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# Enhanced inhibitory control by neuropeptide Y Y5 receptor blockade in rats

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**Conflict of interest disclosure**

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**Abstract**

*Rationale*: The neuropeptide Y (NPY) system acts in synergy with the classic neurotransmitters to regulate a large variety of functions including autonomic, affective and cognitive processes. Research on the effects of NPY in the central nervous system has focused on food intake control and affective processes, but growing evidence of NPY involvement in attention deficit/hyperactivity disorder (ADHD) and other psychiatric conditions motivated the present study. *Objectives*: We tested the effects of the novel and highly selective NPY Y5 receptor antagonist Lu AE00654 on impulsivity and the underlying cortico-striatal circuitry in rats to further explore the possible involvement of the NPY system in pathologies characterized by inattention and impulsive behavior. *Results*: A low dose of Lu AE00654 (0.03 mg/kg) selectively facilitated response inhibition as measured by the stop signal task, whereas no effects were found at higher doses (0.3 and 3 mg/kg). Systemic administration of Lu AE00654 also enhanced the inhibitory influence of the dorsal frontal cortex on neurons in the caudate-putamen, this fronto-striatal circuitry being implicated in the executive control of behavior. Finally, by locally injecting a Y5 agonist, we observed reciprocal activation between dorsal frontal cortex and caudate-putamen neurons. Importantly, the effects of the Y5 agonist were attenuated by pretreatment with Lu AE00654, confirming the presence of Y5 binding sites modulating functional interactions within frontal-subcortical circuits. *Conclusions*: These results suggest that the NPY system modulates inhibitory neurotransmission in brain areas important for impulse control, and may be relevant for the treatment of pathologies such as ADHD and drug abuse.

**Keywords:** Neuropeptide Y; Y5 receptor antagonist; stop signal task; response inhibition; short term depression

## Introduction

Neuropeptide Y (NPY) is one of the most abundant peptides found in the mammalian nervous system (Tatemoto et al. 1982). NPY is involved in many important physiological functions ranging from the modulation of food intake and stress sensitivity to the regulation of the cardiovascular system, circadian rhythms and energy homeostasis (Bi et al. 2012; Dumont et al. 1992; Zhang et al. 2011). The abundance of NPY in cerebral cortex, hippocampus and hypothalamus is consistent with the important role of NPY at the interface between cognitive, emotional and autonomic functions (Grove et al. 2000). NPY is also found in large quantities in several brainstem nuclei where it is co-localized in catecholaminergic neurons, particularly noradrenergic projection neurons (Aoki and Pickel 1990; Everitt et al. 1984; Grove et al. 2000; Hendry et al. 1984). In the cortex and striatum, two important nodes of the circuitry involved in response inhibition, NPY is expressed in aspiny GABAergic interneurons which often co-release nitric oxide and somatostatin (Kawaguchi, 1993; 1995).

There is evidence of NPY system dysregulation in several psychiatric disorders such as depression, schizophrenia, attention-deficit/hyperactivity disorder (ADHD) and post-traumatic stress disorder (Allen et al. 1986; Eaton et al. 2007; Redrobe et al. 2002; Scassellati et al. 2012). Moreover, decreased NPY immunoreactivity has been found in the brain of Alzheimer patients (Martel et al. 1990) and aged rats especially in the hippocampus, brainstem and several cortical regions (Cha et al. 1997; Fuxe et al. 1990; Huh et al. 1998). Preclinical studies have demonstrated NPY involvement in memory (Redrobe et al. 1999) and arousal (Ehlers et al. 1999; Fuxe et al. 1990), but also in attention, impulse control (Greco and Carli 2006) and the behavioral response to drugs of abuse (van den Pol 2012). However, apart from a few studies, NPY-mediated effects on higher cognitive functions have received little attention.

The stop-signal reaction time (SSRT) is a measure of the speed of the inhibitory processes derived from the stop-signal task (Logan 1994) and is retarded in several pathologies characterized by impulsive behaviour such as drug addiction, ADHD, schizophrenia and in patients with frontal lobe damage (Aron et al. 2004; Feil et al. 2010; Lipszyc and Schachar 2010). Stop-signal task performance is dependent on cortico-striatal circuitry and drugs affecting catecholamine neurotransmission improve response inhibition in humans and other animals

(Aron et al. 2014). Furthermore, the same brain regions and neurotransmitter systems involved in stop-signal task performance are found to be abnormal in ADHD patients (Eagle et al. 2008; Robbins and Arnsten 2009). Interestingly, there is evidence for a possible role of NPY in the behavioral phenotype of ADHD individuals (Lesch et al. 2011; Oades et al. 1998; Scassellati et al. 2012) and animal models of ADHD show abnormal functioning of the NPY system (Heilig et al. 1989; Martire et al. 1989b).

NPY Y5 receptors are mainly located in the hippocampus, thalamus, hypothalamus, caudate putamen (CPu), cerebral cortex and brain stem nuclei (Grove et al. 2000; Morin and Gehlert 2006; Parker and Herzog 1999), with particularly high levels in cingulate cortex and locus coeruleus (Grove et al. 2000; Wolak et al. 2003). However, the impact of NPY Y5 receptor activation on projection neurons in these regions and the behaviors that they subserve is poorly understood. Here we investigated the effects of a novel and highly selective NPY Y5 receptor antagonist on response inhibition as measured by the stop-signal task and on the underlying cortico-striatal neural circuitry using electrophysiological extracellular recordings and immediate early gene product (c-*fos*) analysis.

## Methods

### *Materials*

Lu AE00654 was synthesized at Lundbeck Research USA (Fig. 1). NPY Y5 receptor-selective peptide agonist [cPP$^{1-7}$,NPY$^{19-23}$,Ala$^{31}$,Aib$^{32}$,Gln$^{34}$]-h Pancreatic Polypeptide (cPP) was obtained from Tocris Bioscience (Ellisville, MO).

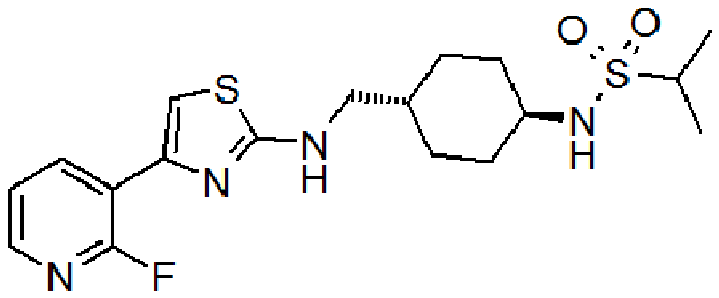


**Fig. 1** Chemical structure of Lu AE00654

**Behavior**

*Stop-signal task subjects and protocol*

Subjects were forty-eight male Lister Hooded rats (Charles River, UK). Their weight ranged between 250-315g at the beginning of the behavioral training (370-450g during drug administration). Animals were housed in groups of four, under an inverted light-dark cycle (lights off 7.30am – 7.30pm) and tested during the dark phase. The rats were maintained at 85% of their free-feeding body weight via provisions of 15g of laboratory chow on rest days and 10g on training/test days plus reinforcer pellets earned during the task (Test Diet, 45mg precision pellets). Food intake was monitored by weighing the animals every other day for the duration of the experiment. All behavioral experiments took place within the University of Cambridge, Department of Experimental Psychology and were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

Twelve operant conditioning chambers (Med. Associated, Vermont, USA) were used as described previously (Bari et al. 2009). The chambers were controlled by the Whisker Control System (Cardinal and Aitken 2001) and custom software written in Visual Basic by A.C. Mar. Rats were trained following a procedure modified from Eagle and Robbins (2003) to perform the SST as described previously (Bari and Robbins 2013b). Briefly, rats gradually learned to press the right lever to receive a reward pellet into the food well. When the animals reliably completed at least two sessions of 100 trials within 30 minutes, they were presented with the left lever and

learned to press it to extend the right one, which if pressed resulted in the delivery of the reward in the central food well. The limited hold (LH) - the time available for the rats to press the right lever after pressing the left lever - was progressively shortened until the rats reliably completed 100 trials with a LH of 5s. Stop trials were then introduced using a stop-signal tone that lasted until the end of the LH period and the number of total trials was set to 210. The LH and stop-signal were made shorter until they were kept constant for each animal (LH range: 1.2 – 1.3 s and the tone length was further shortened to 200 ms). During go trials the rats were rewarded with a food pellet for pressing the left then the right levers in fast sequence before the LH expired. If the rats failed to press the right lever within the LH, they received a time-out period (TO; 5s darkness, no levers available) and the trial was recorded as a go error. Stop trials were delivered randomly on 20% of total trials. Stop trials began in the same manner as a go trial, but after pressing the left lever, an auditory stop-signal (4500 Hz, ~80 dB tone) produced by a sonalert tone generator mounted on the back wall of the chambers was played and the rats were required to refrain from pressing the right lever for the duration of the LH. During training, stop-signals were presented as soon as the rat pressed the left lever (i.e., zero delay). During testing and for the calculation of the SSRT stop-signals were delivered after a pre-determined delay (i.e., stop-signal delay, SSD). If the rats pressed the right lever within the LH in a stop trial, they were punished with a TO and the trial was recorded as a stop error.

Following initial training, the mean go reaction time (mRT) from two zero delay sessions was used to calculate SSDs for each of the 48 rats. Two SSDs (i.e., mRT-100 ms and mRT-300 ms) were used throughout the study. SSRTs were estimated using the protocol described by Logan (1994). SSRT and stop accuracy were adjusted for the presence of omission errors on go trials in order to account for the stop trials in which a correct inhibition could not be attributed to a successful stop, but could be accounted for by distraction or inattention. The adjustment was performed using the correction factor of Tannock et al. (1989): adjusted *p*(inhibit) = observed *p*(inhibit) – *p*(omission) / 1 - *p*(omission). Rats were excluded from the experiment if they displayed at least one of the following characteristics: (1) inverted inhibition function; (2) 100 or 0% stop accuracy; (3) go accuracy below 80%.

*Drug administration*

Lu AE00654 (0.03, 0.3 and 3 mg/kg) was suspended in 1% hydroxypropyl methylcellulose (Sigma-Aldrich) in double distilled water. Drug or vehicle was injected intraperitoneally according to a randomized *latin* square design, at a volume of 1 ml/kg. After each injection, the animals were left undisturbed singly housed in a quiet room for 1 hour before test started. Three days of wash-out were allowed between drug testing sessions. SSDs were introduced only on drug days, while on the other days animals were run on zero delay sessions.

*Data analysis*

Measures analyzed included SSRT, mRT, % stop accuracy, % go accuracy, the standard deviation of the go reaction time (GoRTSD) and reward collection latency (RCL). These two latter variables represent putative measures of sustained attention and motivation, respectively (Bari and Robbins 2013b). Repeated measure ANOVA was used to analyze the data, with dose levels as within-subjects factors. Post-hoc analysis was performed using Sidak's correction for multiple comparisons, where appropriate. Mauchly's test was used to control for violations of the sphericity assumption, where appropriate. Data was analyzed using SPSS and graphs were plotted in Excel.

**Electrophysiology**

*Subjects and procedures*

Electrophysiological recordings were made from Sprague-Dawley (Harlan, Indianapolis, IN, USA) rats. All experimental procedures were conducted under protocols approved by Rosalind Franklin University of Medicine Institutional Animal Care and Use Committee. All animals were housed under conditions of constant temperature (21-23 °C) and maintained on a 12:12 light/dark cycle with food and water available *ad libitum*. Prior to surgery, rats were deeply anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic apparatus as previously described (Sammut et al. 2010). Burr holes (~2-3 mm in diameter) were drilled in the skull overlying the dorsomedial frontal cortex (dmFC; coordinates from bregma: 3.7 mm anterior, 2 mm lateral) and CPu (coordinates from bregma: -0.5 to 2 mm anterior, 2 to 3.5 mm lateral). The dura mater was resected and bipolar stimulating electrodes were implanted bilaterally into the

dmFC using a micromanipulator (coordinates from Bregma: 3.7 mm anterior, 2 mm lateral, and 2 mm ventral). Extracellular recording electrodes were implanted into the CPu ipsilateral to the right cortical stimulating electrode (coordinates: 0.5 mm anterior, 3-4 mm lateral, 3-7 mm ventral). All coordinates were determined using a rat brain stereotaxic atlas (Paxinos and Watson 1986). The level of anesthesia was periodically verified via the hind limb compression reflex and maintained using supplemental administration of anesthesia as previously described (Sammut et al. 2010). Temperature was monitored using a rectal probe and maintained at 37° C using a heating pad (VL-20F, Fintronics Inc., Orange, CT, USA).

*In vivo* single-unit extracellular recordings of striatal medium-sized spiny neurons were performed using microelectrodes filled with sodium chloride (2M) solution (Sammut et al. 2010). Electrical stimuli (duration = 500 μsec, intensity = 200-1400 μA) were generated using a Master-8 stimulator and photoelectric constant current/stimulus isolation units and delivered in single pulses (0.5 Hz) via the electrode implanted ipsilateral to the recording pipette over 100 consecutive trials. Train stimulation (30Hz, 1000 ms train duration, 2 s intertrain interval, ITI) was administered via the electrode contralateral to the recording pipette. After completion of each experiment, rats were deeply anesthetized and perfused transcardially as previously described (Sammut et al. 2010). Brains were then removed and processed for histological assessment of stimulating and recording electrode sites.

*Drug administration and experimental protocol*

Lu AE00654 (0.3-10 mg/kg) was dissolved in 2-hydroxypropyl-β-cyclodextrin solution (20%) and administered by subcutaneous injections. In within-subjects studies, after isolating a cell and recording spontaneous (non-evoked) firing activity for 3 minutes as described previously (Ondracek et al. 2008), stimulation currents were adjusted to approximately 50% maximal responding to stimulation delivered at 0.5 Hz. A pre-train stimulation baseline trial consisting of 100 individual single-pulse stimulations delivered over 200 s was then recorded. Once stable levels of single-pulse-evoked spiking were obtained, a series of train stimulations (25) were delivered to the contralateral cortex (30 Hz, 1000 ms train duration, 2 s ITI for a duration of 50 s). In order to examine the impact of train stimulation on spike probability during the train stimulation trial, single-pulse stimulation was delivered concurrently to the ipsilateral cortex via

a second stimulation channel which was triggered by channel 1 (train) stimulation, but delayed 500 ms from the end of the stimulus train. Immediately following the train stimulation trial, three additional post-train stimulation trials (200 s each) were performed in a manner which was identical to the pre-train stimulation trial (100 single-pulse trials each, no train stimulation). Three post-train trials were chosen based on the observation that train-induced changes in spike probability in control animals were usually observed to return to prestimulation levels during the second or third post-train stimulation trials, recorded approximately 200–600 s after completion of the train stimulation trial (Ondracek *et al.*, 2008). Next, Lu AE00654 or vehicle was administered and the effects of the stimulation protocol as described above were reassessed approximately 20 minutes later. In between-subjects studies, animals were injected with either Lu AE00654 or vehicle at least 20 minutes prior to initiation of the recording session.

*Data analysis*

Electrophysiological data were analyzed in Clampfit as previously described (Sammut et al. 2010). The influence of Lu AE00654 administration was determined by comparing cortically-evoked activity in drug groups to time-matched controls receiving vehicle. Peri-stimulus time histograms were constructed (1 ms bins) for each trial and spike probabilities were calculated by dividing the number of evoked action potentials (either 0 or 1 per pulse) by the number of stimuli delivered. Data were also summarized using spike onset latency and standard deviation of spike onset latency plots as indicated. Excitatory (E) responses observed during train stimulation were operationally defined as an increase in spike probability of > 2 SD above the pre-train stimulation mean. Inhibitory (I) responses observed during train stimulation were operationally defined as a decrease in spike probability of > 2 SD below the pre-train stimulation mean (Ondracek *et al.*, 2008). The statistical significance of drug-induced changes in measures of cell activity was determined using a two-way ANOVA with a Tukey post-hoc test.

**c-Fos immunohistochemistry (IHC) and immunofluorescence (IF)**

*Subjects and experimental protocol*

Adult male Wistar (for IHC experiment) or Sprague Dawley (for IF experiment) rats weighing 200–225 g were obtained from Charles River Laboratories, Inc (Wilmington, MA) and individually housed in standard husbandry conditions (21–22°C, 35-50% relative humidity, 12:12-h light/dark cycle). Rats had *ad libitum* access to drinking water and standard laboratory rat chow. All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and with the approval of the Institutional Animal Care and Use Committee at Lundbeck Research USA.

Rats were stereotaxically implanted with unilateral 26G stainless steel guide cannulae (Plastics One, Roanoke, VA) under isoflurane anesthesia (2.5-5% in oxygen). Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (1986). The incisor bar was set at 3.3 mm below the ear bars and coordinates were as follows: CPu, 1.8 mm posterior to bregma, 4.5 mm lateral to midline and 4.3 mm below the skull surface; dmFC, 2.7 mm anterior to bregma, 0.5 mm lateral to midline and 1.6 mm below the skull surface. Cannulae were secured to the skull with stainless steel screws and dental acrylic cement. Animals were allowed at least one week to recover from surgery with daily handling, prior to receiving injections.

Local injections of saline or 1.8 nmol cPP were given in a 1 µl volume over 60 s with a 33G internal cannula (Plastics One). The injector extended 1 mm beyond the end of the guide cannula. Sprague Dawley rats in the IF experiment were pretreated with Lu AE00654 (10 mg/kg subcutaneously) dissolved in 2-hydroxypropyl-β-cyclodextrin solution (20%), 1 hour before cPP or vehicle injections. One hour after vehicle or cPP injection, animals were deeply anesthetized and perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were removed, postfixed overnight, cryoprotected in 20% sucrose, and frozen in dry ice. Coronal free-floating sections were cut at 40 µm on a sliding microtome and stored in PBS or cryoprotectant until use for IHC or IF.

Coronal sections were taken for Fos IHC in the standard avidin-biotin-peroxidase method. Endogenous peroxidase activity was quenched by incubation for 30 min with 0.3% $H_2O_2$ in

KPBS (0.02M). Sections were incubated for 1h at room temperature in blocking reagent (5% normal goat serum in KPBS). After the initial blocking step, sections were incubated in rabbit polyclonal anti-c-Fos antibody (PC38, EMD Biosciences Inc., San Diego, CA) diluted 1:30,000 in KPBS with 0.4% Triton X-100 and 2% normal goat serum for 1 h at room temperature and then overnight at 4˚ C. After rinses in KPBS, sections were incubated in biotinylated goat anti-rabbit secondary antibody at 1:600 (Vector Laboratories, Burlingame, CA) in KPBS with 0.4% Triton X-100 and 2% normal goat serum for 2 h at room temperature. Sections were rinsed in KPBS and incubated for 1 h in avidin-biotin complex (ABC Vectastain kit, Vector Laboratories) diluted in KPBS with 0.4% Triton X-100. Sections were rinsed with KPBS and the peroxidase reaction was developed with nickel-intensified diaminobenzidine (Vector Laboratories). Sections were mounted on gelatin-coated slides, air-dried, dehydrated with ethanol, cleared in xylene and coverslipped.

For c-Fos IF, coronal sections were incubated at 1h at room temperature in blocking reagent and then with rabbit polyclonal anti-c-Fos antibody diluted 1:10000 in PBS with 0.4% Triton X-100 and 3% normal goat serum for 1 h at room temperature and then overnight at 4˚ C. After rinses in PBS, sections were incubated in fluorescently labeled goat anti-rabbit (Alexa Fluor 488) secondary antibody (1:400, Life Technologies, Grand Island, NY) in PBS with 0.4% Triton X-100 and 3% normal goat serum for 2 h at room temperature. PBS washed sections were mounted with a fluorescence mounting medium and coverslipped.

*Data analysis*

Images were captured using an Axio Imager M1 upright microscope (Zeiss, Thornwood, NY) and an AxioCam MRm digital camera (Zeiss). The number of c-Fos immunoreactive cells was manually counted in sections representing the dmFC and CPu by investigators blinded to the treatments. The number of c-Fos-immunoreactive cells in sections representing the dmFC was manually counted. Results are presented as mean ± standard error for the number of c-Fos-immunoreactive cells per section in each brain area. For c-Fos IHC, cPP-treatment was compared with vehicle using a Student's t test. For c-Fos IF, one-way analysis of variance was

conducted followed by Tukey's multiple comparisons tests. P values < .05 were considered significant. All statistical tests were performed using the Prism statistical package (GraphPad Software Inc, San Diego, CA).

## Results

*Effects of Lu AE00654 on stop-signal task performance*

Twenty subjects were excluded from the final analysis for violating stop-signal task or race model requirements during at least one drug testing session (final N = 28). This criterion for exclusion was chosen to avoid dosing a subset of animals twice with the same drug dose. Lu AE00654 had a small but significant effect on SSRT ($F(3,81) = 2.96$, $p < .05$). Post-hoc tests showed that only at 0.03 mg/kg SSRT estimate was significantly lower than Veh ($p < .05$) after Sidak's correction for multiple comparisons. Lu AE00654 did not affect significantly go accuracy, mRT, GoRTSD or RCL. There was no significant effect of drug on stop accuracy at any SSD (Fig. 2).

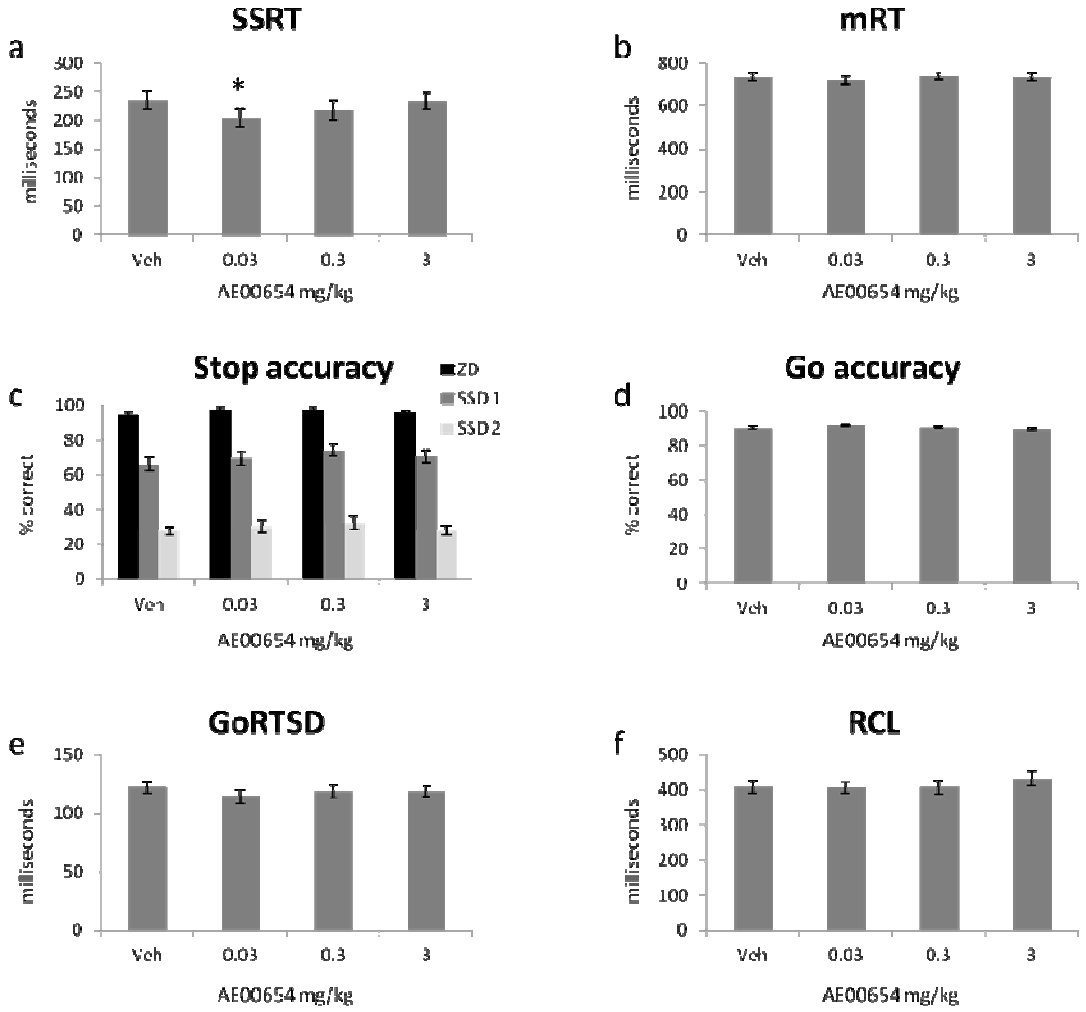


**Fig. 2** Effects of Lu AE00654 on stop-signal task variables. There was a significant effect of the drug on SSRT, which was shorter at 0.03 mg/kg, compared to vehicle injection (* $p < .05$). Abbreviations: SSRT, stop-signal reaction time; mRT, mean reaction time; GoRTSD, standard deviation of reaction time on go trials; RCL, reward collection latency

*Effects of Lu AE00654 on cortically-evoked spike activity in the CPu*

Comparisons of measures performed prior to train stimulation across groups indicated that NPY Y5 receptor antagonism did not significantly alter the responsiveness of putative striatal projection neurons to low frequency stimulation (0.5 Hz) of the ipsilateral dmFC. Thus, no

significant differences were observed between treatment groups in pre-train measures of current intensity required to elicit spike activity ~50% of the time, spike probability, spike latency, or the standard deviation of spike latency (data not shown).

Similar to our previous studies (Ondracek et al., 2008; Sammut et al., 2010), high frequency train stimulation (30 Hz) of the contralateral cortex was found to produce either inhibitory, excitatory or no effects on spike probability and had variable effects on spike activity assessed after the train stimulation trial in both vehicle and Lu AE00654-treated rats (see Fig. 3-4). We first examined the impact of systemic administration of Lu AE00654 (0.3 or 10 mg/kg) on the incidence of excitatory and inhibitory responses evoked during train stimulation of the contralateral cortex. Outcomes from these between-subjects studies indicated that the proportion of cells responding to train stimulation with an E, I or N response was not altered by either dose of Lu AE00654 (Table 1). Furthermore, the magnitude of E and I responses observed during the train stimulation trial was not changed by either dose of Lu AE00654.

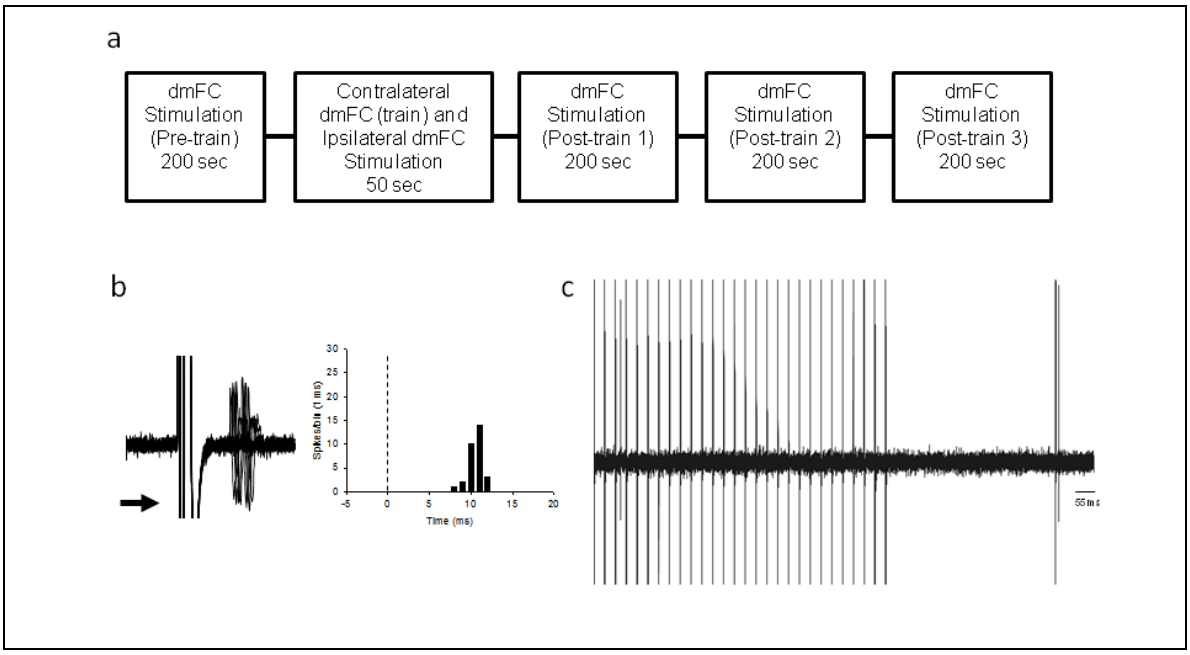


**Fig. 3** Effects of cortical stimulation on medium spiny neurons activity recorded in the CPu. a) Description of the stimulation protocols and pre-, train, and post-train assessment of cortically-

evoked spike activity. b) Left: examples of ten superimposed cortically-evoked spike responses recorded prior to train stimulation. Right: peri-stimulus time interval histogram showing the cumulative response of the same medium spiny neuron. c) Representative trace of spike activity recorded during train stimulation of the contralateral dmFC

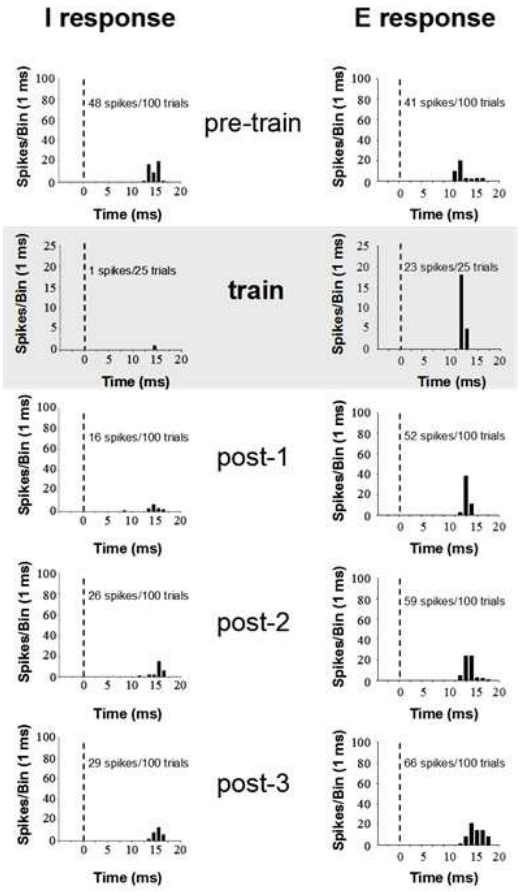


**Fig. 4** Examples of inhibitory (I, left) and excitatory (E, right) responses observed during train stimulation of the contralateral dmFC. I and E responses were operationally defined as a change in spike probability of > 2 SD above/below the pre-train mean. Increases or decreases in spike probability that did not meet this criterion were defined as “no change” (N)

| Response | Treatment | | |
|---|---|---|---|
| | Vehicle | Post-drug (0.3 mg/kg) | Post-drug (10 mg/kg) |
| I | 23.5% (8/34 cells) | 18.8% (3/16 cells) | 36% (9/25 cells) |
| E | 17.7% (6/34 cells) | 25% (4/16 cells) | 16% (4/25 cells) |
| NR | 58.8% (20/34 cells) | 56.2% (9/16 cells) | 48% (12/25 cells) |

**Table 1** Effects of Lu AE00654 on the observed frequency of inhibitory (I) and excitatory (E) responses of striatal cells during cortical train stimulation. Ratios in parentheses indicate the number of cells exhibiting the indicated type of response per number of cells tested. No significant change in the relative incidence of E and I response types was observed following treatment with either dose of Lu AE00654 ($p > 0.05$; Fisher Exact test). Abbreviations: E, excitatory; I, inhibitory; NR, no response

While mixed responses to cortical train stimulation were observed following vehicle treatment, Lu AE00654 administration induced a persistent short-term depression of post-train cortically-evoked activity in the CPu (Fig. 5, top; $F(1,218) = 3.067$, $p < .05$). This dose-dependent effect was observed with the high (10 mg/kg), but not the low (0.3 mg/kg) dose of Lu AE00654 (N = 34 cells/34 vehicle-treated rats, 16 cells/10 Lu AE00654 0.3 mg/kg-treated rats, and 25 cells/18 Lu AE00654 10 mg/kg-treated rats)

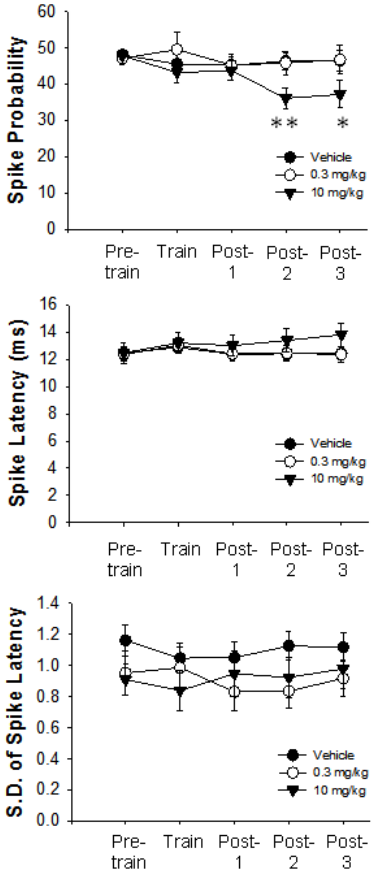


**Fig. 5** Effects of systemic Lu AE00654 administration on cortically-evoked spike activity recorded in the CPu before, during and after high frequency train stimulation of the contralateral dmFC (between-subjects studies). Significant suppression of cortically-evoked activity was observed in drug-treated rats during post-train intervals 2 and 3 following systemic administration of the 10 mg/kg dose, but not the 0.3 mg/kg dose, of Lu AE00654 (top). Error bars indicate S.E.M. of group measures. No significant effects on spike latency (middle) or standard deviation of latency (bottom) were noted. * $p < .05$, ** $p < .01$

In order to confirm the above observations, within-subjects studies were performed in cells recorded prior to, and following systemic administration of Lu AE00654 (0.3 or 10 mg/kg). Results were highly consistent with those observed in the between-subjects studies, indicating that the high dose of Lu AE00654 induced a short-term depression of cortically-evoked activity

in CPu following train stimulation of the contralateral cortex (Fig. 6, top right; $F(1,54)) = 6.665$, $p < .05$; N = 7 cells/7 Lu AE00654 0.3 mg/kg-treated rats, and 8 cells/8 Lu AE00654 10 mg/kg-treated rats).

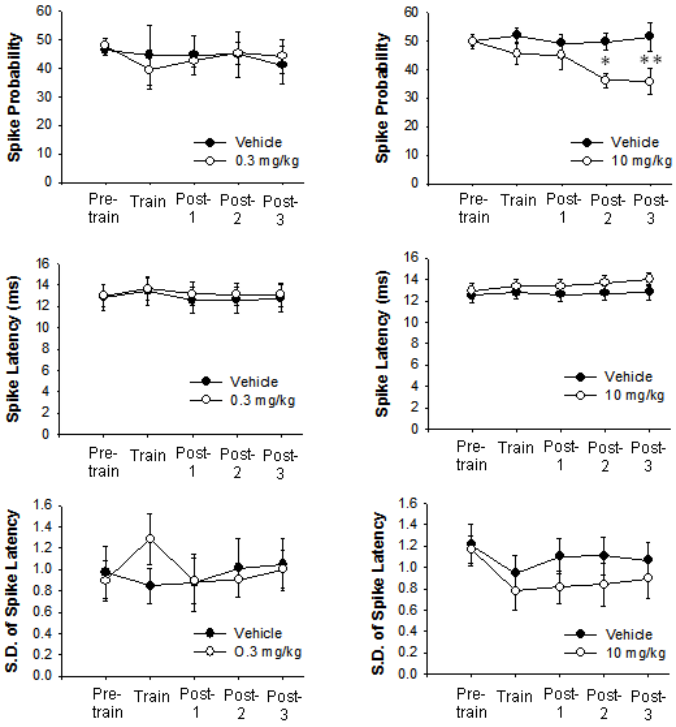


**Fig. 6** Effects of systemic Lu AE00654 administration on cortically-evoked spike activity recorded in the CPu before, during, and after high frequency train stimulation of the contralateral dmFC (within-subjects studies). Significant suppression of cortically-evoked activity was observed in drug-treated rats during post-train intervals 2 and 3 following systemic administration of the 10 mg/kg dose (top right), but not the 0.3 mg/kg dose (top left), of Lu AE00654. Error bars indicate S.E.M. of group measures. * $p < .05$, ** $p < .01$

We next examined the impact of NPY Y5 receptor antagonism on the magnitude and duration of train-induced changes in cell excitability across different response categories (i.e., E, I and N response groups). Cells exhibiting an E response to train stimulation exhibited a dose-dependent

suppression of post-train spike probability following systemic administration of Lu AE00654. This suppression was evident in E responders in the 3rd post-train stimulation trial following administration of 0.3 mg/kg of Lu AE00654 (Fig. 7, top left). Moreover, following systemic administration of 10 mg/kg of this compound, the suppression observed in E responders was present in both the 2nd and 3rd post-train stimulation trials (Fig. 7, top right, $F(1,31) = 13.438$, $p < .05$). Systemic administration of 10 mg/kg of Lu AE00654 appeared to induce a decrease in the standard deviation of the spike latency observed in E responders; however this effect did not reach significance (Fig. 7, bottom right). No significant changes were observed in I or N responders following systemic administration of either dose of Lu AE00654 (data not shown).

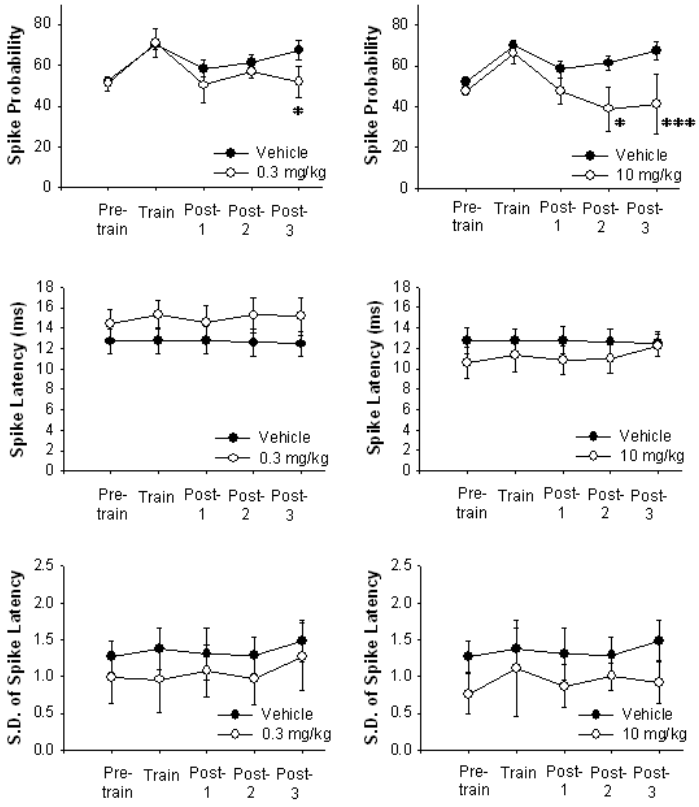


**Fig. 7** Effects of 0.3 mg/kg (left) or 10 mg/kg (right) of Lu AE00654 on excitatory responses (E) in CPu evoked during train stimulation of the contralateral cortex. Significant suppression of spike probability was observed in E responding cells during post-train interval 3 following

systemic administration of the low dose of Lu AE00654 (0.3 mg/kg, top left). The same effect was observed in E responding cells during post-train intervals 2 and 3 following systemic administration of the high dose of Lu AE00654 (10 mg/kg, top right). No changes were observed in cells responding to stimulation with I or N profiles (not shown). * $p < .05$, *** $p < .001$

*Effects of local cPP injection on c-fos expression*

Animals that received cPP injections in the dmFC showed a significant increase in c-Fos-positive cell count in the CPu compared to animals receiving saline (vehicle: 2.2 ± 0.97, n = 5; cPP: 205.7 ± 99, n = 3, t(6) = 3, $p < .05$). Similarly, intra-CPu injection of cPP significantly increased the number of c-Fos-positive nuclei in the dmFC (vehicle: 3.5 ± 2.2, n = 4; cPP: 178.3 ± 58.2, n = 4, t(6) = 2.81, $p < .05$). These data suggest that NPY Y5 receptor activation in the cortex increases activity in the CPu and vice versa (Fig. 8).

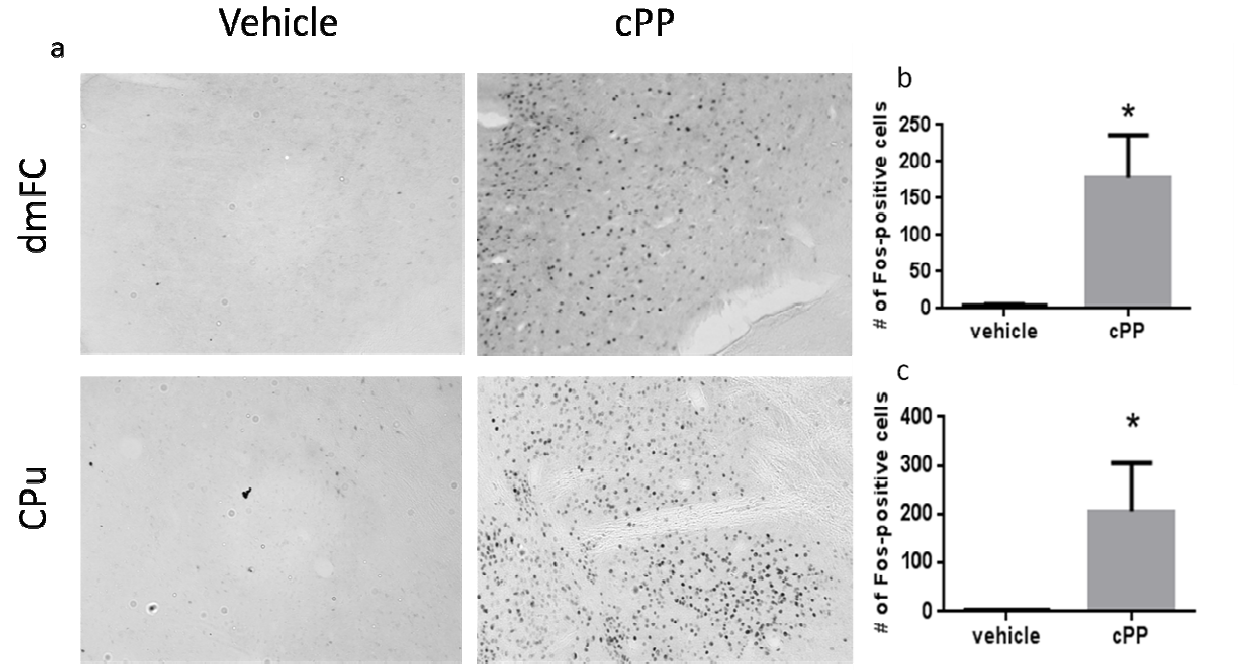

**Fig. 8** Effects of the selective NPY Y5 receptor agonist cPP or vehicle on c-*fos* expression in dmFC and CPu of animals injected in the CPu or dmFC, respectively. a) Representative sections through the dmFC or CPu of rats administered vehicle (left column) or cPP (right column). b) Local infusion of cPP in the CPu increased c-Fos-positive cell count in dmFC. c) cPP infusion in the dmFC increased c-Fos-positive cell count in the CPu (* $p < .05$)

*Effects of Lu AE00654 on cPP-induced c-fos expression*

There was a significant effect of cPP on c-*fos* expression in the dmFC ($F(2,11) = 7.36$, $p = 0.009$). Post hoc comparisons using the Tukey HSD test indicated that the animals that received cPP injections in the CPu showed a significant increase in c-Fos-positive cell count in the dmFC compared to animals receiving saline (vehicle: 5.75 ± 0.48, n = 4; cPP: 56.3 ± 20.2, n = 4). cPP-induced c-*fos* expression was blocked by pretreatment with Lu AE00654 (10.1 ± 1.0, n = 6) (Fig. 9).

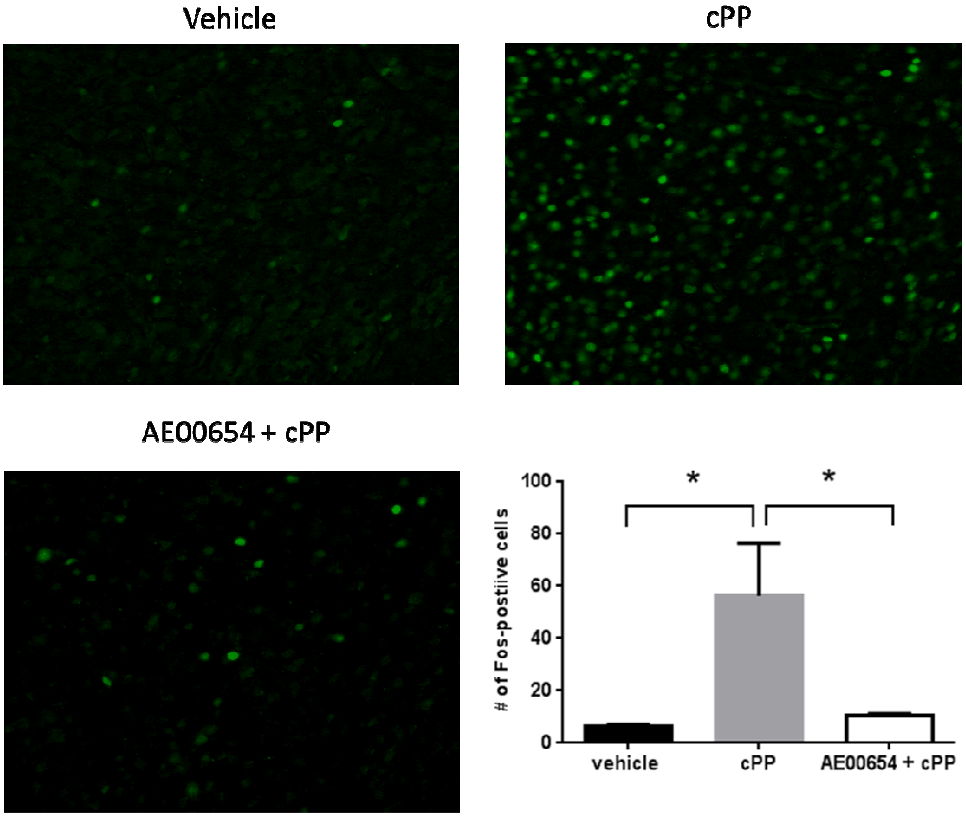

**Fig. 9** c-Fos immunofluorescence in the dmFC from rats treated with vehicle (top left), cPP (top right), or Lu AE00654 and cPP (bottom left). Local infusion of cPP in the CPu increased c-Fos-positive cell count in the dmFC, which was blocked by pretreatment with Lu AE00654 (* $p < .05$)

## Discussion

We have investigated the effects of NPY Y5 receptor modulation on response inhibition and measures of neuronal activation in underlying cortico-striatal pathways in the rat. The novel and highly selective NPY Y5 receptor antagonist Lu AE00654 significantly speeded response inhibition (i.e., shortened SSRT) and enhanced electrophysiological measures of cortically-evoked neural inhibition in the striatum. We also showed that local injection of a NPY Y5 receptor agonist activates neurons in the cortico-striatal circuitry involved in stop-signal task performance. Moreover, the effects of the Y5 agonist were attenuated by pretreatment with Lu AE00654, thus confirming the involvement of Y5 receptors in the above circuit level effects. These findings suggest that systemic blockade of NPY Y5 receptors selectively facilitates the fast inhibition of an ongoing action probably by enhancing short-term cortical inhibition over basal ganglia structures involved in response control.

Lu AE00654 was designed to achieve potent and selective blockade of the human Y5 receptor. *In vitro*, Lu AE00654 binds to the human and rat Y5 receptors with Ki = 1.1 and 0.4 nM, respectively. Lu AE00654 displays >= 8,800 fold selectivity for human Y5 relative to other known drug targets. *In vivo*, Lu AE00654 displays antidepressant-like and anxiolytic activity in the rat (Packiarajan et al. 2010). In the present study, Lu AE00654 selectively improved SSRT estimates with no effects on other variables including stop accuracy and the speed of the go response. This pattern of results suggests that faster response inhibition in treated animals was not due to improvements in sustained attention or motivation, as the variability of the go response and reward collection latency were not affected by the drug.

Effects on SSRT were observed at the lower dose tested (0.03 mg/kg) and corresponding drug concentrations in plasma and brain of 14.8 and 15.5 nM, respectively. There was an effect of the

middle dose of Lu AE00654 (0.3 mg/kg), but this was not significant after correction for multiple comparisons (Sidak), whereas the high dose (3 mg/kg) did not affect stop-signal task performance. However, higher doses of Lu AE00654 were necessary to enhance the inhibitory activity of dmFC on striatal neurons in extracellular recording experiments. This discrepancy might be the consequence of the high sensitivity of the stop-signal task for low doses of several drugs (Eagle et al. 2007). In fact, high doses of certain compounds may increase inhibition to the point that also the go responses are affected, thus masking any beneficial effect on SSRT. Alternatively, this difference may be due to the use of anesthetized preparations for the electrophysiological experiments. Urethane is known to affect multiple neurotransmitter-gated ion channels (Hara and Harris 2002), thus shifting the dose-response curve of several drugs (e.g., Armstrong et al. 1982; Maggi et al. 1984; West 1998).

The NPY system is known to participate in the regulation of food intake (Maniam and Morris 2012; Mercer et al. 2011) and affective states such as stress (Heilig and Thorsell 2002) and anxiety (Kask et al. 2002). Previous experiments that used a NPY Y5 selective antagonist, similar to the one used in the present investigation, reported no change in the quantity of food consumed when administered alone, as well as anxiolytic and antidepressant effects, but only after chronic administration (Walker et al. 2009). At the doses used for the behavioral experiment, Lu AE00654 did not affect putative measures of motivation such as the speed of the go response or the latency of reward collection, thus ruling out possible effects on the incentive motivational properties of the reward used.

Inhibitory performance as measured by the SSRT is dependent on the integrity of cortico-basal ganglia circuitry modulated by ascending catecholaminergic systems, as shown in humans and rodents (Aron 2007; Eagle et al. 2008). In the rat, temporary inactivation of the dmFC (encompassing prelimbic and cingulate cortices) selectively retards SSRT (Bari et al. 2011). Moreover, lesion of the dorsal striatum impairs both response inhibition and execution in the stop-signal task (Eagle and Robbins 2003), which is suggestive of a more general role of this structure in response control (Bari and Robbins 2013a). The rat cerebral cortex is enriched with NPY Y5 receptors located on the soma and proximal dendrites of a subpopulation of GABAergic interneurons, with the densest immunoreactivity found in the cingulate cortex (Caberlotto et al.

1998; Grove et al. 2000). The results of our c-Fos analysis showing that activating Y5 receptors in the dmFC increases neural activity in the CPu, are consistent with their modulatory action on GABA-mediated control of cortico-striatal pyramidal neurons (Wolak et al 2003).

On the other hand, the observation of increased c-*fos* expression in dmFC (including the cingulate area) after Y5 stimulation in the CPu suggests a possible direct modulatory influence of Lu AE00654 subcortically. Our electrophysiological results indicate that phasic stimulation of frontal cortical afferents activates a powerful feed-forward NPY-mediated facilitation which opposes short-term depression of cortico-striatal transmission via activation of NPY Y5 receptors. Thus, it is plausible that blocking this NPY-mediated facilitation with Lu AE00654 acts to decrease spike activity and spike jitter by increasing the impact of ongoing inhibitory processes. These effects are consistent with increasing GABA-A receptor stimulation on striatal projection neurons, potentially via activation of parvalbumin positive, fast-spiking interneurons (Tepper et al. 2004). Indeed, stimulation of these interneurons decreases spike activity and spike jitter in identified striatal projection cells (Mallet et al. 2005). Increased dopamine D2 receptor activation would also be expected to produce changes in projection neuron activity (Mallet et al. 2006) consistent with the electrophysiological outcomes of the current study.

We have shown previously that short-term depression is mediated largely by dopamine D2 receptor activation (Ondracek et al. 2008) and that D2 receptor antagonist infusion in the CPu impairs both response execution and response inhibition (Eagle et al. 2011). Taken together, these observations suggest that NPY-containing interneurons in the CPu (Sammut et al. 2010) act to oppose short-term inhibitory influences of dopamine D2 receptor activation via NPY-GABA dependent mechanisms (Chen and van den Pol 1996). Alternatively or additionally, Lu AE00654 may have blocked presynaptic Y5 receptors thus decreasing local glutamate currents that regulate the membrane excitability of CPu neurons (Acuna-Goycolea et al. 2005). Both hypotheses are compatible with the observation that NPY and NPY Y5 agonists increase extracellular dopamine in striatal regions (Adewale et al. 2007; Heilig et al. 1990; Quarta et al. 2011), whereas Y5 receptor antagonism decreases stimulant-induced dopamine release there (Sorensen et al. 2012). Stop-signal task performance is sensitive to noradrenergic agents and increasing extracellular norepinephrine availability speeds inhibitory processes in rats and

humans (Eagle et al. 2008), whereas α2 inhibitory autoreceptor stimulation impairs performance in this task (Bari et al. 2009; Bari et al. 2011). Thus, another potentially relevant property of the NPY system for the present results is its interaction with the noradrenergic system (see Dumont et al. 1992 for review). NPY is co-localized in noradrenergic neurons of the locus coeruleus and can modulate the release of norepinephrine both locally (Agnati et al. 1983; Fuxe et al. 1984; Illes and Regenold 1990) and at the level of coeruleo-cortical terminals (Martire et al. 1989a; Yokoo et al. 1987). *In vivo* and *in vitro* experiments suggest the presence of NPY-α2 receptor interaction in the rodent locus coeruleus and frontal areas (Fuxe et al. 1990; Illes and Regenold 1990; Widdowson et al. 1991; Yokoo et al. 1987). Interestingly, NPY-α2 receptor interaction is lost in the spontaneously hypertensive rat (Martire et al. 1986; 1989b), which is considered a valid model for some of the behavioral characteristics of ADHD and exhibits impaired behavioral inhibition, inattention and dysregulation of the noradrenergic system (Engberg et al. 1987; Oades et al. 2005; Russell 2002; Viggiano et al. 2004). Thus, future studies are warranted to investigate the possibility that NPY Y5 receptor antagonism improves response inhibition via a norepinephrine-dependent mechanism.

In the present study we have described converging evidence from different experimental approaches on the modulatory action of NPY Y5 receptors on brain systems involved in response control. The improvements in a measure of response inhibition (behavior), the increase in signal-to-noise filtering of cortico-striatal projections (electrophysiology), together with functional neuroanatomical evidence (c-*fos* expression), concur with previous studies suggesting the involvement of the NPY system in conditions characterized by elevated impulsivity, such as ADHD and impulsive aggression (Coccaro et al. 2012; Lesch et al. 2011; Oades et al. 1998). Based on the present data, we hypothesize that the principal mechanism for the NPY-mediated modulation of impulsive behavior is the facilitation of cortical control over subcortical structures, namely the striatum. However, the strength of our multidisciplinary approach is dampened by the use of different rat strains and different modalities of drug administration in the experiment reported here. This is in part the consequence of the involvement of three different laboratories in the present investigation. Nonetheless our results may have a significant impact on the development of new and more efficacious drugs aimed at improving cognitive deficits and decreasing impulsive tendencies in both patients and healthy individuals.

In summary, here we have shown that one of the putative mechanisms for the beneficial effects of Lu AE00654 on impulsivity is the enhancement of cortical inhibition over striatal areas involved in response control. Although previous research has focused on the modulation of affective processes by the NPY system, there is increasing interest in the role of this system in the regulation of higher cognitive functions and the possible treatment of neuropsychiatric disorders (Greco and Carli 2006). Indeed, the present results suggest that modulation of the NPY system may be relevant for the treatment of pathologies characterized by impulse-control deficits such as ADHD and drug addiction.

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