited other kinetic responses to cocaine, were not analyzed further.

Cocaine-induced rises in calcium were not repeatable

As we wished to apply cocaine in the presence of antagonists in order to investigate the mechanism(s) underlying cocaine-induced calcium rises, we first needed to determine whether the different response types of cocaine-induced dF/F rises were repeatable. Recordings exceeding 60 min were executed with application of cocaine at 2.5 min into the recording, and a second application administered 40 min later. However, in all cases where spiking activity was robustly induced in the first application of cocaine, repeat applications were unable to elicit any similar kinetic activity (n = 25; 4 animals; Fig. 2A). And in some cases, even when the first and second application interval exceeded 80 min, activity from the first

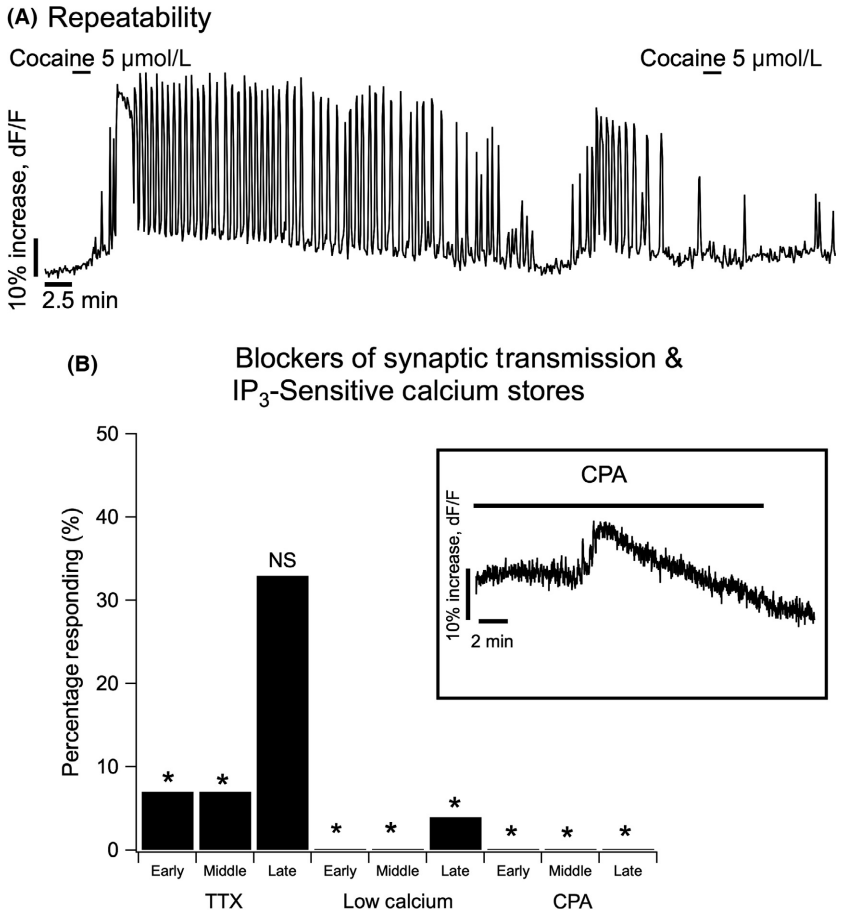

Figure 2. Cocaine responses were not repeatable within the first and second application intervals examined (20–80 min). (A) As shown in this dF/F recording taken from one representative LDT cell, even if spiking induced by a first application of cocaine began to wane, robust repeat responses could not be elicited by a second application of the drug. Often, responses to first applications failed to extinguish even at 1 h post application. Accordingly, determination of effects of antagonists or low calcium external solutions had to be conducted by comparing responses between two different populations of cells. (B) The proportions of cells responding with the early and middle spiking responses were significantly lower in the presence of tetrodotoxin (TTX), which blocks voltage-dependent sodium channels, indicating reliance of these response types on intact synaptic transmission. The early and middle spiking response was eliminated by low calcium solutions. The late spiking response was also reduced in frequency in these conditions, however, this spiking profile was still elicited in a small proportion of cells (7%) raising the possibility that this response type was not entirely dependent upon calcium flux across the membrane. However, cocaine failed to elicit any of the three types of spiking behavior following dumping of the IP_3-sensitive intracellular calcium stores. (B, inset) Success of cyclopiazonic acid in dumping these stores was monitored by fluorescent imaging conducted while this drug washed into the bath, and transient increases in dF/F were indicative of successful dumping of this store. When taken together, these data suggest that mechanisms occurring presynaptic to the postsynaptic imaged cells are involved in the cocaine-induced changes in dF/F resulting in the early and middle latency spiking response, whereas intracellular calcium stores are involved in all three kinetic responses to this stimulant. Asterisks in this, and subsequent figures, denote statistical significance with alpha set lower than 0.05.

application did not appear fully extinguished. The inability to elicit second responses within the time frame examined was consistent for all of the observed response profiles. Accordingly, as it was not possible to do within the same cell experiments relying on repeatability, the next series of experiments, which examined the effects of antagonists on the cocaine-induced spiking, were conducted by utilizing across cell comparisons consisting of a control group of LDT cells and a separate antagonist group. Control and antagonist recordings were interleaved to reduce any contribution of technical or environmental changes within the laboratory, or litter effects, to differences seen between the two groups. Proportions of cells responding with each response type were not significantly different between the different experimental series (Fisher's Exact Test, $P > 0.05$).

Cocaine-induced calcium spiking was attenuated by blockade of voltage-dependent action potentials

To determine the role of action potentials in cocaine induced rises in LDT cells, voltage-dependent sodium channels were blocked by a 5 min incubation of the slice with TTX (500 nmol/L) prior to cocaine application. Of the 83 cells examined, cocaine elicited calcium rises in 36% of these cells (n = 30/83; 3 animals). In those cells responding to cocaine in the TTX condition, cocaine elicited the medium latency type of spiking in only a small fraction of these cells (7%; n = 2/30), which was a response rate significantly different from that obtained when TTX was absent in experiments with a separate population of cells (Fisher's Exact Test, n = 208, P = 0.0038; Fig. 2B). Similarly, fewer cells responded with the early latency response type when TTX was present (7% n = 2/30; P = 0.0382; Fig. 2B). Interestingly, the rate of cells responding with the late response type did not significantly differ between TTX present and TTX absent conditions (33%; n = 10/30; P = 0.0749; Fig. 2B). Further, the plateau response underlying this spiking which was sometimes noted in control conditions, was also present in those cells spiking in TTX conditions, suggesting that this source of calcium is independent of involvement of voltage-dependent sodium channels. When taken together, these data suggest that the mechanisms underlying the three temporally-distinct spiking behaviors are not identical.

Cocaine-induced calcium spiking was abolished by lowering extracellular calcium

To determine if cocaine-induced calcium spiking required flux of calcium across the membrane, we reduced the extracellular concentration of calcium by incubation of the tissue in a low calcium external solution prior to application of cocaine. In the presence of the low calcium solution, cocaine failed to elicit any middle latency spiking (n = 0/55; 3 animals; Fig. 2B), which suggests that the TTX-resistant component of this response type is dependent, in part, on influx of extracellular calcium across the membrane. When taken together with our TTX findings, these data suggest that cocaine is eliciting medium latency calcium rises via actions involving presynaptic mechanisms which could involve somas with terminals left intact in the slice, with a possible contribution from postsynaptic mechanisms, which could include cocaine-induced action potentials, as well. Early latency responses were similarly abolished in low calcium solutions, indicating that the component of this response type which was resistant to block by TTX, was similarly reliant on flux of calcium across the postsynaptic membrane and/or into the presynaptic terminal (n = 0/55; Fig. 2B). Interestingly, cocaine elicited late latency spiking behavior in a few cells, however, the response rate for this type was significantly lower than in regular calcium conditions suggesting that flux across the presynaptic and/or postsynaptic membrane plays a role, but that this source is not exclusively involved in this response profile (n = 4/55; Fisher's Exact Test, P = 0.0228; Fig. 2B). In addition, plateau responses were significantly diminished in low calcium conditions as this kinetic response was only seen in two of the late spiking cells (n = 2/55). These data lend further support to the interpretation that the mechanisms underlying early, middle, and late latency spikers are different.

Cocaine-induced calcium spiking was abolished by mobilization of intracellular calcium stores

To determine whether cocaine-induced calcium spiking were dependent on release of calcium from intracellular stores in the ER, we mobilized IP_3-sensitive intracellular calcium stores with the reversible SERCA-pump inhibitor, cyclopiazonic acid (CPA). LDT cells in this study exhibited rises in dF/F when CPA was applied (Fig. 2B, inset), which we have shown in other studies is indicative of successful dumping of the SERCA-pump-dependent intracellular calcium stores from the ER of LDT neurons (Kohlmeier et al. 2004).

A 20 min wash-in of CPA prior to cocaine application was chosen to effectuate maximal release of intracellular calcium. In the presence of CPA, cocaine failed to induce calcium spiking in any of the cells examined, and plateau responses were also never elicited (n = 38; 3 animals; Fig. 2B). Accordingly, SERCA-pump-dependent intracellular calcium release from the ER is required for all types of spiking behavior induced by acute application of cocaine. When taken together, our data further support the conclusion that cocaine induces at least two responses that are mechanistically different. One is reliant on extracellular flux likely triggered by an intracellular cascade of events dependent on initial rises in calcium, and another response, which can be generated solely by release of intracellular calcium. As these mechanisms are dependent on a complex intracellular cascade, they are likely to occur across different time scales.

Dopamine elevated calcium in the LDT and induced calcium spiking profiles that were similar to cocaine

In order to explore whether an increase in synaptic dopamine (DA) is involved in cocaine-induced calcium

spiking, rather than due to the plethora of other cellular actions of cocaine, our first step was to investigate the effects of DA on dF/F of Fura-2AM loaded LDT cells. Application of DA at a concentration used in other brain slice studies to elicit cellular actions (30 μmol/L, 3 mL) elicited changes in dF/F in half of the LDT cells examined (n = 45/90; 3 animals; Fig. 3A), which is a novel finding. Furthermore, temporal profiles similar to those elicited by cocaine were obtained, suggesting that the underlying mechanisms involved in cocaine and DA-induced rises in calcium in LDT cells are similar (Fig. 3A2). Interestingly, DA-induced responses were not repeatable, which demonstrated a further similarity to actions of cocaine. The proportion of middle latency responses induced by DA was compared to the proportion of this response type elicited by cocaine between two populations of cells. There was no significant difference in the proportion of cells responding with the middle latency spiker response profile to DA from the proportion responding with this response to cocaine (27% responding to DA with middle latency spiking: n = 12/45, Fisher's Exact Test, P = 1.0000). The proportions of cells responding with the early latency spiking and the late latency spiking response to DA were also not significantly different from the proportions responding to cocaine with these response types (Early spiking, 35%, Fisher's Exact Test, P = 0.4853, n = 16/45; late spiking, 22%, Fisher's Exact Test, P = 0.3668, n = 10/45). However, the amplitude of the middle latency response induced by cocaine was found to be significantly greater than the amplitude of this response type elicited by DA. The amplitude of dF/F induced by DA when this response type was elicited was nearly 72% smaller than that induced by cocaine in a matched group of cells (DA: 10.8 $\pm$ 0.8% dF/F, n = 12; cocaine: 36.7 $\pm$ 6.1% dF/F, n = 19; Mann–Whitney Test, n = 25, P = 0.0027; $3A_3$). The early latency response exhibited an average amplitude that was 56% of the response amplitude induced by cocaine, which was a significant difference (DA: 12.0 $\pm$ 2.7, n = 12; cocaine: 27.6 $\pm$ 4.1, n = 19, P = 0.003; $3A_3$). Interestingly, while the average amplitude of the late latency response induced by DA was smaller than that induced by cocaine, the difference was not significant (DA: 13.8 $\pm$ 2.5, n = 10; cocaine: 20.5 $\pm$ 3.1, n = 15, P = 0.139; $3A_3$). Our data could indicate that there is a difference in mechanisms underlying the calcium spiking between the two drugs. Differences between these mechanisms would not be surprising, as actions of cocaine include inhibition of the DA transporter which has membrane actions, including altering the voltage across the membrane which would be expected to result in secondary actions which could influence calcium (Cameron et al. 2013a,b; Kolanos et al. 2013). As a comprehensive dose–response comparative study was beyond the scope of this report, we used a concentration of DA used in other slice studies. Therefore, it remains a possibility that differences in amplitudes in calcium elicited by DA and cocaine can be explained by distinct pharmacological properties induced by differences in effective drug concentrations between cocaine and DA which would be expected to influence receptor occupancy or the amount of synaptic DA achieved. Regardless of the underlying reason behind the difference in amplitudes induced, our data are consistent with the interpretation that spiking behavior stimulated by cocaine is mediated by rises in DA in the LDT.

Amphetamine elevated calcium in the LDT and induced calcium spiking profiles that were similar to cocaine

We wished to extend our examination regarding whether DA was involved in the spiking behavior induced by cocaine. Therefore, our next step was to monitor responses to amphetamine, which exhibits the pharmacological outcome of increasing synaptic DA. However, the mechanisms behind this increase are quite different than those underlying cocaine-stimulated DA rises, or from increases in synaptic DA induced by exogenous application. Amphetamine is taken up into cells by the DA transporter, resulting in DA transporter-mediated inward currents, and a plethora of intracellular actions beyond those induced by cocaine (Cameron et al. 2013a,b; Kolanos et al. 2013). Application of amphetamine (10 μmol/L, 3 mL) elicited changes in dF/F in 43% of the LDT cells examined (n = 32/75; seven animals, Fig. 3A1). As acute actions of amphetamine on LDT cells have never been reported, this is a novel finding. Further, amphetamine-elicited calcium rises could be grouped into early, middle, and late spikers, and the proportion of these response types was similar to those elicited by cocaine (early spikers 9%, middle spikers 21%, and late spikers, 21%, Fig. 3B2), as well as DA. Also reminiscent of effects of cocaine and DA, amphetamine-induced calcium spiking was not repeatable (n = 32/32, data not shown). 1-hour long recordings revealed that spiking induced by amphetamine was persistent.

Amphetamine is known to increase intracellular calcium, which is a demonstrated effect of TAAR1 (trace amine-associated receptor I) activation, associated with DA transporter phosphorylation through a calcium/calmodulin-dependent protein kinase (CAMK)-dependent pathway, resulting in an efflux of DA (Gnegy et al. 2004). In addition, transporter-mediated inward ionic currents induced by amphetamine could be expected to activate voltage-dependent calcium channels (Cameron et al. 2013a,b; Kolanos et al. 2013). Therefore, we hypothesized

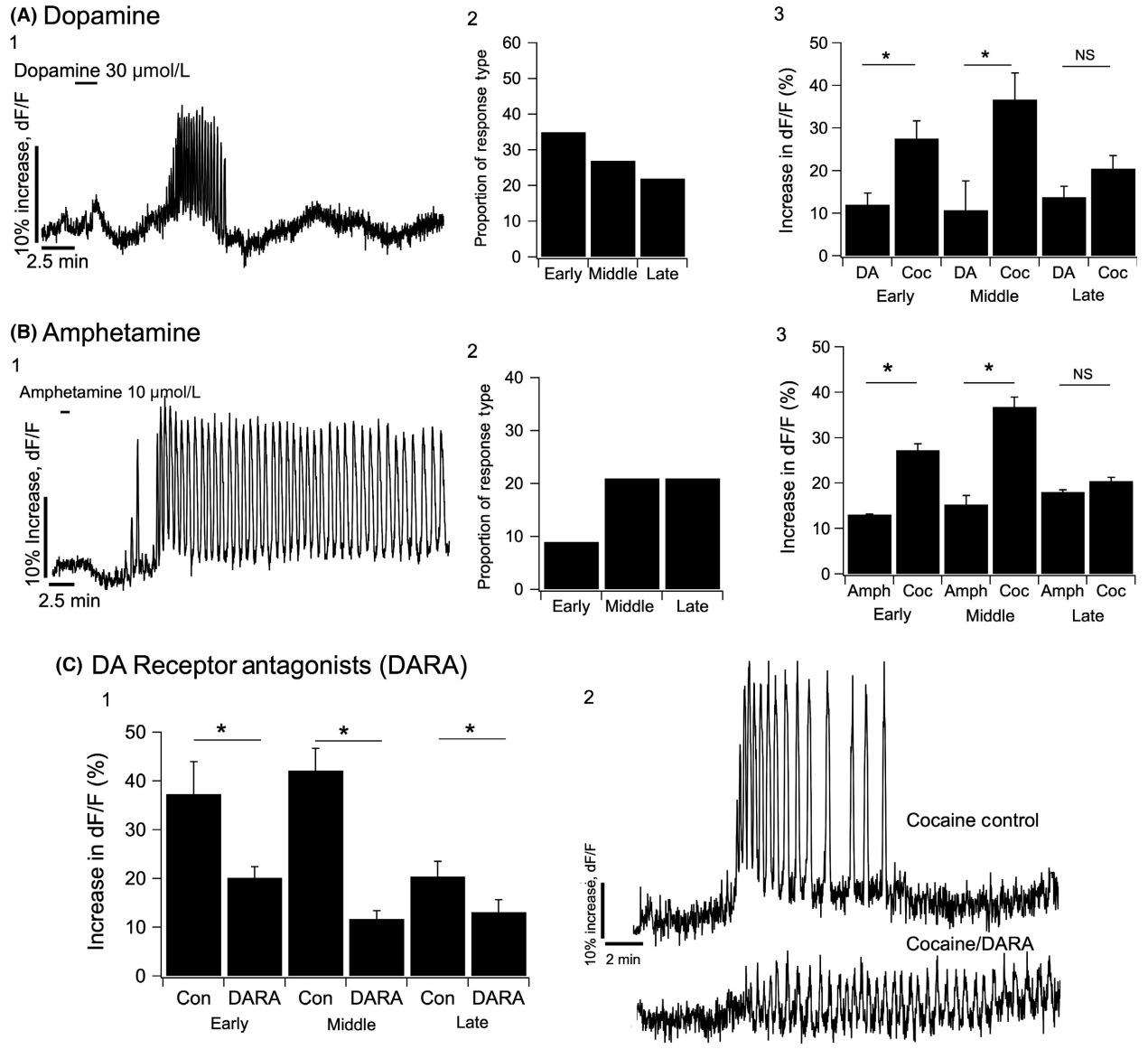

Figure 3. Application of DA (30 μmol/L) and of amphetamine (10 μmol/L) resulted in spiking behavior in Fura-2AM loaded LDT neurons that was qualitatively very similar to that induced by cocaine, and DA receptor blockers attenuated cocaine-induced spiking behavior. (A1) Representative LDT cell, in which a spiking change in dF/F is evoked by DA. (A2) Responses from the population of LDT cells examined revealed that the proportion of spiking behavior elicited by DA was not significantly different than the numbers of cells responding to cocaine, and the proportions of the three spiking response types induced by DA were not different from those elicited by cocaine. (A3) However, at the concentrations utilized, the amplitudes of two of the spiking behaviors (early and middle) elicited by DA were significantly smaller than those induced by cocaine, whereas, the amplitude of the late spiking response was not significantly different. (B1) Amphetamine induced spiking behavior that was also qualitatively similar to that induced by cocaine and to that induced by DA, as shown in this representative example of spiking behavior elicited by amphetamine in one LDT cell in this study. (B2) Population data show that the distribution of response types was not significantly different between cocaine and amphetamine. (B3) However, interestingly, at the concentrations of stimulants used, the average amplitudes of the early and middle spiking behavior induced by amphetamine were significantly smaller than that induced by cocaine. The amplitude of the late latency response was not significantly different between the two compounds. Experiments with DA and amphetamine were interleaved with control recordings and the same control population in which cocaine was applied was used to test significance across both data sets. (C1) Presence of the DA receptor antagonists, SCH-23390 and raclopride (10–20 μmol/L), resulted in cocaine-induced rises in dF/F which were significantly smaller in the population of cells test (DARA) than the amplitudes induced in the absence of these antagonists in another population (Con). (B2) Changes in dF/F induced by cocaine in the absence of DA receptor blockers (top recording, Cocaine Control) and in another cell (bottom recording, Cocaine/DARA), in the presence of the blockers showing a much smaller amplitude of spiking induced by cocaine when the blockers are present. DA, dopamine; LDT, laterodorsal tegmental nucleus.

that calcium induced by amphetamine, as it might result from activation of several different pathways, potentially could exceed that induced by cocaine. However, the amplitude of calcium spikes occurring with the middle latency response type induced by amphetamine were 58% smaller when compared to the amplitude of spikes induced by cocaine in cells recorded in interleaved experiments, which was a significant difference (amphetamine: 15.3 ± 2.0% dF/F, $n = 20$; cocaine: 36.8 ± 6.2% dF/F, $n = 20$, Mann–Whitney Test, $P = 0.0006$; Fig. 3B3). Similarly, the amplitude of the early latency spikers induced by amphetamine was significantly smaller than those induced by cocaine (amphetamine: 13.1 ± 0.2% dF/F, $n = 30$; cocaine: 27.3 ± 1.4% dF/F, $n = 22$ Mann–Whitney Test, $P = 0.0012$; Fig. 3B3). Strikingly, the average amplitude of the late latency spiking induced by amphetamine was quite similar to that induced by cocaine (amphetamine: 18.1 ± 0.5% dF/F, $n = 24$; cocaine: 20.4 ± 0.8% dF/F, $n = 15$ Mann–Whitney Test, $P = 0.8837$; Fig. 3B3). Therefore, contrary to our expectations, we found that the amplitude of calcium rises induced by the concentration of amphetamine used, which is similar to that used in other brain slices studies, were actually smaller than those induced by cocaine, suggesting the possibility that there are differences between the two drugs in mechanisms underlying the calcium rises detected with our imaging.

Cocaine-induced calcium spiking was attenuated by DA receptor antagonists

Based on the pharmacological outcome of the actions of cocaine, that is, increase of synaptic DA, the most parsimonious explanation for rises in calcium induced by cocaine involved some degree of activation of DA receptors in the LDT. Although mRNA studies have revealed message for the DA receptor type 2 (D_2) in an area adjacent to the LDT (Weiner et al. 1991a), it is not currently known whether functional DA receptors are present in the LDT, or if they are present, what subtypes might exist in this nucleus. To investigate whether DA receptors are involved in the cocaine-induced transients, we preincubated a subpopulation of LDT brain slices with the relatively selective D_1- like (D_1/D_5) and D_2-like (D_2/D_3) DA receptor antagonists, SCH-23390 and raclopride. Following this incubation, cocaine was applied in the presence of these DA receptor antagonists, and responses were compared to those elicited by cocaine in control LDT brain slices.

Out of a total of 136 LDT cells examined (9 animals), 60 of these exhibited a spiker response to cocaine in the presence of the DA receptor antagonists. The proportions of cells responding with the middle latency spiking response was significantly lower in the presence of the antagonists than in their absence (20%, $n = 12/60$; Fisher's Exact Test, $P = 0.0001$). The proportion of cells responding to cocaine with the early and late responses was not significantly altered by presence of DA receptor antagonists.

We next determined whether the amplitude of the spiker responses was altered in the presence of blockade of D_1-like and D_2-like receptors. Presence of the DA receptor blockers did significantly reduce the average amplitude of all three types of spiker responses (Fig. 3C1&2). In the presence of DA receptor blockers, the average amplitude of the middle latency response was 73% smaller and the early and late latency responses were smaller by 46% and 36% of control, respectively, which were all significant differences (early, $P = 0.004$; middle, $P < 0.001$; late, $P = 0.038$; Fig. 3C1). Interestingly, in the presence of antagonists, a plateau response was only noted in one cell, suggesting that the DA receptor is involved in this response type ($n = 1/56$; Fig. 3C1). These results indicate that activation of D1-like or D2-like DA receptors are involved in all three of the cocaine-induced calcium spiking profiles.

The amplitude of cocaine-induced calcium spiking was reduced by glutamate receptor antagonists

As even acute cocaine exposure has been shown to result in glutamate receptor trafficking resulting in an upregulation of AMPA receptors in the membrane which could be calcium permeable (Ungless et al. 2001; Saal et al. 2003), we examined the response to cocaine in the presence of blockers of AMPA and NMDA receptors, to determine whether these receptors play a role. In the presence of AP-5 and DNQX to block AMPA and NMDA receptors, respectively, cocaine still induced all three types of spiking behavior. However, the total proportion of cells responding to cocaine with a spiking response in the presence of glutamate receptor blockers was significantly smaller than the proportion responding with this kinetic in their absence (Fishers Exact Text, $n = 116/334$; 10 animals; $P = 0.0006$). Further, the amplitudes of the early and the middle spiking response were significantly smaller (Fig. 4A1&2). In the presence of the glutamate receptor blockers, the amplitude of the early and middle latency dF/F responses were both approximately 13%, which represents a 55% and 66%, respectively, smaller rise than the rise induced by cocaine, which was a significant difference (early latency, $P = 0.0002$, $n = 23$; middle latency, $P = 0.0001$, $n = 55$; Fig. 4A1). The amplitude of the late amplitude response was nearly 18%, which was not significantly different than that in the absence of the blockers

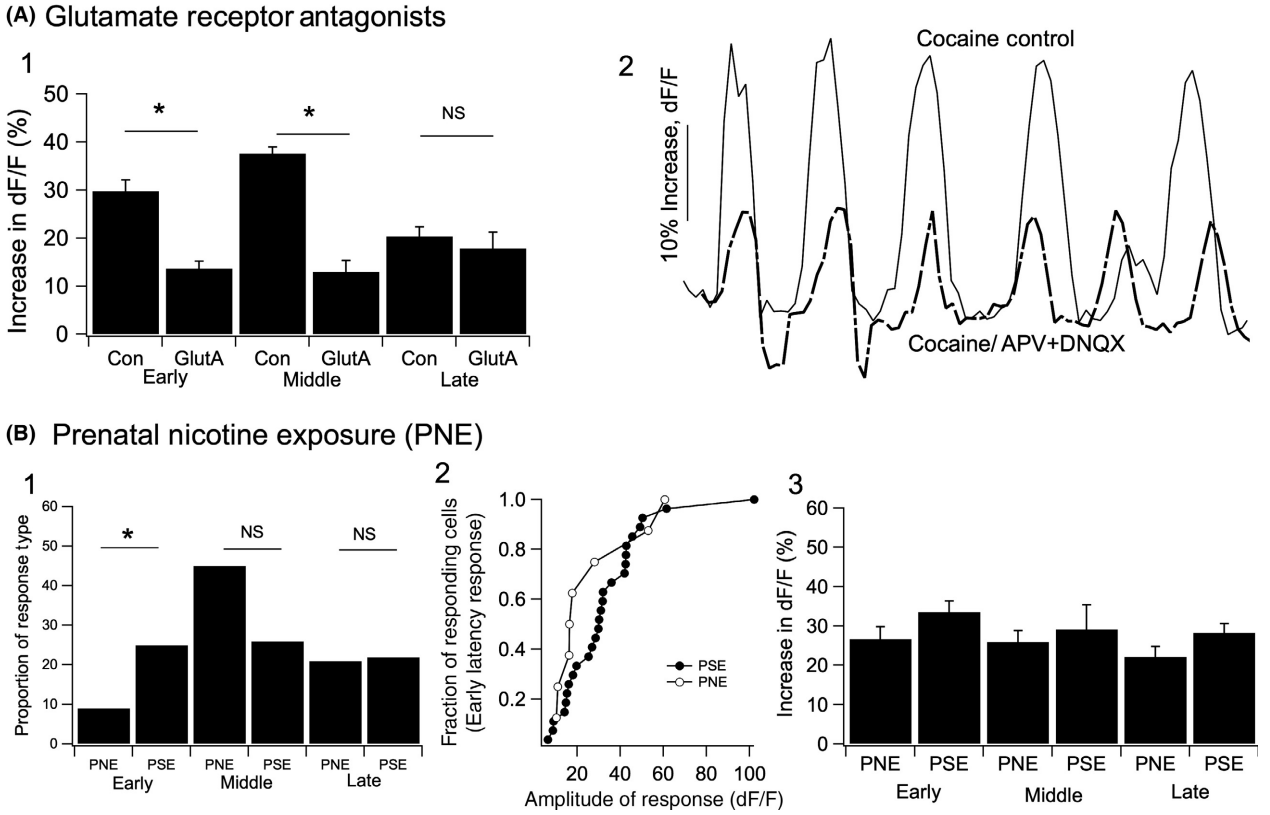

Figure 4. Antagonists of ionotropic glutamate receptors reduced the amplitude of cocaine-induced spiking, however, in a model of prenatal drug exposure shown to influence calcium arising subsequent to activation of glutamate mechanisms, the prenatal nicotine model (PNE), responses to cocaine were only subtly altered. (A1) Population data showing that the amplitude of the early and middle latency spiking responses were significantly attenuated when cocaine was applied in the presence of DNQX (10 μmol/L) and AP5 (50 μmol/L), antagonists of the AMPA and NMDA glutamate receptor, respectively, and noted as GlutA in histograms. However, the amplitude of the late spiking response was not significantly different when these receptor antagonists were present. (A2) Detail of one example of spiking behavior induced by cocaine in control conditions, and as shown in another cell, the typically much smaller amplitude change in dF/F induced by cocaine in the presence of the glutamate receptor blockers. (B) Cocaine-induced changes in dF/F were monitored in LDT slices taken from animals prenatally exposed to nicotine (PNE) and compared to responses induced in saccharine-exposed controls (PSE). There were no significant differences in the amplitude of the early, middle, and late spiking responses between PNE and PSE treatments groups (B1), which was confirmed using the K–S statistic as illustrated by the distribution of amplitudes of dF/F as shown here for the early latency response type (B2). However, the proportion of cells responding with the earliest latency spiking response was significantly lower in the LDT of PNE animals, than the number of cells exhibiting this spiking response type in the LDT of PSE animals. When taken together with our findings of cocaine responses during blockade of synaptic transmission, these data indicate that glutamate receptors, likely located in a presynaptic location are involved in the early and middle latency spiking behavior evoked by cocaine, whereas the late spiking response type is independent of this involvement. In addition, while differences in cocaine-elicited spiking behaviors between the PNE and control LDT cells were slight, our data support the conclusion that calcium resulting from glutamate mechanisms is altered by gestational exposure to nicotine. LDT, laterodorsal tegmental nucleus; PNE, prenatal nicotine exposure; PSE, prenatal saccharine exposure.

($P = 0.6555$, $n = 38$; Fig. 4A1). Therefore, we conclude that while they are not entirely dependent on glutamate receptors, the early and middle latency responses do involve their activation. However, these receptors do not appear to be involved in the longest latency response, which provides one more piece of evidence converging toward the conclusion that the mechanisms underlying the late latency response are different than those generating the early and middle latency response types.

Cocaine-induced early latency calcium spiking was only subtly altered in an animal model of prenatal drug exposure

Gestational exposure to drugs of abuse heightens the risk that later in life an individual will develop drug dependency (Navarro et al. 1988, 1989a,b; Slotkin et al. 1991, 2005, 2006, 2007, 2011; Abreu-Villaca et al. 2004a,b; Levin et al. 2006). This suggests that exposure in the

womb to drugs of abuse alters drug addiction-related neuronal groups or networks, biasing their activation toward continued pathological use of addictive substances upon re-exposure postnatally. Exposure-induced changes occurring during gestation could involve alterations within the LDT, leading to differential responses to drugs of abuse. Indeed, we have previously shown that the outcome of glutamate excitation is altered in an animal model of prenatal drug exposure (McNair and Kohlmeier 2015). As responses to cocaine appeared to involve glutamate transmission in naive animals, we examined whether cocaine elicits differential responses in LDT cells between gestationally drug-exposed mice, and control animals. Accordingly, calcium responses induced by cocaine were conducted in the LDT of mice exposed prenatally to nicotine (PNE) via maternal drinking water and these responses were compared to responses elicited by cocaine in LDT cells from control animals, which had received the drinking solution laced with the flavor enhancer, saccharine, minus nicotine (PSE).

Response profile proportions (%) of total responding cells in PNE (nPNE = 96) and PSE (nPSE = 111) groups were compared for potential differences between the two treatment groups. We found that early latency responses occurred at a significantly lower rate in PNE animals than in PSE controls among cells exhibiting a cocaine-induced change in dF/F (PNE, 9% n = 8/96 vs. PSE 25%, n = 27/111; P = 0.004; Fig. 4B1). The response rate of cells exhibiting middle latency spiking (Chi Square Test, P = 0.6094; nPNE = 30, nPSE = 23) and late spiking behavior (Chi Square Test, P = 1.0; nPNE = 17, nPSE = 20) was not significantly different between PNE and PSE animals (Fig. 4B1). As had been determined in recordings from the LDT in naive animals, effects of cocaine in eliciting spiking behaviors were not repeatable in cells from either PNE (nPNE = 21) or PSE (nPSE = 30) animals.

We then compared the average amplitude of the middle latency calcium spikes between the two treatment groups, but found no significant difference in the amplitude of this spiking response between the nicotine-exposed and control animals (PSE: 28.47 ± 6.06% dF/F, nPSE = 22; PNE: 25.9 ± 4.9% dF/F, nPNE = 27; Mann–Whitney Test, P = 0.5198; Fig. 4B2). Similarly, there was no difference between the amplitude of the late latency spiking profiles between the two groups of animals (PSE: 28.3 ± 2.4% dF/F, nPSE = 15; PNE: 22.2 ± 2.7% dF/F, nPNE = 15; Mann–Whitney Test, P = 0.0968; Fig. 4B2), suggesting that PNE has not altered the mechanisms underlying the medium or late latency cocaine-induced calcium spiking in cells of the LDT.

We then examined the amplitude of the spikes from cells exhibiting the early response and found that, while the proportion of cells responding with this profile was significantly reduced in the PNE animal, the amplitude of this response type in those PNE cells which responded with this kinetic was not significantly different in cells from the PSE animals (PSE: 33.6 ± 5.0% dF/F, nPSE = 20; PNE: 26.7 ± 6.9% dF/F, nPNE = 8, Mann–Whitney Test, P = 0.5142; K-S test, P = 0.2345; Fig. 4B2&3). When taken together, our data suggest that prenatal exposure to nicotine alters the response of LDT cells to cocaine under the experimental conditions utilized, however, the alteration only affects one component of this response and once initiated, the mechanisms underlying the extent of the rise in calcium are not altered by early-life nicotine exposure.

Discussion

We report for the first time that exogenously applied, acute applications of cocaine induced changes in fluorescence of Fura-2AM loaded LDT cells, which provides the first direct evidence that acute exposure to cocaine alters cellular activity in the LDT. Confirmation that cells recorded in this study were within the LDT was obtained by conducting bNOS immunohistochemistry on slices from which data sourced. We and others have shown that the presence of bNOS serves as a reliable marker for definition of the perimeter, and extent, of the LDT (Vincent et al. 1983, 1986; Hope and Vincent 1989; Hope et al. 1991; Veleanu et al. 2016). The rises in fluorescence of Fura-2AM, which serve as indirect measures of rises in calcium, exhibited two distinctly different kinetic profiles: (1) a spiking-like behavior characterized by a rapid rise and decay, and (2) a plateau-like behavior characterized by a gently rising slope, which reached a plateau and then gradually decayed. The plateau rises likely arose secondarily to calcium spiking and were probably reflective of residual calcium which escaped calcium buffering capacity and accumulated, as this profile did not occur in isolation from the spiking behavior. As it was the primary response type, our focus was on characterizing the mechanisms underlying the spiking behavior. For this study, the spiking behavior was divided into three different profiles based on latency of effect. The division was confirmed to be mechanistic as the kinetics displayed a differential sensitivity to pharmacological blockade. A subset of the spiking rises were sensitive to blockade of synaptic transmission (TTX and low extracellular calcium), however, all spiking involved intracellular calcium stores. All three spiking profiles were antagonized by DA_1 and DA_2-like receptor antagonists suggesting a role for DA activation of the DA receptor. Providing further evidence for a role of DA in spiking behavior, application of either DA or amphetamine, which also likely result in rises in

synaptic levels of DA, induced similar spiking and plateau behavior. Finally, activation of ionotropic glutamate receptors was involved in the early and middle spiking behavior, whereas activation of these receptors was not largely involved in generation of the spiking profile exhibiting the longest latency kinetic. Rises in calcium were subtly altered in the prenatally nicotine exposed animal (PNE), a mouse model of the heightened risk of addiction associated with gestational exposures to drugs of abuse in which we have demonstrated reductions in effects on rises in calcium following stimulation with AMPA receptor agonists.

Other studies show calcium rises by cocaine, DA, or amphetamine

This is not the first study to show that cocaine can induce rises in calcium in cells. And, similar to findings obtained in the LDT, in other studies, cocaine-induced rises can be of a long duration postdrug exposure. Cocaine applied in nmol/L and μmol/L concentrations induced rapid rises in calcium in cultured canine smooth muscle cells, which appeared kinetically quite similar to cocaine-induced spiking behavior in LDT cells (Zhang et al. 1996). Calcium rises in smooth muscle cells were suggested to involve intracellular release triggered by initial entry of calcium across the membrane (Zhang et al. 1996). Data acquired in smooth muscle cells are consistent with our LDT findings indicating that calcium arising from SERCA-pump mediated stores is integral to all three types of spiking behavior responses, but that flux across the membrane is also involved. In cultured smooth muscle cells, it was not possible to restore responses, even following 30 min of observation post first application, suggesting that effects of single exposures of cocaine linger even after the drug has been removed and metabolized (Zhang et al. 1996). In vivo imaging of the rat cortex revealed that acute application of cocaine induced long-lived, large amplitude rises in intracellular calcium which did not plateau until 40 min post application and persisted >60 min after the injection of cocaine (Du et al. 2006). As the plasma concentration of cocaine was nearly immeasurable after 1 h, persistence of the effect on calcium led to the suggestion that calcium rises, at least in part, were due to metabolites of cocaine. When taken together with other studies, our findings of a long duration of spiking behavior triggered by cocaine exposure raise the possibility that spiking behavior persisting in LDT cells for more than 40 min post application of cocaine could involve metabolites of cocaine, suggesting actions of cocaine in vivo might outlive presence of the parent compound. Screening for presence of these metabolites in our LDT slices, and the mechanisms they could activate, remains a topic of interest for future studies.

In our study, calcium rises induced in LDT cells by amphetamine exhibited similar kinetics to those induced by cocaine. As it was not the focus of the study, we did not explore the underlying mechanisms of the amphetamine-induced rises. However, other studies examining the effects of amphetamine on intracellular calcium have been conducted. Amphetamine was shown to induce rises in calcium in PC12 cells, which were dependent on intracellular calcium release and efflux of calcium across the membrane similar to the mechanisms leading to rises induced by cocaine in the same cells (Kantor et al. 2004). Further evidence that cocaine and amphetamine rises are dependent on the same intracellular calcium pools was provided by findings that rises in calcium in HEK cells were occluded by cocaine-induced rises (Gnegy et al. 2004). In HEK cells, IP_3-mediated intracellular stores of calcium were likely involved in amphetamine-induced rises in calcium, and were found to be involved in DA transporter-mediated efflux of DA into the synapse, likely involving a PKC-mediated phosphorylation of the DA transporter (Gnegy et al. 2004). Although these data are suggestive that within the LDT, amphetamine and cocaine rises shared some of the same IP_3-sensitive calcium-dependent mechanisms, future studies need to be conducted to identify the source of calcium induced by amphetamine in LDT cells, and determine whether the same calcium pool is targeted by cocaine.

Although we did not investigate the mechanisms underlying amphetamine-induced calcium rises, it is well-known that amphetamine results in substantially higher rises in synaptic DA than those induced by cocaine. While cocaine works by inhibition of the DA transporter, amphetamine promotes the nonexocytoctic release of DA, resulting in much higher synaptic levels of DA. Accordingly, as cocaine-induced rises in LDT cells were found to involve activation of DA receptors, we expected to see larger changes in dF/F stimulated by amphetamine than those induced by cocaine. Therefore, we were surprised to find that amphetamine induced smaller rises in dF/F than those induced by cocaine. Interestingly, these smaller rises were only noted when the early and middle response types were elicited. As these two kinetic responses were found to involve ionotropic glutamate receptors, it is tempting to consider the possibility that differential rises in calcium in LDT cells indicate that there are differences between these two stimulants to the extent by which they engage the glutamatergic system in the LDT. However, despite the fact we applied concentrations of both compounds used in other brain slice studies, the smaller calcium rises induced by amphetamine may simply be due

to noncomparable pharmacodynamic and kinetic profiles between the two drugs. This interpretation is supported by the finding that low concentrations of amphetamine which induce detectable calcium rises were found to require calcium flux across the membrane, but apparently at higher concentrations, rises could be detected which were independent of this flux (Siciliano et al. 2014). Further, biphasic responses to cocaine were also suggested as higher concentrations of this drug (10^{-5}) resulted in reduced calcium rises from those obtained with concentrations in the nanomolar range (Zhang et al. 1996). Accordingly, it is premature to make conclusions regarding the levels of calcium induced by one compound when compared to that activated by another. Therefore, comprehensive amphetamine and cocaine concentration curves need to be generated in the LDT to fully understand the profile of effects on cellular calcium that could be expected following amphetamine or cocaine exposure. Nevertheless, although it was beyond the scope of this study to construct such curves, our data do indicate that amphetamine, like cocaine, at concentrations used in many brain slice studies conducted in other brain regions, induces calcium rises in the LDT similar in kinetic profile to those induced by cocaine.

In our study, DA also led to rises in intracellular calcium in LDT cells. Further, DA receptor antagonists were effective in reducing the actions of cocaine. Using in vivo electrophysiology, DA failed to exert a detectable effect on the membrane excitability of LDT cells (Koyama and Sakai 2000), and to the best of our knowledge, there are no reports of DA inducing rises in intracellular calcium in the LDT. Therefore, our data showing that DA does have a cellular effect on LDT cells represents a novel finding. Further, while indirect, these data lead us the suggestion that DA transmission could occur in the LDT. Studies localizing the dopamine transporter have not been conducted with a focus on the LDT (Ciliax et al. 1995), nor has presence of mRNA for D_1or D_2 receptors been shown specifically within the LDT in rat studies (Weiner et al., 1991b). However, based on data using retrograde and anterograde axonal transport methods, it is likely that DA-containing afferents are directed to the LDT as projections from the DA cell-rich regions of the VTA, substantia nigra, and retrorubral fields were identified, but not immunohistochemically characterized (Cornwall et al. 1990; Semba and Fibiger 1992). Therefore, our data showing actions of DA and functional presence of DA receptors is relevant to considerations of effects of DA input on processes in which the LDT plays a role. While effects on calcium of DA have never been presented in the LDT, DA has been shown to elicit rises in intracellular calcium in other cell types following activation of DA receptors. In these other regions such as the striatum, DA-stimulated rises in calcium have been suggested to play a major role in DA-induced synaptic plasticity, and therefore, actions induced by DA in the LDT could be involved in alterations in synaptic strength in the LDT (Thoenen 1995). Although our data did suggest a role for the D_1 and D_2 receptor in the spiking responses to cocaine, future studies should be conducted to determine specifically which receptor subtypes are present in the LDT, which could include the non-D1 or D2 type. Further, it will be of interest to determine whether heterodimerization of DA receptors played a role in DA, and stimulant-induced calcium rises, as has been shown in other cell types (Calabresi et al. 1987; Lee et al. 2004; So et al. 2005; Rashid et al. 2007), and therefore, whether such a mechanism operates upon putative, endogenous DA input directed to the LDT.

In our study, glutamate receptors were found to play a role in two of the calcium spiking responses to cocaine. The finding of a role of glutamate in cocaine-induced rises was not surprising. In other cell types, DA-mediated rises in calcium have been shown to arise secondarily to DA enhancement of glutamate-activated currents via actions at D_1-like receptors (Smith et al. 1995), and acute exposure to cocaine and amphetamine have been shown to induce changes in synaptic strength via insertion of AMPA receptors into the membrane (Ungless et al. 2001; Faleiro et al. 2003, 2004; Saal et al. 2003). Either of these mechanisms could underlie the role of glutamate in the early and middle latency calcium spiking behavior induced by cocaine. Further, although it remains speculative, since the early and middle latency responses were sensitive to glutamate receptor blockers and inhibited by blockers of synaptic transmission (TTX and low extracellular calcium), as well as D_1 and D_2 receptor antagonists, when taken together, our findings could indicate a role in calcium rises of D_1/D_2 receptors located outside the terminal, and on inputs directed to imaged cells. Activation of these upstream inputs could result in an enhancement of glutamate actions, either pre- or postsynaptically. However, the lack of sensitivity of the calcium rise exhibiting the late kinetic to glutamate receptor antagonists or its lack of elimination by blockers of synaptic transmission, coupled with the sensitivity of this response to D_1 and D_2 receptor blockers and CPA, suggests that it is due almost entirely to postsynaptic actions involving D_1/D_2 receptors linked to release of intracellular calcium stores. Accordingly, it not only remains an interesting question which DA receptor subtypes are functionally present within the LDT, but also to determine their precise pre- or postsynaptic localization.

Acute cocaine-induced calcium rises in the LDT– not unexpected

Our findings of acute actions of cocaine on calcium in LDT neurons are novel. Although no studies have reported acute actions of cocaine on LDT cells, studies conducted in animals previously exposed to cocaine showed behavioral and cellular actions involving the LDT, which were indicative that acute actions must have been induced in this nucleus, and effects seen were suggestive that rises in calcium were involved in these actions (Geisler et al. 2008; Schmidt et al. 2009; Kurosawa et al. 2013; Kamii et al. 2015; Steidl et al. 2015). Glutamate release was enhanced within the LDT in the brain slices obtained from rats who had received an injection of cocaine 24 h earlier, which was an effect enhanced by 5 days of exposure to cocaine (Kurosawa et al. 2013). This cocaine-induced effect was shown to be due to alterations in presynaptic mechanisms in the LDT. However, postsynaptic actions of cocaine in the LDT were demonstrated as well since in animals exposed to 5 days of experimenter-delivered cocaine, upregulation of a persistent sodium current was noted in cholinergic cells of this nucleus, which resulted in heightening cellular excitability (Kamii et al. 2015). Enhancements in presynaptic release of glutamate following cocaine exposure in the LDT involved mechanisms known to involve calcium (Kamii et al. 2015), and involvement of calcium is supported by findings of cocaine-induced enhancements in presynaptic glutamate release noted in the hypothalamus, which likely involved intracellular rises in calcium (Yeoh et al. 2012). Similarly, increases in persistent sodium currents could have arisen following cocaine-induced calcium rises, as in other studies, enhancements of this current were believed to involve calcium-stimulated intracellular messengers (Soderling 1999; Lampert et al. 2006; Hargus et al. 2013; Liang et al. 2013; Kamii et al. 2015). Calcium spikes have been associated with membrane plasticity in other studies and therefore, it remains likely that changes in synaptic strength and membrane plasticity seen in the LDT in vitro following in vivo administration of cocaine involved cocaine-induced rises in calcium (Dolmetsch et al. 1998; Li et al. 1998).

Lastly, anatomical and histochemical studies also provide indirect support that rises in calcium in LDT cells must occur upon cocaine exposure. Following 6 days of self-administration of cocaine in rats, heavy labeling with markers of presence of cFOS was found in the LDT within cells identified with a retrograde tracer as sending projections to the VTA (Geisler et al. 2008). As induction of cFOS is a calcium-dependent process, these data provide further evidence that cocaine activates the LDT, and this activation involves rises in calcium. When taken together, a strong body of evidence presaged that cocaine has cellular actions on LDT cells, and the actions seen suggested that rises in calcium were involved. However, our data provide the first direct evidence that acute exposures of calcium do indeed lead to rises in calcium in LDT cells. Our data provide support to the interpretation that first time exposures not only have actions in the LDT, but also, that they could induce plasticity within the LDT, which would be expected to promote cellular and behaviorally relevant actions persisting beyond exposure to the drug. Future studies should be conducted to determine whether plasticity shown to occur in the VTA, and other brain regions, by acute cocaine exposure also occurs in the LDT (Ungless et al. 2001; Saal et al. 2003).

Caveats

Imaging intracellular calcium with cell-permeant indicator dyes allows bulk loading of cells, which confers the distinct advantage of allowing monitoring of changes in fluorescence from multiple cells, and of reducing the dialysis of intracellular contents which occurs with more invasive recording methodologies, such as patch clamping. The distinct drawback of this technique is the inability to identify single cells from which recordings were obtained. Astrocytes have been suggested to play a role in drug-induced behaviors, suggesting cellular actions of stimulants on glial cells (Miguel-Hidalgo 2009; Miguel-Hidalgo et al. 2010) and it remains a possibility that some recordings in this study sourced from astrocytes. However, we believe that the majority of our recordings sourced from neurons as we have shown that Fura-2AM is taken up by neurons in the LDT, as identified by elicitation of action potentials from Fura-2AM loaded cells (Kohlmeier et al. 2004). Our extensive experience with Fura-2AM loading in the LDT allows us to reliably select neurons based on anatomical features, such as clearly visible processes and size, as glial cells in the LDT smaller than 10 μm. Therefore, in this study, all data derived from cells larger than 10 μm, which represents the cholinergic, glutamatergic, and GABAergic population (Wang and Morales 2009). However, as size is an imperfect predictor of neuronal phenotype in the LDT (Boucetta and Jones 2009; Boucetta et al. 2014), we were not able to determine from which subpopulation of LDT cells responses to cocaine were elicited. Therefore, more invasive patch clamp recordings with concurrent calcium imaging will need to be conducted so as to confirm the phenotype of LDT cells responding and to further elucidate acute actions of cocaine on neurons in this nucleus.

The concentration of cocaine (5 μmol/L), amphetamine (10 μmol/L), and DA (30 μmol/L) used was chosen so that data could be compared to other studies, which have

used these drugs at these concentrations in brain slices. It would have been interesting to conduct dose–response studies, but in preliminary experiments, we found that repeat effects were not possible in the brain slices, precluding multiple applications of the same drug to the same slice. Further, conducting dose–response curves across multiple brain slices remains problematic as there is little control of final concentrations at the relevant effectors or a control for across slice variability in ability of the drug to reach its target. However, effects of these DA-acting compounds have been shown to be concentration-dependent in isolated, cell culture systems. Higher and lower concentrations of cocaine have been shown in some cell types to induce effects of opposite polarity (Zhang et al. 1996). In addition, in other studies, detection of calcium rises induced by low concentrations of amphetamine (2 μmol/L) was only possible in the presence of extracellular flux, whereas higher concentrations (100 μmol/L) were solely dependent on intracellular stores (Gnegy et al. 2004; Siciliano et al. 2014). Therefore, while the scope of this report was to examine whether these drugs had acute actions, which had been suggested by chronic actions, future studies should be designed which examine the potential for differential actions, with distinct underlying mechanisms, by using higher and lower concentrations.

In our study, we were unable to elicit second rises in calcium induced by cocaine or amphetamine following rises in calcium induced by first applications. This raises the concern that repeatability of cocaine actions may not have been possible due to calcium-triggered ischemia and subsequent, cell death. Consistent with this interpretation, the large calcium rises in cortical cells induced by cocaine levels similar to those used by cocaine abusers recreationally were found to be greater than those recorded during ischemic events which are associated with neuronal death (Du et al. 2006). However, in other studies, cocaine is not associated with cell damage following acute applications, suggesting that it is other processes occurring in ischemia, and not just calcium rises, which lead to apoptotic events. Further, our cells appeared to be healthy following imaging, and use of immunohistochemistry allowed us to confirm the presence of normal appearing bNOS-positive cells in the LDT, which on gross inspection appeared no different in profile, or numbers, than in cocaine-naive conditions. Therefore, we do not believe that lack of repeatability was due to cell death. Inability to elicit repeat responses was likely due to incomplete wash-out, receptor tachyphylaxis, exhaustion of mechanisms specifically activated by cocaine and amphetamine, or lingering effects of metabolites, rather than general reductions in cell health. However, as large rises in calcium can be associated with adverse outcomes, such as seizure, calcium-spiking behavior elicited in the LDT could be clinically relevant in adverse pathological events associated with cocaine toxicity.

Functional significance

Our data present for the first time, evidence that acute applications of cocaine, DA, and amphetamine result in cellular actions on LDT cells. The LDT sends glutamatergic, cholinergic, and GABAergic afferents to the VTA (Omelchenko and Sesack 2005, 2006; Wang and Morales 2009), and in fact, the LDT provides the major extrinsic source of cholinergic input to mesoaccumbal DA cells (Dautan et al. 2016a). A consensus of studies indicate that ACh and glutamate output from the LDT to the VTA participates in drug addiction-related behaviors via excitation of DA-containing VTA cells which send projections to nAc targets. Initial evidence of this was provided by in vivo chronoamperometry and electrophysiology-based studies (Forster and Blaha 2000; Forster et al. 2002a; Grace et al. 2007). Very recently, optogenetic techniques have provided further convincing evidence that LDT afferents exert cellular actions on DA VTA cells encoding reinforcement, and thereby exerting control over appetitive behaviors governed by the VTA-nAC circuitry (Lammel et al. 2012; Steidl and Veverka 2015; Dautan et al. 2016b; Xiao et al. 2016). When taken together, the functional and anatomical data suggest that output from the LDT participates in shifting firing of the DA VTA neurons from a tonic pattern to a phasic, burst firing profile, which results in the temporally meaningful large efflux of DA in the nAc which signals reinforcement upon use of drugs of abuse like cocaine and amphetamine, incentivizing or giving reward to, the eliciting stimulus (Grace et al. 2007). Cocaine and amphetamine exhibit high abuse potential, and evoke large rises in DA in the nAC via direct actions on DA-VTA cells. However, in addition to exerting direct actions on neurons in the mesoaccumbal circuitry, these stimulants could activate VTA cells indirectly via actions on VTA-projecting LDT cells. Indeed, supporting this conclusion, our data show that cocaine and amphetamine have actions on LDT cells that are consistent with a high degree of activation which would be likely to alter output to target regions, such as the VTA. Further, neurons of the LDT are involved in mechanisms underlying cortical arousal and sleep generation (Baghdoyan et al. 1984; Jones 1993; Steriade 1999a,b; Douglas et al. 2004) and the striking cellular actions of cocaine elicited in LDT cells is likely involved in the behaviors induced by this stimulant drug, including elicitation of heightened arousal, suppression of sleep, and development of drug dependency.

As prenatal exposure to nicotine confers a vulnerability to drug dependency during postgestational life, alterations in neurons involved in reinforcement of reward learning are likely induced by the early-life experience. Consistent with this interpretation, we have reported reductions in calcium rises induced by stimulation of AMPA receptors in the LDT (McNair and Kohlmeier 2015). We speculated that calcium responses to cocaine would be altered in the LDT of the PNE animal. Under the experimental conditions we used, we did not see a large difference in cocaine-induced calcium rises in this animal model, which does not preclude that there are other differences in responses to cocaine, which are not related to calcium, that were altered. Although we did not see a large difference in cocaine-induced calcium transients in the PNE animal, we did note a reduction in the frequency of occurrence of the early latency calcium response. Interestingly, this early latency response type was reduced in amplitude by presence of glutamate receptor blockers. Our data showing a reduction in cocaine-induced calcium in PNE animals are therefore consistent with the interpretation that ionotropic glutamate receptors are involved in the cocaine-induced rises in calcium, and their activation by cocaine or other stimulants, results in smaller calcium rises in PNE LDT cells. At this time, it is impossible to know whether reductions in cocaine-induced calcium transients in those prenatally exposed to drug of abuse could play a role in behavioral outcomes; however, our data provide further information supporting the conclusion that early-life exposure to nicotine influences calcium-involved, cellular processes within the LDT.

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Author Contribution

MØL and THI performed the studies and analyzed the data. MØL and KAK designed the experiments and wrote the MS.

Disclosure

The authors disclose that they have no conflict of interest with respect to this manuscript.

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