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Research report

Involvement of serotonin 2A receptor activation in modulating medial prefrontal cortex and amygdala neuronal activation during novelty-exposure

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HIGHLIGHTS

- Ketanserin blocks novelty-induced neuronal activation in medial prefrontal cortex.
- This is accompanied by decreased neuronal activation in the basolateral amygdala.
- Ketanserin makes basolateral amygdala more reactive towards the anxiogenic stimulus.
- 5-HT2A blockade does not affect activation of striatal projecting amygdala neurons.

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ABSTRACT

The medial prefrontal cortex (PFC) plays a major role in executive function by exerting a top-down control onto subcortical areas. Novelty-induced frontal cortex activation is 5-HT_{2A} receptor (5-HT_{2A}R) dependent. Here, we further investigated how blockade of 5-HT_{2A}Rs in mice exposed to a novel open-field arena affects medial PFC activation and basolateral amygdala (BLA) reactivity. We used c-Fos immunoreactivity (IR) as a marker of neuronal activation and stereological quantification for obtaining the total number of c-Fos-IR neurons as a measure of regional activation. We further examined the impact of 5-HT_{2A}R blockade on the striatal-projecting BLA neurons. Systemic administration of ketanserin (0.5 mg/kg) prior to novel open-field exposure resulted in reduced total numbers of c-Fos-IR cells in dorsomedial PFC areas and the BLA. Moreover, there was a positive correlation between the relative time spent in the centre of the open-field and BLA c-Fos-IR in the ketanserin-treated animals. Unilateral medial PFC lesions blocked this effect, ascertaining an involvement of this frontal cortex area. On the other hand, medial PFC lesioning exacerbated the more anxiogenic-like behaviour of the ketanserin-treated animals, upholding its involvement in modulating averseness. Ketanserin did not affect the number of activated striatal-projecting BLA neurons (measured by number of Cholera Toxin b (CTb) retrograde labelled neurons also being c-Fos-IR) following CTb injection in the ventral striatum. These results support a role of 5-HT_{2A}R activation in modulating mPFC and BLA activation during exposure to a novel environment, which may be interrelated. Conversely, 5-HT_{2A}R blockade does not seem to affect the amygdala-striatal projection.

1. Introduction

The serotonin 2A receptor (5-HT_{2A}R) has received considerable interest as an important potential target in the treatment of neuropsychiatric disorders such as schizophrenia, obsessive compulsive disorder and borderline personality disorder [1–4]. The 5-HT_{2A}R is highly expressed in the prefrontal cortex (PFC) [5] and exerts an important role in modulating PFC activity and neural oscillations [6–11].

The PFC is a key structure for executive functions [12,13], including cognitive processes such as reasoning, judgment and value-based decision-making, by integrating inputs from multiple brain regions and exerting top-down control on these regions

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in a coordinated manner [14,15]. Exposure to a novel environment engages this circuitry [16–18] with the medial PFC (mPFC) playing a central role in the processing and risk-evaluation of novelty, as seen in humans [19] and rodents [20,21]. Emotional valuation is integrated in the decision-making circuitry through the mPFC (risk assessment and inhibitory control), the basolateral amygdala (BLA) (emotional learning) and the ventral striatum (VS) (integrating information about reward, context and motivational drive); all parts of an evaluation system [22–25] where excitatory transmission from BLA to VS facilitates reward-seeking behaviour [26,27]. Further, there is a functional dissociation across mPFC subregions, with activation of the dorsal parts being related to the risk/uncertainty and the ventral parts with the subjective gain/loss valuation associated with a decision [28].

Rodent studies have reported c-Fos immunoreactivity (IR) and mRNA levels to be increased in the PFC after exposure to novelty [29–33]. C-Fos is an immediate-early gene widely used as a marker of general neuronal activity [34,35]. The rapid increase in immediate-early gene expression acts as first-response proteins [35,36], leading to a number of events involved in long-term functional adaptations of the PFC such as synaptic plasticity and memory consolidation [37]. The strongest c-Fos induction occurs during the early phases of behavioural training, when a learning process is commonly taking place integrating memory-dependent processes in the brain [31,33,38–40].

We have previously reported that novelty-induced *c-fos* expression in the PFC is blocked by systemic ketanserin administration and is 5-HT_{2A}R, not 5-HT_{2C} receptor, dependent [29]. This suggests a crucial role for this receptor in the PFC-mediated top-down control of risk-assessment and processing of reward-seeking behaviour during a novelty-exposure. As shown by functional imaging studies there is an inverse relationship between density of PFC 5-HT_{2A}Rs and amygdala reactivity in humans exposed to fearful stimuli [41]. Further, ketanserin in humans leads to more risk-aversive behaviour [42]. The aim of the present study was to explore the role of the different subregions of the mPFC and 5-HT_{2A}R activation in relation to amygdala reactivity during novelty-exposure. Also we wanted to investigate whether the 5-HT_{2A}R had a modulatory role on the activation of BLA neurons projecting to the VS.

Mice receiving systemic injections of ketanserin or vehicle were exposed for five minutes to an open-field arena in order to induce mPFC activation as done previously [29]. Further, the time spent in border versus centre was monitored during the five minutes exposure. A well-validated and strongly reliable stereological counting method [43,44] was applied for the quantification of total c-Fos-IR neurons in the mPFC, differentiating between the dorsomedial (dmPFC) and ventromedial (vmPFC) PFC, and in the BLA. In order to establish the involvement of the mPFC, animals received a priori a unilateral NMDA or sham lesion into the mPFC. Moreover, all animals were injected one week before the novelty assay with a retrograde tracer (Cholera toxin subunit b (CTb)) into the VS, enabling us to identify and quantify the fraction of c-Fos expressing BLA neurons projecting to the VS.

2. Experimental procedures

2.1. Animals

The animal care and experimental procedures were performed in accordance with the European Community Councils Directive of Nov 24th 1986 (86/609/EEC) by The Danish National Committee for Ethics in Animal Research under the Danish Ministry of Justice (License number 2010/561-1834). Female C57Bl mice (N = 48), around 12 weeks-old (21–25 g), were group-housed (eight in each cage) and kept in a controlled environment with a 12/12 h light/dark cycle, provided with standard rodent diet and water *ad libitum*. The animals were allowed to acclimatise in the animal facility for at least one week prior to the day of the first surgery and they were randomly assigned to experimental groups.

2.2. Surgery

First, we performed excitotoxic/sham lesions. After one week, retrograde tracing was performed on all mice. At the time of surgery, the mice were anesthetized with a solution of ketamine (2.1 mg/ml) and dexmedetomidine (0.016 mg/ml) (20 ml/kg, i.p.). The mice were placed in a stereotaxic frame (Stoelting, Wood Dale, IL), the skull was exposed and a hole was drilled for the unilateral intracerebral injections. For the excitotoxic lesions, 0.5 ul of NMDA (22 mg/ml; Sigma, St. Louis, MO) dissolved in sterile 0.1 M phosphate-buffer (PB) (pH 7.4) or vehicle (sterile 0.1 M PB, pH7.4) were injected into the mPFC at the following coordinates: +2.9 mm anterior to Bregma; −0.25 mm lateral to the midline; and dorsoventral −2.0 mm below the dura. For the retrograde tracing, 0.2 ul 0.4% CTb (# 104, List Biological Laboratories, Campbell, CA, USA) were injected at the following coordinates for the VS: + 2.2 mm anterior to Bregma; −0.75 mm lateral to the midline; and dorsoventral −4.25 mm below the dura. All coordinates were referenced to Bregma according to a standard Paxinos mouse brain stereotaxic atlas and further adjusted in pilot studies. The drugs were delivered with a 5 μl Hamilton syringe (75 RN; 34s/15/3; Hamilton, Bonaduz, Switzerland) slowly through 3 min and the syringe was left in place for another 5 min in order to prevent spreading of the drug along the needle track. All animals were given 1 ml of saline solution i.p. post-surgery and returned to cage placed on a heating element (28°) to aid recovery. Just before wake-up, meloxicam (20 ml/kg, s.c.) was administered for analgesic effect.

2.3. Novelty paradigm and locomotor activity assessment

One week after retrograde tracing, all mice were exposed to a novelty paradigm. All mice were handled for three days prior to the open-field test by the experimenter. The mice were acclimatized to the experimental room overnight. At the time of open-field testing, the mice were injected with ketanserin tartrate (0.5 mg/kg; 20 ml/kg, i.p.) (Sigma-Aldrich, St Louis, MO, USA) or its vehicle (sterile distilled water (20 ml/kg, i.p.)) and returned to their home-cage. Each treatment group was placed in separate cages to prevent the response of one group to influence the response of another. After 30 min the mice were placed in the middle of the open-field arena (35 × 35 × 40 cm) situated in a dimly lit room. After 5 min the novelty-exposed mice were returned to their respective home-cage for another 30 min before receiving an overdose of ketamine/dexmedetomidine solution and transported to a separate room for blood collection and perfusion fixation of the brains. As a control for basal versus open-field induced c-Fos-IR levels four mice were held in a home-cage in the same room while the open-field testing took place and handled shortly at a similar time point as the open-field group, but without being exposed to the open-field.

Behavioural activity during the open-field exposure was recorded by a webcamera located on the ceiling above the open-field arena. The total distance moved by the animals and the total time the animal spent in the centre of the arena (31.5 × 31.5 cm) versus the border (3.5 cm) was analysed and calculated with the video-tracking software EthoVision (version XT 9, Noldus, Wageningen, the Netherlands).

2.4. Plasma corticosterone levels

In order to monitor whether ketanserin or mPFC lesioning had an effect on the stress response to novelty-exposure, we measured plasma corticosterone levels in the exposed mice. Blood was collected through cardiac puncture once the mice were anesthetized. Due to technical challenges we only succeeded for a subgroup of animals. Blood was collected in K2-EDTA-coated tubes and centrifuged for 10 min at 3000*g* to obtain plasma. Plasma corticosterone was measured with a commercial ELISA kit (# KA0468, Abnova,Walnut, CA, USA). In short, samples were diluted 1:200 and added in duplicate along with standards on polyclonal corticosterone antibody pre-coated 96-well microtiter plates and incubated with competing biotinylated corticosterone. Following two hours of incubation, the plate was washed and then incubated with streptavidin-horseradish conjugated peroxidase for 30 min. Plates were subsequently washed and a chromogen substrate was added. The reaction was stopped with 0.5 N HCl and OD was immediately obtained at 450 nm and corrected at 620 nm on a Thermo Scientific Multiskan FC. The sample concentrations were calculated from the standard curve (0–100 ng/ml) using a four parameter logistic curve fit. Two intra-assay controls (n = 4) were included for two randomly selected subjects, proving the coefficient of variance (CV) to be less than 10% for both.

2.5. Transcardial perfusion fixation and tissue preparation

When deeply anaesthetized and after blood collection, the mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH7.4) for 2 min followed by 4% paraformaldehyde in PBS for 8 min. Brains were dissected, post-fixed overnight at 4 °C and submerged in 30% sucrose in PBS at 4 °C until saturation. At the time of sectioning, whole brains were frozen on dry ice and embedded in Tissuetek® (Sakura Finetek, Zoeterwoude, the Netherlands) and cut into 60 μm coronal sections using a Vibratome Ultrapro 5000 Cryostat (GMI Inc., Ramsay, Minnesota, USA). Following systematic random sampling principles (Gundersen et al., 1999), sections were collected in three parallel series through the most frontal part of the brain until Bregma level +0.02 mm (obtaining 6–7 sections containing the entire mPFC per mouse for lesion characterisation and c-Fos-IR quantification and the VS for tracing characterisation) and two parallel series of the more posterior part of the brain (−0.1 mm to −2.80 mm from Bregma; (obtaining 6–7 sections containing the entire BLA per mouse)) before being stored in wells of cryoprotectant at −20 °C.

2.6. Colorimetric immunohistochemistry

The free-floating 60 μm sections were rinsed for 3 × 10 min in PBS, incubated for 20 min in 1% H_2O_2 in PBS to block endogenous peroxidase activity and subsequently incubated for 50 min in PBS with 5% normal donkey serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 (TX) to block nonspecific binding sites. The sections were then incubated at 4 °C for 48 h with the primary antiserum in PBS with 1% BSA and 0.1% TX. After incubation in primary antiserum, the sections were washed for 3 × 10 min in PBS with 0.1% TX (PBS-TX) and incubated for 2 h in biotin-conjugated secondary antibody diluted 1:1000 in PBS-TX with 1% BSA, washed for 3 × 10 min in PBS-TX and transferred to an avidin-biotin complex solution (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in PBS-TX for 3 h. After washing for 3 × 10 min in PBS, the sections were incubated in SG chromogen solution or 0.01% DAB for 10 min followed by incubation in either SG peroxidase solution (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions or 0.01% diaminobenzidine (DAB; Sigma Aldrich, St. Louis, MO, USA) with 0.03% H2O2 in PBS, respectively for 10 min and then washed 3 × 10 min in PBS. For double-labelling, the two primary antibodies were co-incubated.

The DAB reaction (orange-brown) was applied for all single-labelling procedures (c-Fos, CTb and NeuN labelling) and for CTb-labelling in double-labelling procedures. The SG peroxidase reaction was applied for c-Fos-labelling in double-labelling procedures producing a blue-black colour localised to the nucleus.

The following primary antibodies were used: rabbit anti-NeuN monoclonal antibody (# MABN140, 1:16000; Milipore, Hellerup, Denmark), rabbit anti-c-Fos polyclonal antibody (# H-125, 1:1000; Santa Cruz, Heidelberg, Germany) and goat anti-CTb (# 703, 1:16000; List Biological Laboratories, Campbell, CA, USA). Sections were mounted on Superfrost® Plus glass slides (Menzel, Braunschweig, Germany) and dried before being dehydrated and coverslipped (Hounisen, Aarhus, Denmark). The CTb single stained sections for tracing characterisation were also stained with cresyl violet (Sigma-Aldrich) before the dehydration steps.

2.7. Lesion and tracing assessment

Lesions were histologically confirmed on NeuN-stained sections by an observer blind to the experimental groups. Mice with NMDA-induced excitotoxic lesions extending from cingulate, prelimbic, infralimbic and anterior medial orbitofrontal cortex (see Fig. 1A for lesion area) were included in the analysis. One mouse with mPFC lesions extending into the secondary motor cortex (M2) and dorsal peduncular cortex (DP) was also included in the analysis. Mice with weak excitotoxic effect in outer cortical layers of the contralateral side were included, since the lesioning of the targeted hemispheres were very prominent in comparison. CTb tracing sites were confirmed histologically by CTb-labeling and cresyl violet staining making neuroanatomical structures more visible. CTb tracing sites were mainly located in the nucleus accumbens (NAc) core and/or shell. In a few animals CTb injection sites also extended into the ventral caudate putamen (CPu) and structures located adjacent to the NAc (the diagonal band (DB)), dorsal endopiriform nucleus (DEn), dorsal peduncular cortex (DP), dorsal tenia tecta (DTT), lateral septal nucleus (LS), medial forebrain bundles (mfb), ventral pallidum (VP), septohippocampal nucleus (SHI), semilunar nucleus (SL) (see Fig. 1B for tracing area).

2.8. Regions of interest delineation

Cell countings were performed for the entire mPFC, comprising cingulate, prelimbic, medial orbitofrontal and infralimbic cortex, followed by cell countings performed for the vmPFC only, comprising medial orbitofrontal and infralimbic cortex (Fig. 2). Total c-Fos-IR neuron number in the dmPFC was calculated by substracting vmPFC cell countings from total mPFC cell countings. The counting in these areas was performed in the sham-lesioned animals only. For the mPFC, vmPFC and for the anterior part of the BLA, countings embraced both hemispheres. Double-labelled CTb + c-Fos-IR neurons were only counted in the BLA ipsilateral to the NMDA or sham lesion – the same hemisphere as the tracing in the NAc. To control for background c-Fos-IR, not related to the open-field-induced c-Fos expression related to decision-making, we performed additional c-Fos-IR cell countings in the somatosensory cortex (SSC), since we assume this area not be involved in the decision-making circuitry. The counting area was delineated using the NewCAST software (Visiopharm, Hoersholm Denmark) based on a standard Paxinos mouse brain atlas (Paxinos and Franklin, The mouse brain, 2nd, 2001). The boundaries for the mPFC and vmPFC extended from Bregma 2.80 mm to the section before the emergence of corpus callosum at Bregma 1.10 mm (Fig. 2A). The boundaries of the anterior BLA extended from Bregma −0.94 mm

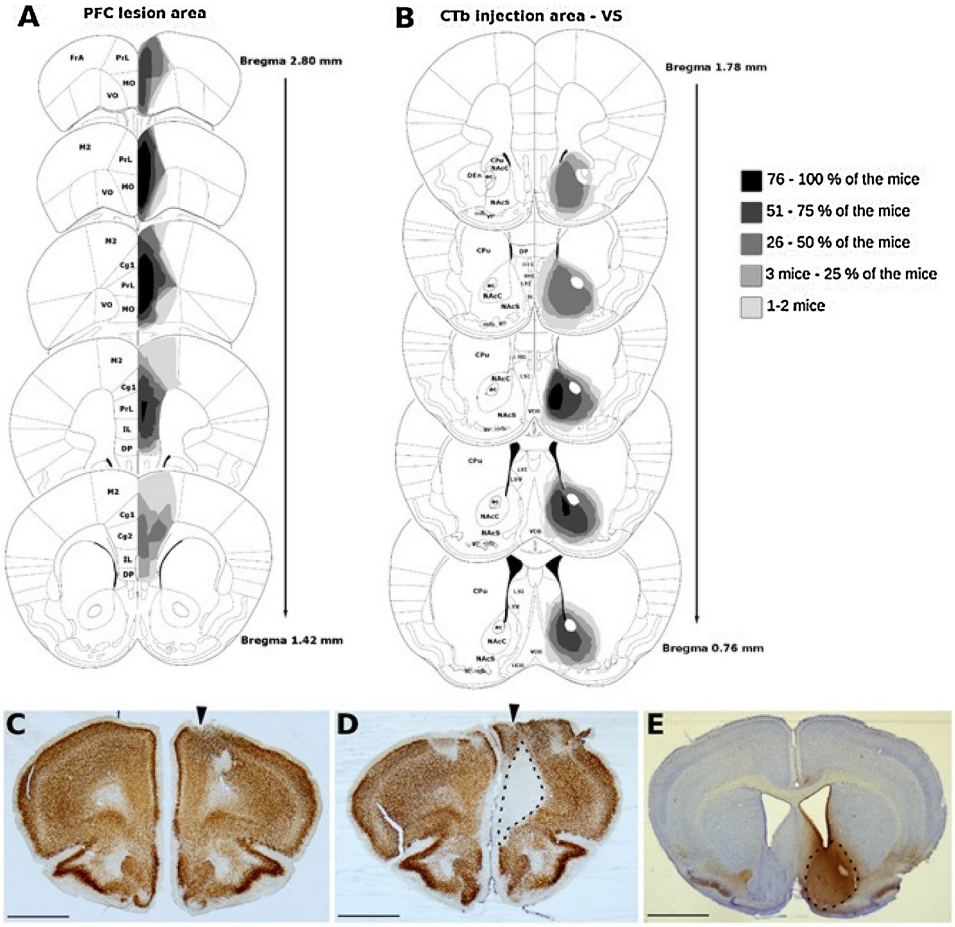

Fig. 1. Lesion and tracing sites. (A) Diagram of the extension of mPFC lesions shown in five coronal section diagrams extending from Bregma +2.80 mm (most anterior) to Bregma +1.42 mm (most posterior). (B) Diagram of the extension of CTb tracing sites in the VS shown in five coronal section diagrams extending from Bregma +1.78 mm (most anterior) to Bregma +0.76 mm (most posterior). Brain section diagrams are modified from Paxinos and Franklin (2001) (A-B). Representative coronal brain sections with sham lesion (C) and excitotoxic mPFC lesion (delineated with black dots) (D). Black arrows show injection needle entrance (C–D). (E) Coronal brain section with representative tracing site in the ventral striatum (delineated with black dots). Scale bars, 1000 μm. ac, anterior commissure; Cg, cingulate cortex; CPu, caudate putamen; CTb, Cholera Toxin subunit b; DB, the diagonal band; DEn, dorsal endopiriform nucleus; DP, dorsal peduncular cortex; DTT, dorsal tenia tecta; FrA, frontal association cortex; IL, infralimbic cortex; LS, lateral septal nucleus; M2, secondary motor cortex; mfb, medial forebrain bundles; MO, medial orbitofrontal cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PrL, prelimbic cortex; VO, ventral orbitofrontal cortex; VP, ventral pallidum; VS, ventral striatum; SHI, septohippocampal nucleus; SL, semilunar nucleus.

to the section before the emergence of posterior BLA at Bregma −1.82 mm (Fig. 2B).

2.9. The stereological design and precision of estimates

The total number of c-Fos positive nuclei, CTb labelled neurons and neurons both c-Fos and CTb positive were estimated unilaterally and bilaterally for the BLA and bilaterally for the mPFC and vmPFC using the optical fractionator sampling design as described previously (e.g. Fabricius et al., 2008). Estimates of the reference volume (V_{ref}) for BLA and mPFC/vmPFC were obtained by employing a point-counting method based on Cavalieri's principle combined with systematic random sampling [45,46]. The precision of total cell number (N) and volume (V_{ref}) estimates was provided in the coefficient of error (CE(N) and CE(V_{ref}) respectively) according to (Gunderse et al., 1999) (see Tables 1–3). The sampling is considered optimal when CE is approximately half or less of the observed CV (=standard deviation/mean), which was the case in this study (see Tables 1–4).

The counting procedure was performed using the NewCAST software (Visiopharm, Hoersholm, Denmark) superimposing counting frames on the image and a Nikon Eclipse 60i microscope (Nikon Nordic AB, Copenhagen, Denmark). The microscope was equipped with a Heidenhain electronic microcator measuring the z-axis (section thickness) with a precision of 0.1 um. The x-y position was monitored with a ProScanTM II motorized stage system (Prior Scientific Instruments Ltd., Cambridge, UK). Sections were analysed using a 100X oil immersion objective with a high numerical aperture (NA = 1.40) allowing focus in a thin focal plane within a thick section (Olympus, Ballerup, Denmark). Digital live microscope images were visualized by a high-resolution Olympus DP72 camera (Olympus), with a final magnification of 2400X.

2.10. Counting criteria

The total number of c-Fos-IR cells was counted manually by an investigator blind to the treatment groups in the counting area as defined above for the dmPFC, vmPFC, SSC and the BLA (Fig. 6 A–H). Round or oval-shaped nuclei with blue–black immunostaining darker than background were counted as c-Fos-IR nuclei. Furthermore, in the BLA, cells with orange–brown stained cytoplasm were counted as CTb-IR cells. Cells with orange–brown staining of the cytoplasm and blue–black staining of the nuclei were counted as CTb + c-Fos double-IR cells (Fig. 8H–J).

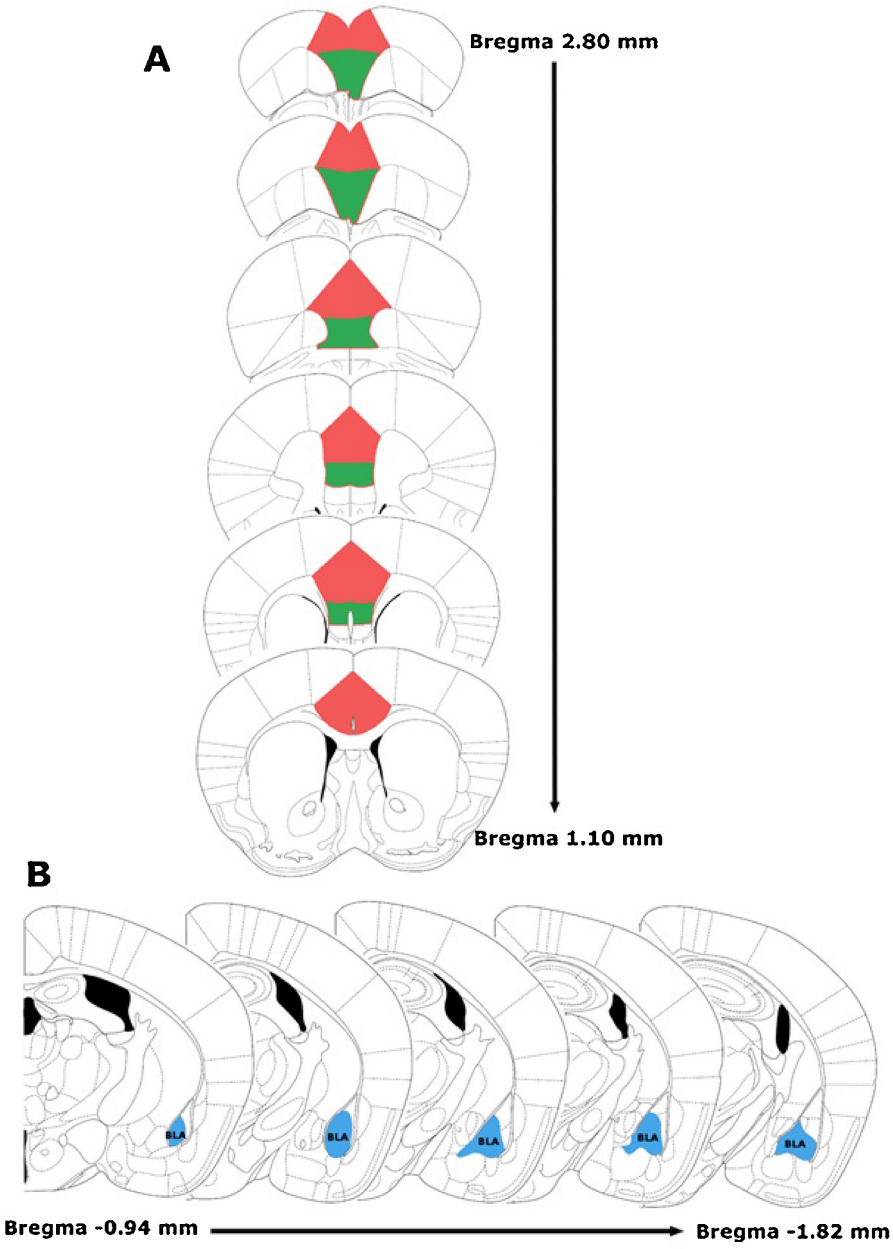

Fig. 2. The basolateral amygdala and prefrontal cortex area from which sections were sampled. (A) Coronal brain section diagrams of (A) the dorsomedial prefrontal cortex (red area), comprising both cingulate and prelimbic cortex, and ventromedial prefrontal cortex (green area), comprising medial orbitofrontal and infralimbic cortex, ranging from Bregma 2.80 mm (most anterior) to Bregma 1.10 mm (most posterior) and (B) the basolateral amygdala, anterior part, (BLA) from Bregma −0.94 mm (most anterior) to Bregma −1.82 mm (most posterior) from which sections were systematically sampled for analyses. Brain section diagrams are modified from the Paxinos mouse brain atlas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Precision of estimates – c-Fos-IR in the mPFC.

	Mean (N)	Range (N)	CE (N)	Mean (Q^-)	Range (Q^-)	Mean (P)	Range (P)	CE (V)	CV
mPFC:									
Ket/sham	8460	[3996–11401]	0.07	200	[139–271]	191	[54–427]	0.023	0.27
Veh/sham	11664	[8055–16021]	0.07	199	[145–312]	85	[48–123]	0.032	0.25
vmPFC:									
Ket/sham	2014	[978–2652]	0.08	159	[120–195]	101	[60–155]	0.03	0.3
Veh/sham	2452	[1971–3976]	0.08	170	[134–246]	88	[60–116]	0.028	0.28
SSC:									
Ket/sham	35	[0–120]	0.53	4.4	[0–15]	107.6	[96–129]	0.02	0.09
Veh/sham	41	[0–78]	0.39	5.4	[0–10]	100.4	[71–130]	0.02	0.17

Table 2
Precision of estimates – c-Fos-IR in the BLA.

	Mean (N)	Range (N)	CE (N)	Mean (Q^-)	Range (Q^-)	Mean (P)	Range (P)	CE (V)	CV
Veh/lesion	1310	[843–1844]	0.075	196	[125–260]	322	[220–616]	0.015	0.31
Ket/lesion	1198	[809–2012]	0.080	175	[109–249]	266	[199–330]	0.016	0.32
Veh/sham	1336	[794–1952]	0.078	182	[114–279]	230	[141–335]	0.016	0.27
Ket/sham	950	[707–1616]	0.090	132	[111–203]	321	[215–463]	0.015	0.29
Veh/non-sham	1160	[772–1797]	0.086	147	[110–177]	231	[160–317]	0.017	0.26
Ket/non-sham	993	[554–1495]	0.087	147	[86–186]	323	[191–555]	0.024	0.31
Homecage	787	[519–1116]	0.089	138	[92–179]	461	[233–836]	0.013	0.26

Table 3
Precision of estimates – CTb-IR in the BLA.

	Mean (N)	Range (N)	CE (N)	Mean (Q^-)	Range (Q^-)	Mean (P)	Range (P)	CE (V)	CV
Veh/sham	4539	[1958–6714]	0.082	170	[108–300]	483	[315–621]	0.013	0.39
Veh/lesion	5479	[2876–7644]	0.077	190	[121–234]	452	[403–513]	0.013	0.33
Ket/sham	4244	[1894–8547]	0.086	147	[109–227]	535	[447–626]	0.013	0.58
Ket/lesion	4302	[2525–6387]	0.077	188	[144–297]	516	[412–695]	0.013	0.37

Table 4
Precision of estimates – CTb + c-Fos-IR in the BLA.

	Mean (N)	Range (N)	CE (N)	Mean (Q^-)	Range (Q^-)	Mean (P)	Range (P)	CE (V)	CV
Veh/sham	294	[120–670]	0.12	85	[38–202]	455	[312–545]	0.013	0.51
Veh/lesion	328	[113–516]	0.11	99	[32–160]	454	[400–523]	0.013	0.39
Ket/sham	236	[55–584]	0.14	60	[12–158]	534	[442–622]	0.013	0.70
Ket/lesion	277	[80–492]	0.13	79	[25–131]	519	[416–697]	0.013	0.55

CE, coefficient of error; CTb, Cholera toxin subunit b, CV, coefficient variation; Ket, Ketanserin; N, total number of cells; P, number of frames counted; Q^-, number of cells counted Veh, vehicle; V, volume.

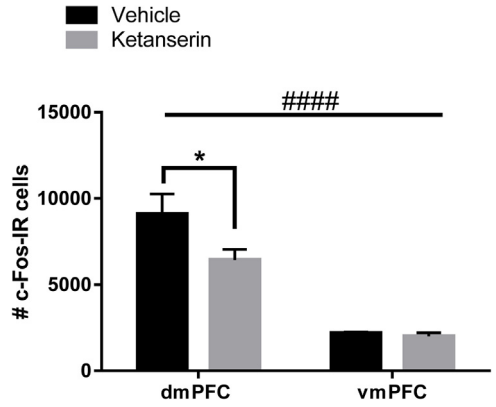

Fig. 3. Effect of ketanserin treatment on total numbers c-Fos positive neurons in the dmPFC and vmPFC in sham-lesioned mice after novelty exposure. Two-way ANOVA analysis shows a significant effect of treatment by region with a significant effect of the ketanserin treatment on the total number of c-Fos-IR neurons in dorsomedial prefrontal cortex (dmPFC), but not the ventromedial prefrontal cortex (vmPFC) area in the sham-lesioned animals. $^{####}p < 0.0001$, $^{*}p < 0.05$. Data are presented as mean ± SEM.

2.11. Statistical analysis

Repeated measures two-way ANOVA followed by post-hoc Sidak's multiple comparisons tests were applied for within-subject comparison of c-Fos-IR in the mPFC and vmPFC. For analysing c-Fos-IR and CTb + c-Fos-IR in the BLA, one-way ANOVA followed by post-hoc Sidak's multiple comparisons test was used in order to compare vehicle and ketanserin treated animals within the sham-lesioned and the NMDA-lesioned groups. Two-way ANOVA was applied in order to analyse a potential general effect of the ketanserin treatment on explorative behaviour and glucocorticoid levels, followed by post-hoc Sidak's multiple comparison test for comparing vehicle and ketanserin treated animals within the sham-lesioned and NMDA-lesioned groups. For analysis correlation, Pearson correlation test was applied. All statistical tests and graphs were generated using GraphPad Prism version 6.02 for Windows (GraphPad Software, San Diego, USA). Grubbs' outlier test was run before each analysis and significant outliers taken out. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Ketanserin blocks the dmPFC activation induced by novelty-exposure

We know from our previous study that *c-fos* expression is induced in the PFC by novelty [29]. Supporting our previous study, ketanserin administration prior to the open-field exposure blocked the induced mPFC activation, here measured by number of c-Fos-IR neurons. When comparing c-Fos-IR in dmPFC versus vmPFC in the sham-lesioned animals only, there was a significant region effect and region x treatment interaction in the total number of c-Fos-IR neurons (region effect $F(1, 13) = 107.6$, $p < 0.0001$; treatment effect $F(1, 13) = 4.353$, $p = 0.057$; interaction $F(1, 13) = 0.0418$, $p < 0.05$) (Fig. 3). Post-hoc analysis revealed a significant effect of the ketanserin treatment for the dmPFC ($df = 26$, $p < 0.05$) (Fig. 3). We counted c-Fos-IR neurons in the SSC as a control for basal levels of c-Fos-IR induced by the open-field exposure, as this is an area presumably not involved in the decision-making circuitry. Indeed, we found substantial lower numbers of c-Fos-IR cells in this area (an average of 35–40 c-Fos-IR neurons were counted on a subsample of 10 brains) compared to dmPFC and vmPFC regions. Counting data are shown in Table 1.

3.2. Ketanserin reduces the BLA activation induced by novelty-exposure, but makes BLA more reactive to the anxiogenic stimulus

Five minutes of open-field exposure increased the total number of c-Fos-IR cells in the BLA (as counted in both hemispheres) in

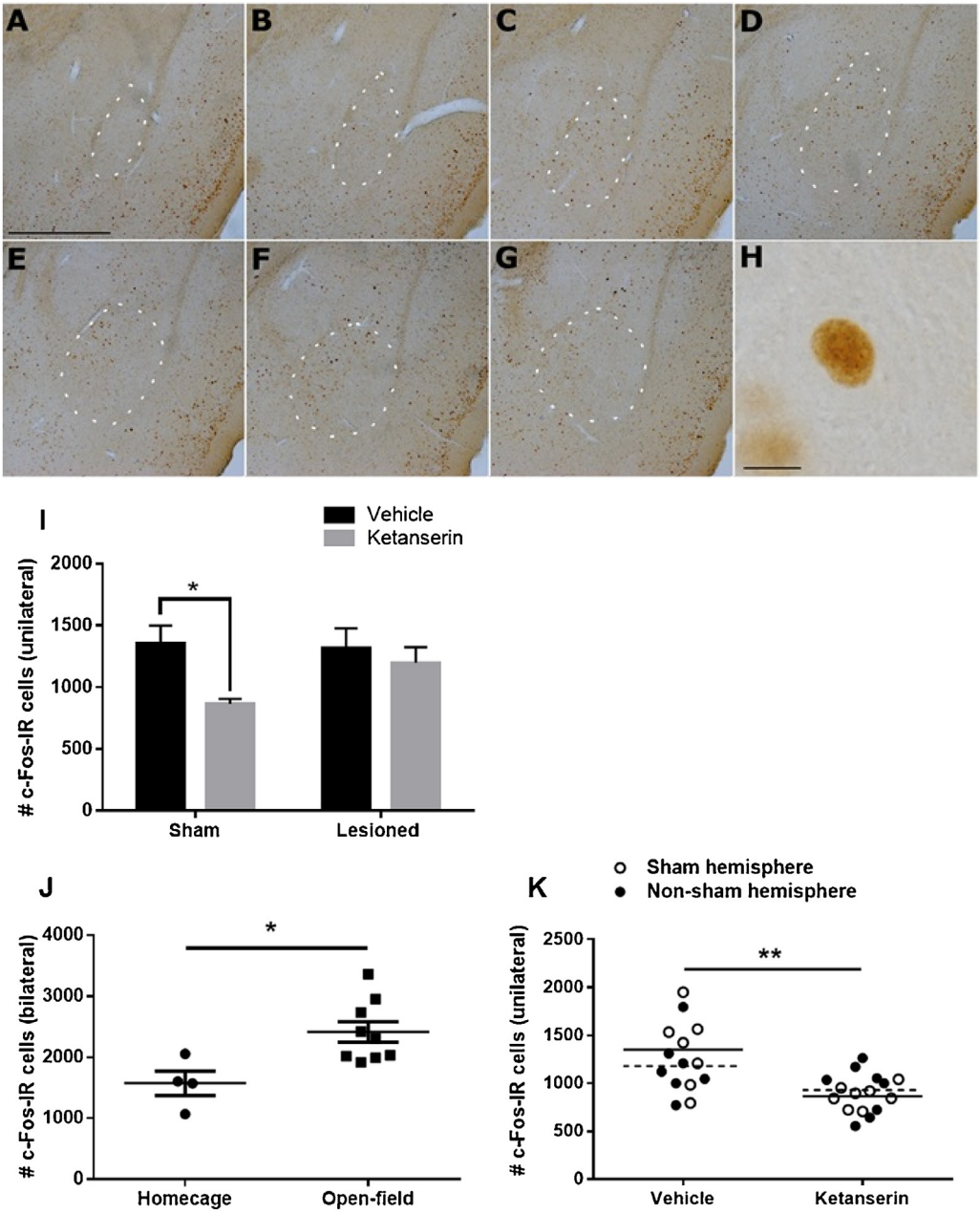

Fig. 4. Effect of ketanserin treatment and mPFC-lesion on total numbers c-Fos positive neurons in the BLA after novelty exposure. (A–G) Consecutive images of brain slices from one mouse brain comprising the BLA (white stippled lines) (scale bar, 550 μm) included for the stereological quantification of c-Fos-IR nuclei (H; scale bar, 10 μm). (I) Ketanserin significantly reduced the total number of c-Fos-IR neurons induced by the novelty-exposure in the BLA of the sham-lesioned side (one-way ANOVA, $p < 0.05$). Sidak's post-hoc multiple comparisons test shows a significant effect of ketanserin in the sham-lesioned group ($^{*}p < 0.05$). The effect of ketanserin was abolished by the excitotoxic mPFC lesion. Data are presented as mean ± SEM. (J) Scatterplot of total number of c-Fos-IR cells as calculated from countings in both hemispheres, comparing homecage control mice ($n = 4$) and vehicle-treated mice exposed to the open-field arena ($n = 9$) showing that novelty-exposure induces c-Fos-IR in the BLA (Student's *t*-test, $^{*}p < 0.05$). (K) Total number of c-Fos-IR neurons in the BLA for both the sham and non-sham hemisphere in sham-lesioned animals only showing a significant effect of ketanserin on c-Fos-IR numbers (Student's *t*-test, $^{**}p < 0.01$). Median values for the total number of c-Fos-IR neurons in both the ketanserin and vehicle-treated groups are indicated by horisontal lines in respectively the sham (solid line) and non-sham hemisphere (stippled line); IR, immunoreactivity.

the novelty-exposed versus home-cage animalsl ($df = 11$, $p < 0.05$) (Fig. 4J). Ketanserin administration prior to the open-field exposure affected the number of c-Fos-IR neurons in the BLA of the sham-lesioned hemisphere ($F(3, 26) = 3.169$; $p < 0.05$), with post-hoc multiple comparisons analysis showing a significant reduction in total number of c-Fos-IR neurons in the ketanserin-treated animals, compared to the vehicle-treated group ($df = 26$, $p < 0.05$) (Fig. 4I). When adding the BLA c-Fos-IR neuron counting from the other hemisphere, the significant effect of ketanserin was confirmed ($df = 19.62$, $p < 0.01$) (Fig. 4K). In the NMDA-lesioned group, the attenuating effect of ketanserin on c-Fos-IR in the BLA ipsilateral to the lesion was blocked, indicating that the effect of ketanserin on BLA most likely is mediated through the mPFC (Fig. 4I). In the sham-lesioned group, ketanserin treatment generated a positive correlation between the number of c-Fos-IR neurons in the BLA and the relative time spent in the centre versus border + centre ($r = 0.7992$, XY pairs = 8, $p < 0.05$) of the open-field arena (Fig. 5A). This correlation was abolished by the mPFC lesion ($r = 0.1843$, $p > 0.05$) (Fig. 5B). For a mouse in the open field arena, stay along the open-field border is considered the safe area, whereas cen-

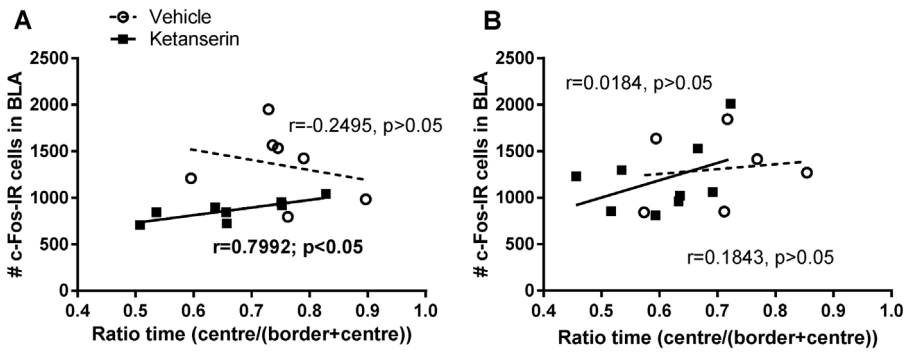

Fig. 5. Correlation between number of c-Fos-IR neurons in the BLA and open-field behaviour after 5-HT_{2A}R blockade. (A) In the sham-lesioned ketanserin-treated group there was a significant correlation between ratio of time spent in the centre versus border + centre and total number of c-Fos-IR neurons in the BLA (Pearson, r = 0.7992, p < 0.05). No correlation was observed for the vehicle-treated animals (Pearson, r = −0.2495, p > 0.05). (B) This effect was abolished in the ketanserin-treated mPFC-lesioned animals (Pearson, r = 0.1843, p > 0.05) and no correlation was either observed for the vehicle-treated mPFC lesioned animals (Pearson, r = 0.0184, p > 0.05). Countings for c-Fos-IR cells in BLA is done unilaterally for the sham and lesioned hemisphere respectively.

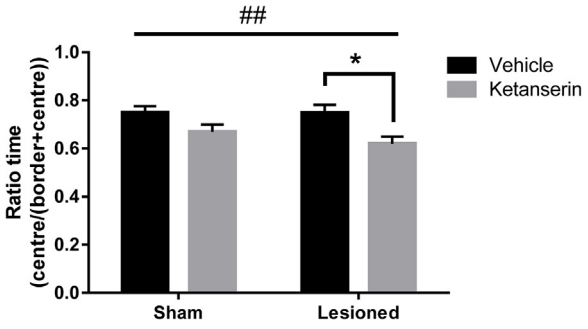

Fig. 6. Ratio of time spent in the centre versus total time spent in the border and centre during 5 min exposure to the novel open-field arena. A significant effect of ketanserin was observed for relative time spent in centre versus border + centre (two-way ANOVA, ##p < 0.01) for both sham-lesioned and NMDA-lesioned group. Sidak's post-hoc multiple comparisons test shows a significant difference between the effects of ketanserin and vehicle administration in the mPFC-lesioned animals for the relative time spent in the centre versus total border + centre time (*p < 0.05). Data are presented as mean ± SEM.

tre stay is considered to be more anxiogenic. No correlation was observed for the vehicle-treated group for the relative time spent in centre versus total border + centre time in either the sham-lesioned (r = −0.2495, XY pairs = 7, p > 0.05) or the mPFC lesioned animals (r = 0.0184, p > 0.05) (Fig. 5A, B). Only countings from the sham-lesioned and NMDA-lesioned hemispheres were included. Counting data are shown in Table 2.

3.3. Ketanserin induces aversive behaviour and mPFC lesioning intensifies this effect

Ketanserin had a significant effect on the time the mice spent along the border versus in the centre of the open-field arena. For both the sham-lesioned and NMDA-lesioned group, during the 5 min exposure to the open-field arena, the ketanserin-injected mice spent less relative time in the centre versus border + centre (treatment effect F(1,39) = 12.04 p < 0.01) as compared to vehicle-treated mice. There was no interaction effect of the lesion with the treatment, but post-hoc multiple comparisons test revealed a significant difference between ketanserin and vehicle treatment for the mPFC lesioned mice in time spent in the centre versus border + centre (df = 39, p < 0.05) (Fig. 6).

3.4. Ketanserin induces increased plasma corticosterone levels and more locomotor activity

Levels of plasma corticosterone were within the range 186.1 ng/ml (for the vehicle-injected sham group) and 230.9 ng/ml (for the ketanserin-injected sham group). Two-way ANOVA showed a significant effect of ketanserin (treatment effect F(1,24) = 4.967, p < 0.05) on corticosterone levels, with ketanserin increasing the glucocorticoid response induced by the novelty-exposure (Fig. 7A). Ketanserin also had a significant effect on the total distance travelled in the open-field by the mice, with ketanserin-injected animals presenting longer distance moved (treatment effect F(1,40) = 7.695, p < 0.01) (Fig. 7B). There was no

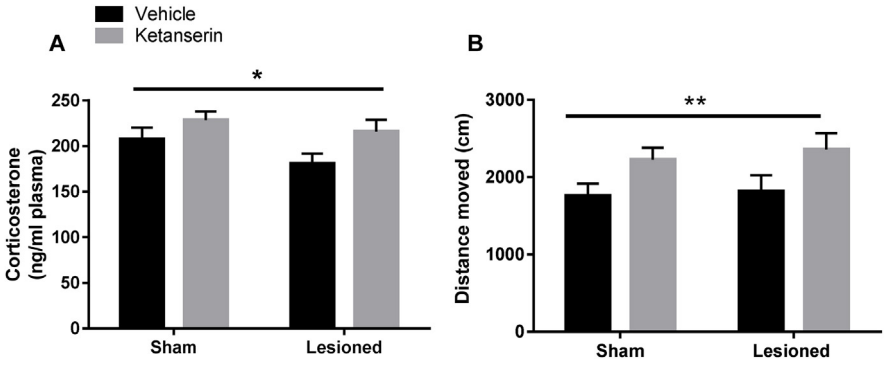

Fig. 7. Plasma corticosterone levels and exploratory behaviour during the novelty-exposure. In the ketanserin-treated group a significant increase in plasma corticosterone levels induced by the novelty-exposure was observed (two-way ANOVA, *p < 0.05), with no group differences between the sham-lesioned and mPFC-lesioned groups after post-hoc multiple comparisons test (A). Treatment had also a significant effect on exploratory behaviour, with ketanserin administered animals showing longer distance moved during the 5 min open-field exposure (two-way ANOVA, **p < 0.01). No group differences were observed between the sham-lesioned and mPFC-lesioned groups after post-hoc multiple comparisons test. Data are presented as mean ± SEM.

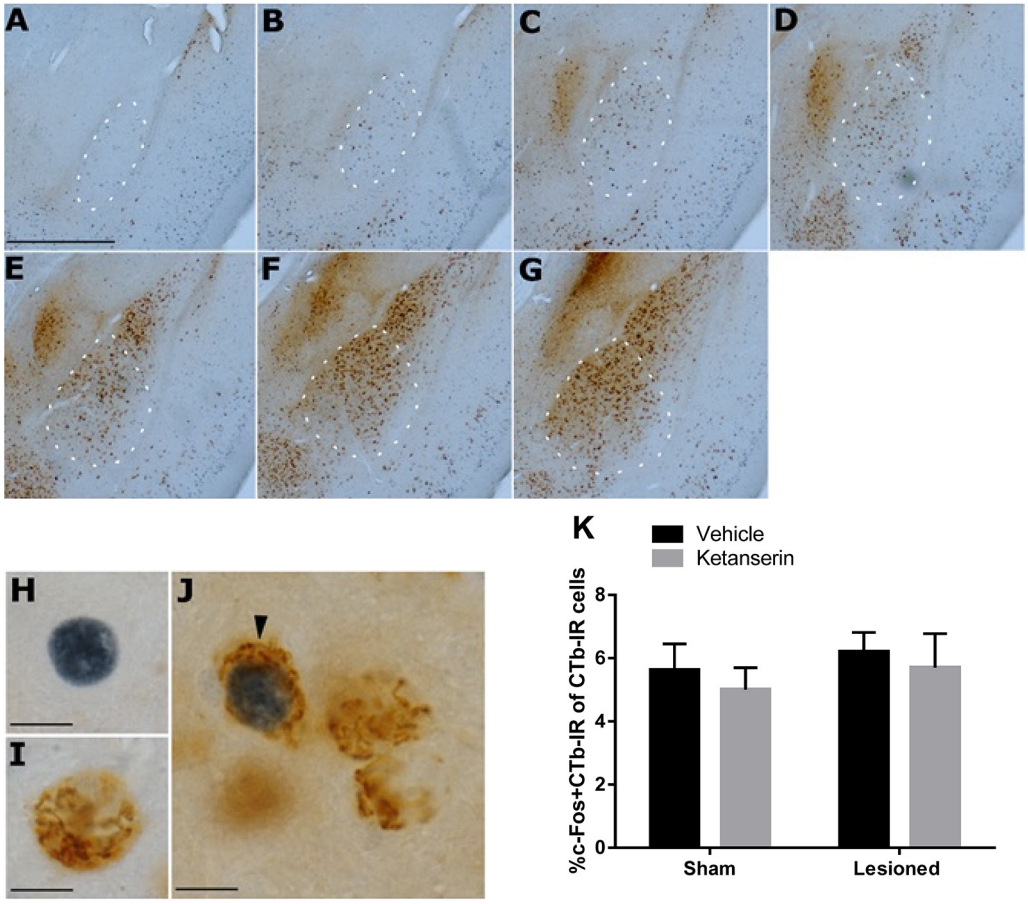

Fig. 8. No significant effect of ketanserin or the mPFC lesion on number of activated of VS-BLA projecting neurons. (A–G) Consecutive images of brain slices from one brain comprehending the BLA (white stippled lines) (scale bar, 550 μm) included for the stereological quantification of CTb-IR neurons (I; brown-stained neuron) and CTb-IR neurons also being c-Fos-IR (J); brown-stained neuron with blue/grey-stained nucleus; (H) c-Fos-IR nucleus (blue/grey stained) (scale bars, 10 μm). (K) CTb + c-Fos-IR cells were stereologically quantified in sham-lesioned and mPFC-lesioned animals to investigate for an effect of ketanserin treatment on VS-BLA projecting neurons activated by the novelty-exposure. No effect of ketanserin or the mPFC lesion was observed (One-way ANOVA, $p > 0.05$). A small fraction of CTb-toxin labelled neurons were activated during novelty-exposure (5.2–6.1%). IR, immunoreactivity; CTb, Cholera Toxin subunit b. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction effect of the lesion with the treatment and no significant differences between ketanserin and vehicle treated animals within the sham-lesioned and mPFC-lesioned groups.

3.5. Activation of BLA neurons projecting to VS is not affected by 5-HT$_{2A}$R blockade

The proportion of BLA neurons projecting to the VS (identified by CTb-IR) activated by the novelty-exposure (identified by CTb + c-Fos-IR) was for the vehicle-treated group, 6.0% [range 3.9–8.3%] in the mPFC-lesioned mice and 6.4% [range 4.0–10.0%] in the sham-lesioned mice; and for the ketanserin-treated group, 6.1% [range 2.9–8.9%] for the mPFC-lesioned mice and 5.2% [range 2.2.–7.4%] for the sham-lesioned mice. One-way ANOVA analysis did not show a significant effect of ketanserin in either sham-lesioned or mPFC-lesioned group ($F(3,24) = 0.368$, $p > 0.05$) on the number of activated BLA-VS projecting neurons (Fig. 8K). Counting data are shown in Tables 3 and 4.

4. Discussion

Mapping the effect of 5-HT$_{2A}$R activation on neural substrates involved in specific cognitive tasks is highly relevant for understanding the role of this receptor in executive function. Here we show how pharmacologically blocking this receptor attenuates the neuronal activation induced in the mPFC and BLA by exposure to a novel environment, and how this is associated with an increased stress response and more anxiogenic-like behaviour in mice. Further, we show that this does not involve a change in the activation of the amygdala-striatal projection. Our results add support to the hypothesis that the 5-HT$_{2A}$R, through modulation of mPFC activation, exerts a top-down control on amygdala activation when exposed to a novel environment. The 5-HT$_{2A}$R mediated top-down control does not seem to involve BLA neurons projecting to VS.

The 5-HT$_{2A}$R antagonist used in this study, ketanserin, has also affinity for the 5-HT$_{2C}$ receptor, so we cannot exclude an involvement of this receptor in the effect observed. However, the affinity of ketanserin for the 5-HT$_{2A}$ receptor is 14-fold higher than for the 5-HT$_{2c}$R, and since we used a low dose (0.5 mg/kg) we do not expect a strong involvement of the 5-HT$_{2C}$ receptor. Furthermore, the dose applied was selected based upon our previous study [29], where we moreover excluded an involvement of the 5-HT$_{2C}$ receptor on the effect of ketanserin [29]. Further supporting this, functional autoradiography studies on rat brain slices by 5-HT$_{2A/2C}$ agonist-stimulated [^{35}S]GTPγS binding present a stronger involvement of 5-HT$_{2A}$R than 5-HT$_{2C}$ receptor in mPFC regions [47].

c-Fos expression as a measure of PFC and amygdala activation is a well-validated approach [30–32] and proven to be independent of the exploratory activity [33], the physiological stress-response or sensory stimulation induced by the novel environment [48]. In

our previous study we showed how the novelty-induced c-Fos gene expression in the PFC is 5-HT_{2A}R-dependent [29]. The present study confirmed these observations of a blocking effect of ketanserin on the novelty-induced PFC activation (as represented by number of c-Fos-IR neurons), and we further extended these results with a subregion-specific analysis showing that during novelty the ketanserin-induced effect on c-Fos-IR in the mPFC resides in the dmPFC and not the vmPFC. This is consistent with prelimbic PFC, and thereby dmPFC, being the mPFC region with strong projections to the BLA as opposed to the infralimbic cortex, with only few, if any, projections to the BLA [49]. The present study further included the other neural substrates involved in the emotion-based network. Based on human neuroimaging studies this is believed to involve the frontal-striatal-amygdala circuitry [50]. By use of a well-validated and high standard unbiased stereological methodology [45] that allows for accurate estimations of total c-Fos-IR cell numbers in well-defined brain regions, we could detect a decrease in neuronal activity of the BLA in the ketanserin-treated animals – an observation which was further supported when taking c-Fos positive BLA nuclei from both hemispheres into account.

The 5-HT_{2A}R is also expressed locally in the BLA neurons, primarily on GABAergic parvalbumin-expressing interneurons [51,52], so we cannot exclude a local effect of ketanserin on the neuronal activation measured in the BLA. However, the effect of ketanserin on BLA c-Fos expression was diminished in the mPFC lesioned animals, thereby supporting the involvement of this region in the modulatory effect of 5-HT_{2A}R blockade on BLA neuronal activation. Ketanserin treatment made the amygdala more sensitive to the aversive exposure, as shown by the positive correlation between relative time spent in the centre and number of c-Fos positive neurons. Thus, turning off 5-HT_{2A}R mediated mPFC activation made the BLA more reactive. When totally disconnecting the mPFC by the lesion, this effect was abolished. In the vehicle group, even though not significant, there was a tendency to the contrary.

Previous findings support a modulatory effect of cortical 5-HT_{2A}R activation on emotional responses to a novel environment in rabbits [53] and mice [54]. Human functional imaging studies have moreover shown an inverse relationship between the density of 5-HT_{2A}Rs in the mPFC and amygdala reactivity during aversive responses [41]. Thus, the results obtained in the present study are in line with these observations and our presumption that lower 5-HT_{2A}R activation in PFC is associated with more risk averseness [55]. Nevertheless, we expected the stronger BLA reactivity induced by the blockade of 5-HT_{2A}R to be revealed by a general increase in c-Fos-IR in BLA in the ketanserin-treated group and not a decrease as observed. An explanation to this can be that different subsets of neurons get activated in the amygdala during the process of novelty exploration. As our experimental setup only allows us to visualize a specific time-point we may be overseeing that the main difference relies on the transition between the neuronal ensembles activated during the novelty-exposure. Functional connectivity between mPFC-BLA is bidirectional – amygdala activation first functions as a bottom-up incoming signal to the mPFC, where it gets integrated with other incoming inputs and assigned a signal valence that is sent back to the amygdala in order to modulate its activation [56]. BLA neuronal firing is therefore an expression of both fear and safety signals [57,58], with a subset of neurons specifically firing during diminished fear [57]. In the ketanserin-treated animals there may be a delay in the activation of this group of neurons in the BLA explaining the reduced c-Fos expression and the more aversive behaviour. The switch between fear and safety signals is mediated by gamma oscillation synchronization in the BLA and mPFC and its amplitude phase coupling to theta waves originating in the mPFC [57]. This process involves the 5-HT_{2A}R through activation of fast-spiking parvalbumin containing interneurons in the mPFC [6,59]. Interestingly, the amygdala input to the mPFC projects specifically onto parvalbumin interneurons [60] and blockade of the 5-HT_{2A}R causes desynchronization of gamma waves in the mPFC [59]. These observations together point to a role of the 5-HT_{2A}R in modulating communication between mPFC and BLA and thereby extend/pace of habituation to a new stimulus. Disruption of this communication by the mPFC lesion exacerbates the anxiogenic-like behaviour.

The enclosed space model used in this study involves avoidance responses rather than anxiety responses more characteristic of an elevated open-field without surrounding walls [61]. The animal's behaviour therefore reflects the conflict between exploring the centre of the arena or stay at the borders, which the mouse perceives as safer. Remaining against the walls of the open-field arena resembles passive avoidance in humans [61], revealing a stronger risk-averseness. Our finding that blockade of the 5-HT_{2A}R by ketanserin induces a longer stay along the walls of the open-field arena is supported by a previous pharmacological fMRI study, where ketanserin ingestion made participants more risk-averse in a gambling task [42]. Partly lesioning the mPFC exacerbated the effect of ketanserin on risk-averseness supporting an involvement of this area in the behavioural effect. Solely lesioning the mPFC unilaterally did not have an effect, indicating that the 5-HT_{2A}R blockade is necessary for inducing the risk-aversive behaviour. It may be that 5-HT_{2A}R blockade hinders the compensatory effect of the intact side in the partially lesioned mPFC animals, intensifying the effect that switching off mPFC activation has on the behaviour. Other serotonin receptors, like the 5-HT_{1A} receptor [62], and neurotransmitter systems, like the dopaminergic [63], are known to be involved in regulating the BLA-PFC pathway. Thus lesioning the mPFC may affect a broader set of systems involved in the modulation of behaviour than solely the 5-HT_{2A}R, and therefore we have to be cautious not to interpret the anxiogenic-like behaviour only in relation to the blockade of 5-HT_{2A}R and change in amygdala activation. Further, other pathways and neural substrates, like the hippocampus, are also involved in fear responses to novelty-exposure [64], and are likely affected by the mPFC lesion. The key conclusion from our observations is that 5-HT_{2A}R is involved in modulating mPFC and BLA activation and to some extent the risk-aversive behaviour during novelty-exposure.

The BLA is connected to the VS, and together with the mPFC comprises the emotional system engaged during behavioural responses [22,24]. The VS refers to a part of the striatum that mainly consists of the nucleus accumbens and is involved in integrating information about reward, context, and motivational drive and through that gate the formation of action intention in the dorsomedial striatum [24]. Input from the mPFC [65] and the BLA [26,27] to the VS is involved in modulating reward-seeking behaviours and by that also in driving impulsive behaviours [66]. We and others have reported 5-HT_{2A}R activation to be associated with increased impulsivity [67–69] with reward-seeking or risk-aversive behaviours probably modulated by PFC 5-HT_{2A}R activation level [55]. Therefore, we wanted to examine in the present experimental set up whether the 5-HT_{2A}R modulatory effect upon BLA reactivity extended on neurons projecting from the BLA to the VS. Our observations do not support a role of the 5-HT_{2A}R or the mPFC in modulating activity of the BLA-VS projecting neurons during novelty-exposure. The most plausible explanation is that the animal is not presented to reward cues or incentives during the exploration of the open-field that may engage the BLA-VS or VS-mPFC projection. Indeed, only a small proportion – around 6% of the VS projecting BLA neurons were activated during the novelty-exposure.

In summary, our data adds further support to the belief that the 5-HT_{2A}R plays a role in modulating the mPFC and subcortical areas involved in the emotion-based circuitry and activated during novelty-exposure. A likely contribution of this receptor may be in

regulating the signalling strength between mPFC and BLA during risk-assessment, as we found no evidence for the BLA-VS projection to be involved in the risk-aversive behaviour or to be affected by the 5-HT_{2A}R blockade.

Conflict of interest

None.

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