



The use of laser capture microdissection to identify specific pathways and mechanisms involved in impulsive choice in rats



Shirisha Meda^a, Nadja Freund^{a,e}, Kevin J. Norman^a, Britta S. Thompson^a, Kai-C. Sonntag^{b,c,d}, Susan L. Andersen^{a,c,d,*}

^a Laboratory of Developmental Neuropharmacology, McLean Hospital, Harvard Medical School, USA

^b Laboratory for Translational Research on Neurodegeneration, Program for Neuropsychiatric Research, McLean Hospital, Harvard Medical School, USA

^c Basic Neuroscience Division, McLean Hospital, Harvard Medical School, USA

^d Department of Psychiatry, McLean Hospital, Harvard Medical School, USA

^e Experimental and Molecular Psychiatry, Ruhr-University, Germany

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ABSTRACT

Background: Microinjections, lesions, viral-mediated gene transfer, or designer receptors exclusively activated by designer drugs (DREADDs) can identify brain signaling pathways and their pharmacology in research animals. Genetically modified animals are used for more precise assessment of neural circuits. However, only a few of the gene-based pathway modifications are available for use in outbred rat strains.

New method: Behaviorally characterized Sprague-Dawley rats undergo tract tracing through microinjection of fluorospheres, followed by laser capture microdissection (LCM) and qPCR for detecting mRNA of pathway-associated gene products. Correlations between mRNA expression and behavior identify specific involvement of pharmacologically relevant molecules within cells of interest. Here, we examined this methodology in an impulsive choice paradigm and targeted projections from the orbital and medial prefrontal cortex.

Results: In this proof of concept study, we demonstrate relationships between measures of impulsive choice with distinct neurotransmitter receptor expression in cell populations from four different signaling pathways.

Comparisons with existing methods: Combining behavior, tract tracing, LCM, and gene expression profiling provides more cellular selectivity than localized lesions and DREADDs, and greater pharmacological specificity than microinjections and viral-mediated gene transfer due to targeting identified neurons. Furthermore, the assessment of inter-individual pathways provides insight into the complex nature of underlying mechanisms involved in typical and atypical behavior.

Conclusions: The novel combination of behavior, tract tracing, LCM, and single gene or potential whole genome transcriptome analysis allows for a more targeted understanding of the interconnection of neural circuitry with behavior, and holds promise to identify more specific drug targets that are relevant to behavioral phenotypes.

1. Introduction

Researchers have an arsenal of techniques to characterize the inter-relationship between behavior, pharmacology, and neuroanatomy. Microinjections, excitotoxic lesions, optogenetics, and transgenic animals are frequently used to study neuronal pathways involved in behavior each with unique advantages and disadvantages. Microinjections of agonists, antagonists, combinations of the two, or cell-permeable peptides that can be used to manipulate intracellular mechanisms can alter the activity at a target receptor that is located regionally (e.g., on all pathways), but provide little information about multiple pharmacological

markers within an individual pathway. Disconnection approaches are also used (Churchwell et al., 2009), where microinjections on contralateral sides are used to activate or deactivate a region to determine the region's role in behavior; disconnection approaches are complicated and indirectly characterize the pathway. All of the microinjection approaches rely on the availability of specific ligands for study. Viral vectors may further our understanding of how specific neuronal cells/pathways are involved by regionally overexpressing or silencing genes using cell-specific promoters (Sonntag et al., 2014). Similar to microinjections and lesions, viral vectors also cannot distinguish among different projection pathways, and few cell type-specific promoters are available.

* Corresponding author.

E-mail address: sandersen@mclean.harvard.edu (S.L. Andersen).

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Optogenetics is ideal for determining functional connections. This approach, however, fails to inform about the pharmacology of the stimulated pathway, can be non-specific (see (Witten et al., 2011)), and requires ~6 weeks for functional expression in pathways, which makes it not feasible for developmental studies.

Here, we offer an alternative approach to these methodologies: laser-capture microdissection (LCM). In comparison to immunohistochemistry or *in situ* to identify a limited number of signaling mechanisms within a cell type or pathway, LCM can be used to simultaneously characterize transcriptional profiles across multiple cells or pathways (Baskin and Bastian, 2010; Mauney et al., 2018; Pietersen et al., 2011). These cellular changes are then related to specific behaviors. The combination of behavior, identification of pathway-specific targets, capture, and qPCR could prove to be a useful methodology to determine how different pathways mediate unique aspects of behavior. In the current paper, we apply this approach to the neurobiological basis of impulsivity—much of which has been characterized and thus allowing us to compare our findings to those obtained by other methods.

Impulsivity is a complex phenomenon that is comprised of motor or choice impulsivity (Dalley and Robbins, 2017; Fineberg et al., 2010) and can be evaluated in rodents. Delay discounting measures intolerance to delayed reward that occurs when a subject chooses a smaller reinforcer sooner rather than waiting for a larger reinforcer that is received later (Evenden and Ryan, 1996). Impulsive choice is investigated with multiple techniques. Each of these approaches reveals more information at the regional, circuit, and pharmacological levels as the field progresses. Early studies show that lesions of the nucleus accumbens (NAc) or the basolateral amygdala (BLA) increase delay discounting (Cardinal et al., 2001; Winstanley et al., 2004). Increased discounting is found after whole orbital frontal cortex (OFC) excitotoxic lesions (Kheramin et al., 2003; Mobini et al., 2002). Other studies implicate other receptors and brain regions in other types of impulsivity (e.g. Dellu-Hagedorn et al., 2018).

Cortical innervation of both the NAc and the BLA plays a critical role in delay discounting, with glutamatergic input into NAc believed to be a point of convergence (Fineberg et al., 2010; Simon et al., 2013; Winstanley et al., 2004). Pharmacologically, delay discounting is partly modulated by dopamine in the NAc (Saddoris et al., 2015), where decreased dopamine D2/D3 receptors are associated with more discounting (Dalley et al., 2007). Decreased D2 receptors in the NAc are observed following elevated D1 receptors on glutamatergic projections from the prelimbic prefrontal cortex (plPFC) implicating involvement of the plPFC→NAc pathway in impulsive behavior (Freund et al., 2016). Regional dissection of the plPFC shows a significant correlation between discounting and D1 receptor expression (Loos et al., 2009); similar relationships were found with D1 receptor overexpression by viral mediated transfer (Sonntag et al., 2014). Disconnection of regions (by the GABA agonist muscimol) in a second pathway, the plPFC→BLA, also increases discounting (Churchwell et al., 2009), although the pharmacological mechanism involved via this pathway is not known.

Different aspects of delay discounting are mediated by the lateral and medial subregions of the OFC (Abela and Chudasama, 2013; Mar et al., 2011; Moschak and Mitchell, 2014; Winstanley et al., 2004) in most, but not all, studies (Stopper et al., 2014). Besides dopamine, both serotonergic and noradrenergic systems are involved in delay discounting (Dalley et al., 2008). Whole brain depletion of 5-hydroxytryptamine (5-HT; serotonin) increases discounting (Mobini et al., 2000). When probed more specifically, the 5-HT_{1α} receptor agonist 8-OH-DPAT increases impulsivity when microinjected into the OFC (Yates et al., 2016). Noradrenergic receptors (Adra2a) are targeted for the treatment of impulsivity (Nishitomi et al., 2018). Microinjections of the noradrenergic Adra2a receptor agonist into the medial prefrontal cortex did not (Pardey et al., 2013), whereas microinjections into the hippocampus did decrease impulsivity (Abela and Chudasama, 2014).

Here, animals were first characterized for delay discounting (Fig. 1) and we used LCM to capture fluosphere-labeled tract-traced projection

neurons from the plPFC and the OFC as they project to the NAc and the BLA (Fig. 2). We then examined mRNA expression of dopaminergic (Freund et al., 2014; Kheramin et al., 2004; Winstanley et al., 2005a), noradrenergic (Abela and Chudasama, 2014), and serotonergic receptors (Yates et al., 2014) as they have all been implicated in delay discounting (Dalley et al., 2008). The expression of individual receptor mRNA within specific pathways were then correlated with the level of delay discounting to identify their involvement in impulsive behavior.

2. Results and discussion

The delay discounting curves for each of the groups for the two target regions (NAc and BLA) are found in Fig. 1B. The DDT₅₀ for each individual subject was computed based on the curve across all five delays for every animal (described in the statistical analysis section above). The average fit of the individual data that was used to calculate the DDT₅₀ was 0.93 ± 0.02 . Correlational analyses with bootstrap post-hoc adjustment were performed between DDT₅₀ values and qPCR values for all of the receptors and the results are listed in Table 1.

Additional parameters need to be considered for our approach. First, behavioral manipulations would have helped differentiate other aspects of the characterization. Specifically, Mar et al. (2011) suggest that these modifications include the assessment of reward, anticipatory responding, and even individual learning differences. Second, multiple correlations that were performed within a single subject can increase Type 2 error and require post-hoc corrections. Power analyses suggest that an $n = 9$ is the minimum recommended number of subjects with adjustments made for bootstrapping. Third, issues of regional specificity could still remain with LCM. While LCM is more specific than the abovementioned approaches, multiple populations may still exist within the captured tract-traced cells.

With these issues in mind, we show that our methodology has replicated and extended results from other pharmacological analyses. Graphical representation of our analyses for Adra2a and D1 from OFC and plPFC are presented in Fig. 3. Correlational relationships between the DDT₅₀ and Adra2a or D1 receptors in the OFC→BLA were not significant after bootstrap correction for multiple comparisons (Fig. 3A). The ratio of Adra2a/D1 receptors, likely reflecting the co-localization of Adra2a and D1 (Arnsten, 2009), correlated with the DDT₅₀ ($r = 0.88$, $p = 0.008$). A higher ratio of Adra2a/D1 within the OFC→BLA pathway is related to less impulsivity, which is consistent with drug effects of guanfacine or clonidine (Abela and Chudasama, 2014). The specificity of this observation adds further support for the use of LCM to identify potentially complex relationships within multiple signaling mechanisms.

There were no significant correlations observed in the OFC→NAc between our selected markers and DDT₅₀. Less dopamine D1 mRNA in the plPFC→NAc pathways was associated with more discounting ($r = 0.7$, $p = 0.02$; Fig. 3B). We note that the correlation of D1 in the plPFC→BLA pathway was $r = 0.44$, however, removal of an outlier (square in Fig. 3B) that was two standard deviations from the mean of D1 mRNA values changes the correlation to $r = 0.82$, $p = 0.002$. Fig. 3B shows the fit when the “outlier” subject is not included. The presence of outliers can weaken discovery of a relationship between the molecule and behavior. Fig. 4 shows all of the data points for two different pathways for the sake of transparency, but also to demonstrate the strong reliance on the use correlations to characterize receptor involvement. In Fig. 4A, the elevated point in Fig. 3 is removed and the correlation weakens immensely. Fig. 4B shows the addition of the point removed (the square) and how it weakens the relationship, but the effect is far less dramatic. The identification of an outlier can be detected by correlational analysis and can subsequently be used for further study of aberrant populations. Such identification is not possible with microinjection or virus studies due to other factors (e.g., missed injection; too little volume).

The advantage of measuring mRNA relationships to behavior is the specificity of the mRNA when selective ligands are not available (e.g., for D4 or D5 receptors). The D4 receptor correlated with impulsive choice in

pIPFC→NAc projections ($r = 0.64, p = 0.046$). The D4 receptor has been associated with novelty-seeking (Ebstein et al., 1996), working memory impairment (Zhang et al., 2004), and risk for substance abuse (Ebstein and Belmaker, 1997). Activation of the D4 receptor inhibits pyramidal cell activity by GABA activity (Rubinstein et al., 2001; Zhong and Yan, 2014). Finally, within pIPFC→BLA projections, the dopamine D5 receptor mRNA correlated strongly with the DDT_{50} , where less D5 mRNA was associated with greater discounting. Loos et al. (2009) found that the D1

antagonist SCH-23390 injected into the pIPFC increased discounting behavior, which in turn correlated with tissue levels of D5 receptor. Dopamine D1 receptor within the pIPFC→NAc pathway also correlated with discounting ($r = 0.7, p = 0.02$).

The 5-HT1a receptor mRNA from fluosphere identified neurons can provide additional information about its role in impulsivity by localizing its function. Early studies show that systemically administered 8-OH-DPAT, a 5-HT1a receptor agonist, increases impulsive choice

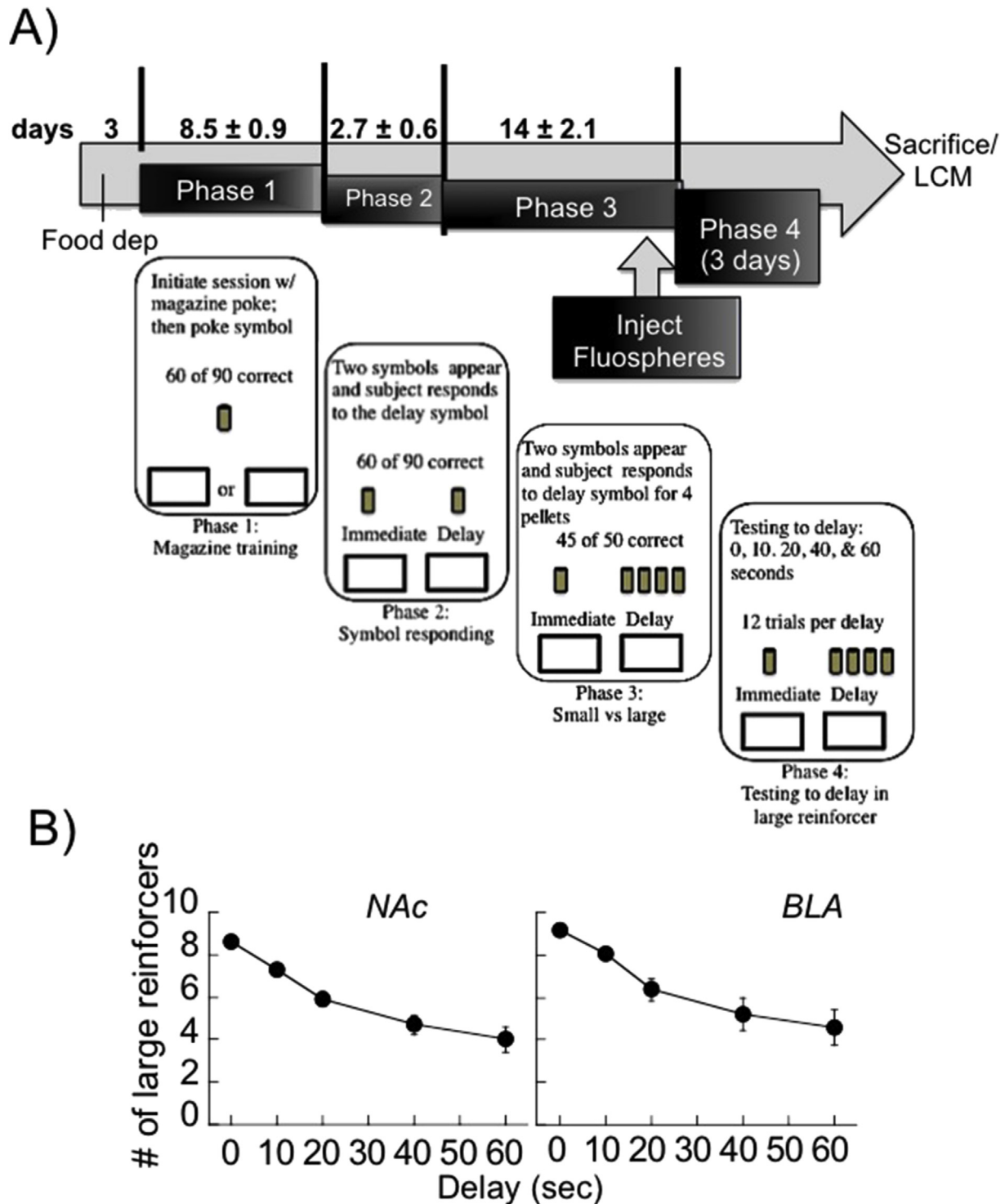


Fig. 1. Workflow of the methodology used and the timeframe. A) A timeline for training and testing of subjects for the delay discounting procedure using a touch screen. B) Group differences for the two cohorts of subjects that received tracer in the nucleus accumbens (NAc) or the basolateral amygdala (BLA) in the delay discounting paradigm. Means ±SE for n = 10 and 12, respectively.

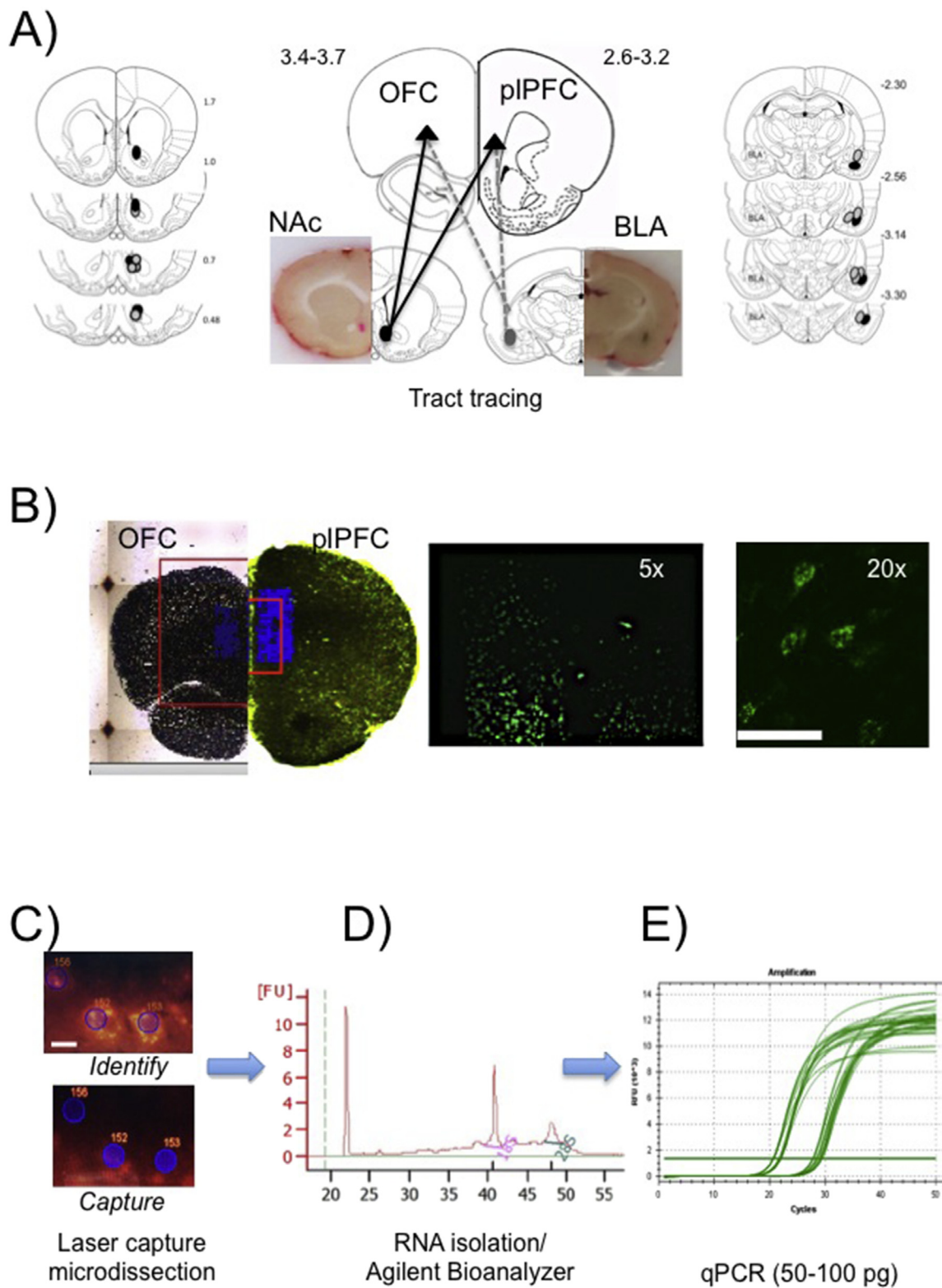


Fig. 2. A) Upon completion of the delay discounting procedures, two separate groups of subjects received a retrograde tracer into either the nucleus accumbens (NAc) or the basolateral amygdala (BLA) to label glutamate neurons in the prelimbic prefrontal cortex (pIPFC) and the medial orbital frontal cortex (OFC) and were sacrificed five days later. Localization of tracer within the nucleus accumbens (NAc; left) and the basolateral amygdala (BLA; right) are shown with bregma coordinates where tracer was found. The different shades were used to highlight the different and often overlapping sites. B) Identification of regions for capture in 8µm sections; representative labeling of tracer found in pyramidal cells in the pIPFC at 5x and at 20x (right). White bar is 50µm. C) Fluoresphere-labeled projections that were traced (blue circle) and captured are shown here at 40x; the white bar is a 20µm scale. D) Quality verification of RNA integrity with an Agilent Bioanalyzer before; E) determination of mRNA with qPCR.

Table 1
Correlations between receptor mRNA and impulsivity score@.

	OFC				PFC			
	BLA	p value	Nac	p value	BLA	p value	Nac	p value
Adra2a	-0.48	0.20	0.50	0.15	0.38	0.25	0.44	0.20
D1	-0.63	0.07	-0.32	0.37	0.46/.82+	0.16	0.70*	0.02
D2	-0.64	0.07	0.22	0.54	-0.56	0.07	0.07	0.84
D3	-0.49	0.18	0.00	1.00	-0.33	0.32	-0.47	0.17
D4	&		&		&		0.64*	0.05
D5 \$	-0.10	0.81	-0.03	0.94	0.81*	0.00	0.58	0.08
5-HT1a	0.68*	0.05	-0.34	0.33	0.80*	0.00	0.24	0.50
N	9		10		11		10	

@ impulsivity scores is the DDT50: negative relationship is high receptor, high impulsivity.

+ significant when the outlier is dropped from the analysis.

*Significance is set at $p < 0.05$.

Significance is adjusted following Bootstrap analysis of 1,000 replications.

& Ct Scores were greater than 35, and thus considered unreliable.

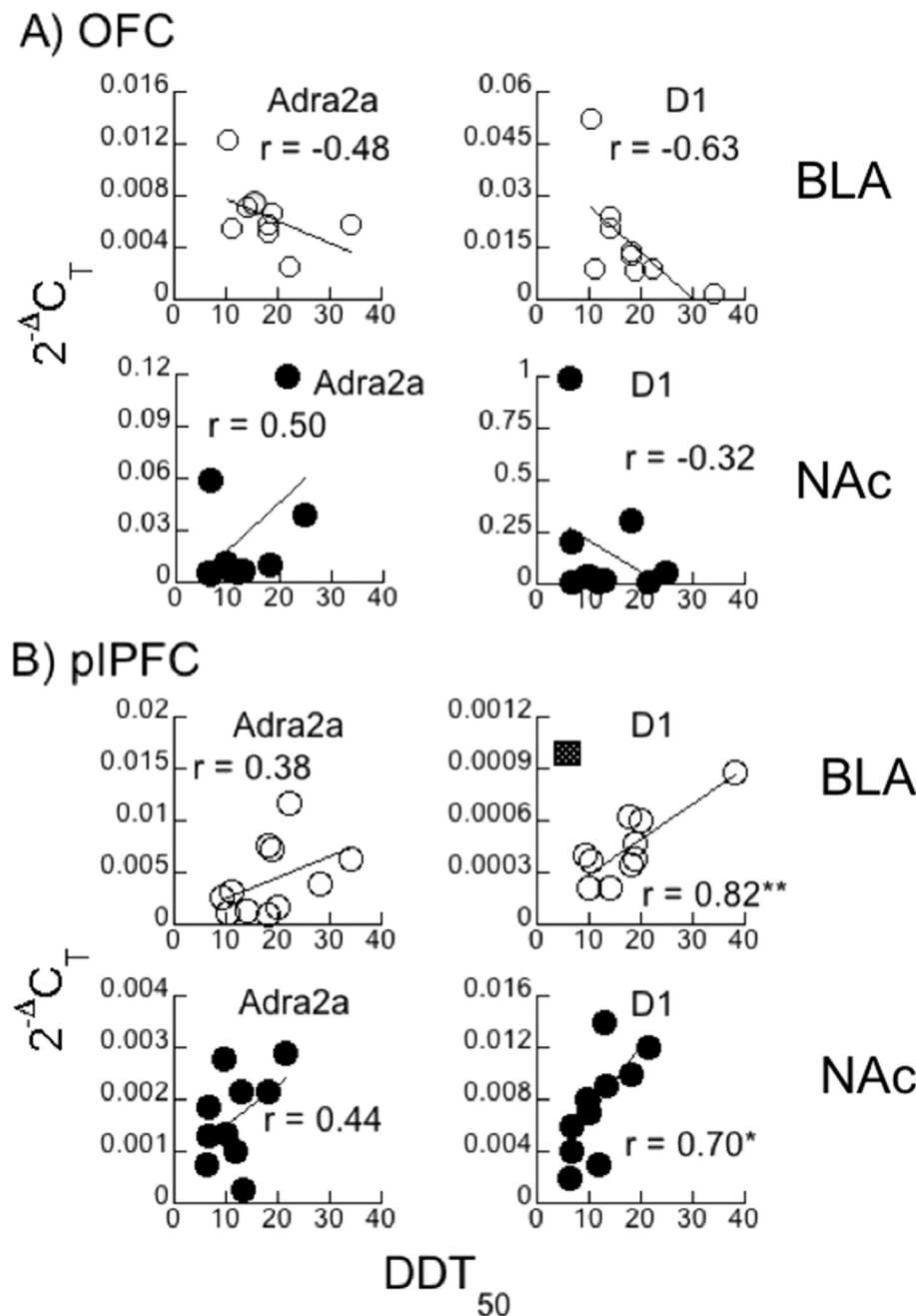


Fig. 3. Correlation analyses of qPCR values for Adra2a and D1 receptors in the A) orbital frontal cortex (OFC) and the B) prelimbic PFC (pIPFC) as a function of each subject's impulsivity score. The delay discounting 50 (DDT₅₀) refers to the delay period when the choice between large and small reinforcers is 50% (Lukkes et al., 2016). Lower DDT₅₀ values reflect greater impulsivity. Four different pathways are represented: innervation from the OFC→basolateral amygdala (BLA; OFC→BLA; open circles) or the nucleus accumbens core: OFC→Nac (closed circles); innervation from the pIPFC→BLA (open circles) or pIPFC→Nac (closed circles). Two data points were not included in the analyses for the BLA→OFC due to insufficient mRNA; an outlier is shown as a stippled circle for D1 in the pIPFC→BLA based on whether it significantly impacted the fit of the line. Pearson's correlation coefficient and significance, following bootstrap correction * $p < 0.05$ are presented.

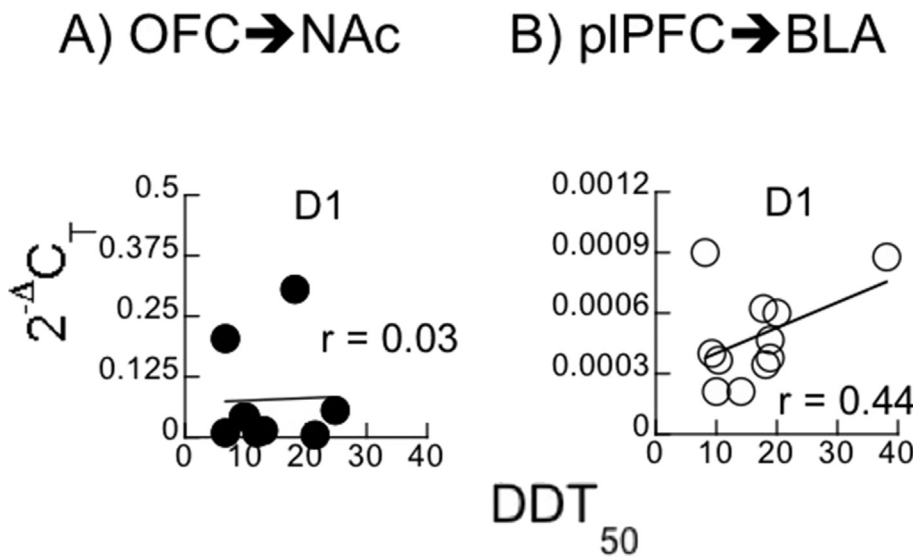


Fig. 4. Correlation analyses of qPCR values for D1 receptors in the projection of the A) orbital frontal cortex (OFC) to the Nucleus accumbens core (OFC→NAc) and B) prelimbic PFC (pIPFC) projections to the basolateral amygdala (pIPFC→BLA) as a function of each subject's impulsivity score. The delay discounting 50 (DDT₅₀) refers to the delay period when the choice between large and small reinforcers is 50% (Lukkes et al., 2016). Lower DDT₅₀ values reflect greater impulsivity. A) One data point is not included in the analysis for the OFC→NAc pathway, and its removal significantly weakens the relationship. B) One data point that was previously removed in Fig. 2 (the square) is now included in the analysis for the pIPFC→BLA pathway. Pearson's correlation coefficient is presented; neither correlation was significant.

(Winstanley et al., 2005b). Next, microinjected 8-OH-DPAT into other regions of the (medial/lateral) OFC, but not the pIPFC, increased discounting (Yates et al., 2014). In the current study, we show that mRNA levels of 5-HT1a receptors significantly correlated with impulsivity (the DDT₅₀) in both projection systems to the BLA (Fig. 5). Low levels of 5-HT1a receptor mRNA in both the OFC→BLA and the pIPFC→BLA pathways are positively associated with more impulsivity. The 5-HT1a receptor functions both as an autoreceptor and heteroreceptor, however, by sampling from mOFC pyramidal cells (and those in the pIPFC) it is

more likely that the measured 5-HT1a receptor mRNA may identify a heteroreceptor (Altieri et al., 2013).

One potential issue raised by this analysis is that LCM captures some terminals in addition to the targeted cell bodies. Adjusting the margin of surrounding the target can increase the specificity of the capture. A second approach is to capture multiple cell types to better characterize the surrounding milieu. LCM is capable of such resolution. Similarly, there is the issue of receptor co-localization, for which two possibilities exist. First, not all of the neurons identified by retrograde tracers or

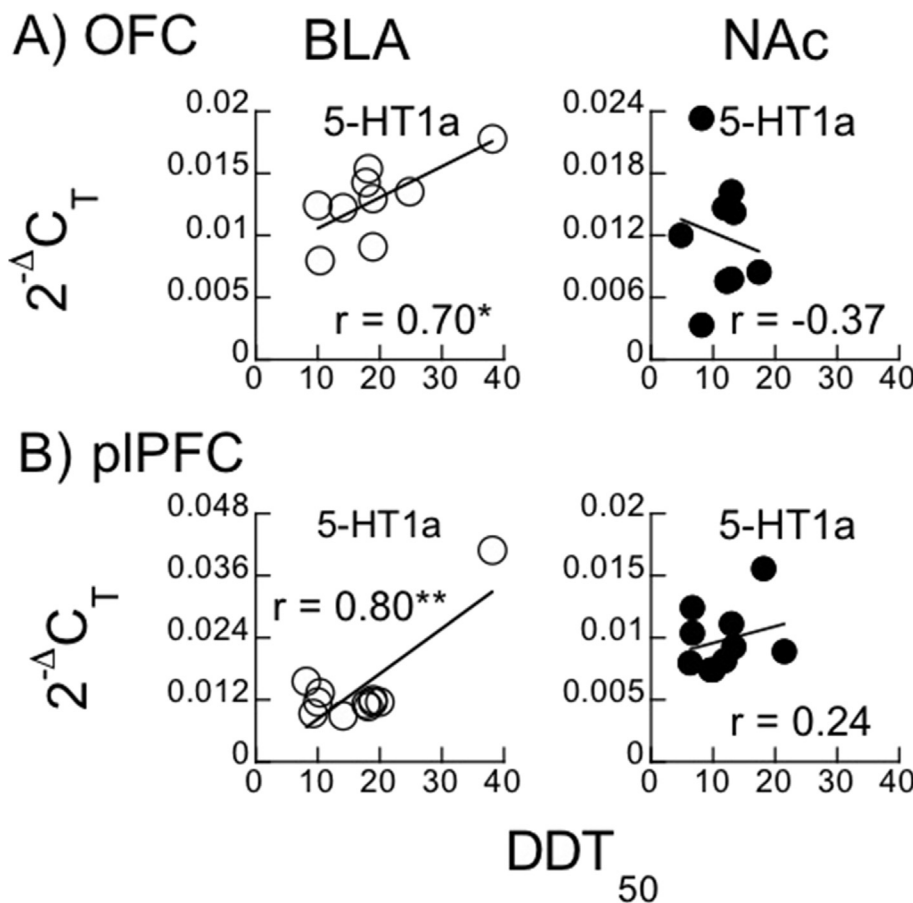


Fig. 5. Correlation analyses of qPCR values for 5-HT1a receptors in the orbital frontal cortex (OFC; A) and the prelimbic PFC (pIPFC; B) as a function of each subject's impulsivity score. The delay discounting 50 (DDT₅₀) refers to the delay period when the choice between large and small reinforcers is 50% (Lukkes et al., 2016). Four different pathways are represented: innervation from the OFC→basolateral amygdala (BLA; OFC→BLA; open circles) or the nucleus accumbens core: OFC→NAc (closed circles); innervation from the pIPFC→BLA (open circles) or pIPFC→NAc (closed circles). Pearson's correlation coefficient and significance, following bootstrap correction *p < 0.05 is presented.

immunohistochemistry are homogenous. Different projections may subtly innervate small groups of cells within a region. Second, co-localization of receptors can be found within a projection group. For example, a significant positive correlation in OFC→BLA projections was observed between Adra2a: D1 receptors and DDT₅₀, suggesting that either increases in Adra2a mRNA or decreases in D1 mRNA are related to an increase in the DDT₅₀. The positive relationship of Adra2a: D1 receptors with less delay discounting in the OFC makes sense when taking the effects of noradrenergic agonists into account. A higher ratio of Adra2a: D1 receptors within the OFC→BLA pathway is related to less impulsivity, which is still consistent with drug effects. This observation adds further support for the use of LCM to identify potentially complex relationships amongst multiple signaling mechanisms. Additional analysis with either RNAScope or immunohistochemistry should be used to confirm co-localization.

3. Conclusions

The combined utility of behavior, tract tracing, LCM, and gene expression profiling may best be applied to determining the underlying differences in neuronal signaling between groups of behaviorally characterized animals. More importantly, this new methodology coupled with data mining approaches could identify novel mRNAs, miRNAs, or other molecules using more advanced whole genome transcriptome analyses approaches. The ability to determine whether the same receptor has different influences on behavior depending on its location within a specific pathway also offers novel insight. Upon closer examination of the graphs, different relationships (e.g., high/low expression) or clustering of mRNA may be detected within the same pathway. Different clustering may suggest that the targets need further refinement and in turn, can identify a unique population of pathways within the region. Finally, the use of LCM with transcriptome analyses increases specificity, as mRNA is more precise than a number of available antibodies or drugs. Together, LCM coupled with immunohistochemical analysis or tracing, qPCR, and behavior provides a novel approach for behaviorally relevant pathway analysis.

4. Materials and methods

4.1. Subjects

Male Sprague-Dawley rats ($n = 22$ in total) that were 90 days of age (350–375 g at the start of the experiment) were obtained from Charles River Laboratories (Boston, MA) and group-housed with three subjects/cage. Subjects were maintained on a 12:12 h. Light/dark cycle (lights on at 0700) with experimental sessions conducted between 0700 and 1200 h. Food and water were available *ad libitum* upon arrival, and subjects were food restricted (90% of their free-feeding weight) four days later when behavioral studies began. Following the completion of the behavioral study and track tracing, animals were rapidly sacrificed by decapitation, their brains immediately frozen, and mounted in TissueTek. All experimental procedures were approved by the IACUC at McLean Hospital and were consistent with the Principles of Laboratory Animal Care (NIH Publication no. 85-23).

4.2. Behavioral apparatus

Trapezoidal-shaped operant conditioning chambers (Lafayette Instruments, Lafayette, IN) with a Bussey-Saksida Touch Screen (Indianapolis, IN) for responding displayed two symbols within a sound attenuating box with white noise. Training and experimental responses were detected by nose poke contact within the presented symbol on the touch screen and reinforced responses were recorded by breaking a photo beam that was located in the magazine. Custom programs were written in MatLab (Mathworks; Natick, MA) (Rose et al., 2008).

4.3. Delay discounting task

Subjects were food deprived to a target goal of 90% of free-feeding weight, consistent with other studies (Winstanley et al., 2004; Zeeb et al., 2010). The original paradigm was based on Evenden and Ryan (1996) and modified by (Lukkes et al., 2016) and is shown in Fig. 1A. Subjects ($n = 10$ –12/group) began training to measure increases in impulsive choice with a delay discounting task shown in the schematic in Fig. 1. Subjects were first trained to initiate each session by poking their nose in the food hopper. This poke produced a square symbol on the right or left of the touch screen (whose position was counterbalanced across subjects) and the presentation of one food pellet (Grain-Based Dustless Precision Pellets; 45 mg, Bio-Serv, Frenchtown, NJ); this sequence constituted a single trial. Phase 1 lasted 8.5 ± 0.9 days. Once the animal reached a criterion of 60 rewards in 90 trials, the rat advanced to Phase 2, which required 2.7 ± 0.6 days on average to progress to Phase 3. Here, they learned which symbol was associated with the larger reward and needed to correctly respond to the larger reward 60 out of 90 trials. Phase 3 taught the subject to differentiate between immediate and delayed reinforcers and took the longest (14 ± 2.1 days). Two symbols appeared: one signaling immediate reward and the other signaling the delayed reward. The side of presentation of the immediate or delay symbol was counterbalanced across subjects, but stayed constant for each individual subject. To assure sufficient learning of what the symbols represented (immediate vs. delayed reinforcer presentation), the criterion was set to responding 45 times out of 50 for the delay square for 2 consecutive days. The final phase of testing (Phase 4) was initiated when the rat poked his nose in the central food well and then both symbols were presented. Selection of the delay square resulted in the delivery of four pellets after a delay of 0, 10, 20, 40, or 60 s presented in ascending order. A total of five blocks of 12 trials were conducted within each daily session that lasted 100 min. At the beginning of each block, the time of the delay was introduced in two forced choice trials (with only the delay square presented at the beginning of each block of 12 trials) to signal a change in delay condition. After the subject poked the screen and received the pellet (small = 1 pellet or large = 4 pellets), the magazine lights turned off, the symbol was removed from the screen, and a 100 s delay before the symbols reappeared on the screen to initiate the next trial. Subjects were tested for three consecutive days on Phase 4. The average number of large reinforcers from the 10 trials at each delay (data were not used from the two forced trials) was averaged across these three days and used for data analysis following the methods of (Mar and Robbins, 2007). We have found that this multi-phased training session resulted in stable learning and performance within 10 days as measured by the average number of large reinforcements received at each across the three days (Fig. 1B).

4.4. Pathway labeling with fluorescent microspheres

Two different cohorts of subjects were used for the retrograde tracing studies: $n = 10$ for NAc and $n = 11$ for BLA, although one subject could not be used due to poor placement of the tracer. Rats were anesthetized with ketamine/xylazine (80/12 mg/kg, i.p.) and a gas-tight Hamilton syringe was used to inject 1 μ l of 565 λ fluospheres (Molecular Probes; Life Technologies) into each of the regions. Specifically, retrograde tracer was stereotaxically injected into either the NAc (AP: 1.6, ML: 1.2, DV: -6.8) or the BLA (AP: -2.8, ML: 4.8 1.2; DV: -8.5) to selectively label projections from the pPFC (Brenhouse et al., 2008) and the OFC (~AP: 3.8; Fig. 1B). Stereotaxic coordinates are based on (Paxinos and Watson, 1986). Fluospheres are not taken up by fibers of passage so only direct projections are stained (Katz et al., 1984). Fluosphere bolus spread and intensity was measured to verify consistency in the amount of tracer in all animals and was $\pm 5\%$ of the mean, as before (Brenhouse et al., 2008). Only subjects that had localized injections of the tracer were used (placement is shown in Fig. 2A).

Table 2
Primers used for mRNA assay.

Receptor	Forward Primers	Reverse Primers
Adra1a	5'- GCG AAT CCA GTG TCT TCG CAG -3'	5'- ACC ATG TCT CTG TGC TGT CCC -3'
Adra2a	5'- CTG TTC ACC GTG TTT GGC AAC -3'	5'- AAA GGG AAT GAC CAG CGT GG -3'
D1	5'- AGA TGA CCC CCA AAG CAG -3'	5'- ACG TCC TGC TCA ACC TTG -3'
D2	5'- CAG ACC ATG CCC AAT GGC -3'	5'- CAC ACC GAG AAC AAT GGC -3'
D3	5'- AAG CGC TAC TAC AGC ATC TGC -3'	5'- GGA TAA CCT GCC GTT GCT GAG -3'
D4	5'- CCT GAT GTG TTG GGA CGC CTT TC -3'	5'- TGG TGT AGA TGA TGG GGT TGA GGG -3'
D5	5'- AAA GAC TGG CTT CCC TTG TGT -3'	5'- CTG ATG TTT ACC GTC TGC ACT -3'

4.5. Laser capture microdissection

A total of $n = 21$ subjects were behaviorally characterized for delay discounting originally, although due to poor placement of the tracer in the NAc one subject was excluded for LCM. The approach is visualized in Fig. 1C-E and followed previously published procedures (Mauney et al., 2018 #17358). Briefly, 10 μm sections were cryostat sectioned at -18°C . Tissue sections were acetone fixed and dehydrated in a series of ethanol dilutions and CitriSolv. An average of 1400 ± 71 cells were captured from the pPFC and 1500 ± 50 cells were captured from the OFC; cells were obtained from two or three sections per subject. Traced cells were captured onto a CapSure LCM Cap (Arcturus, San Diego, CA) followed by incubation at 42°C for 30 min in 50 μl picopure RNA extraction buffer. Total RNA was isolated using a PicoPure RNA isolation kit (Arcturus, San Diego, CA) and RNA quality was determined with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to others (Boone et al., 2015; Torres Mena et al., 2014; Wang et al., 2010) and our own assessment criteria where the RNA integrity number was 7 or higher (Sonntag et al., 2016). Two samples from the BLA projections to the OFC were excluded due to poor yield. Next, 1 ng RNA was reverse transcribed to cDNA using Superscript® IV First-Strand Synthesis System for RT-PCR Kit (Life Technologies, Beverly, MA) by random hexamers, or cDNA was synthesized from 250 pgs RNA using the SMARTer Ultra Low Input RNA Kit - v3 to yield more product (Clontech, Mountain View, CA). Fifty to one hundred pgs cDNA per reaction was then PCR amplified using the iTaq SYBR Green Supermix (BioRad, Hercules, CA) and 50 nM gene-specific primers (Table 2), according to our published protocols (Lukkes et al., 2016). Replicates whose C_T value was more than one away from the average were excluded; the average raw C_T values for the receptors were normalized to the housekeeping gene of GAPDH and quantified based on the $2^{-\Delta C_T}$ method by Livak-Schmittgarden method (Livak and Schmittgen, 2001). Any sample with a C_T value >35 were considered to be a negative call and were excluded from analysis (Boone et al., 2015).

4.6. Statistical analyses

The delay when 50% of the large reinforcers (referred to as DDT_{50} for delay discounting at 50% of the large rewards received) was then determined from the best fit of the line between delay and the number of large reinforcers received for each individual subject. This line was often non-linear in nature and we required that R^2 was greater than 0.8 to assure goodness of fit (Lukkes et al., 2016). The calculated fit of the line to estimate the DDT_{50} using a non-linear curve fitting approach is more appropriate than estimating the delay at which 50% of large reward responding occurred based on a linear relationship (Doremus-Fitzwater et al., 2012). This approach also takes into account the range of reinforcers consumed. Data were analyzed for significant correlations between individual behavior (DDT_{50}) and receptor mRNA expression within the four identified pathways. Statistical significance was determined with a Pearson's correlation, using the two-tailed test (SPSS, v. 20) and adjusted for a bootstrap post-hoc analysis of 1,000 replications. Significant differences were accepted at $p < 0.05$.

Declarations

Author contribution statement

Shirisha Meda, Nadja Freund, Kevin J. Norman, Britta S. Thompson: Performed the experiments.

Kai-C. Sonntag: Designed and performed experiments; Contributed reagents, materials, analysis tools or data; Interpreted data; Wrote the paper.

Susan L. Andersen: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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