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Effect of Neuropeptide Y Y2 Receptor Deletion on Emotional Stress-Induced Neuronal Activation in Mice

NGOC KHOI NGUYEN,[1] SIMONE B. SARTORI,[1] HERBERT HERZOG,[2] RAMON TASAN,[3] GÜNTHER SPERK,[3] AND NICOLAS SINGEWALD[1]*

[1]*Department of Pharmacology and Toxicology, and Center for Molecular Biosciences, University of Innsbruck, Innsbruck, Austria*

[2]*Neuroscience Program, Garvan Institute of Medical Research, Sydney, Australia*

[3]*Department of Pharmacology, Innsbruck Medical University, Innsbruck, Austria*

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ABSTRACT In different behavioral paradigms including the elevated plus maze (EPM), it was observed previously that deletion of the neuropeptide Y Y2 receptor subtype results in potent suppression of anxiety-related and stress-related behaviors. To identify neurobiological correlates underlying this behavioral reactivtiy, expression of c-Fos, an established early marker of neuronal activation, was examined in Y2 receptor knockout ($Y2^{-/-}$) vs. wildtype (WT) mice. Mice were placed on the open arm (OA) or closed arm (CA) of the EPM for 10 min and the effect on regional c-Fos expression in the brain was investigated. The number of c-Fos positive neurons was significantly increased in both WT and $Y2^{-/-}$ lines after OA and CA exposure in 51 of 54 regions quantified. These regions included various cortical, limbic, thalamic, hypothalamic, and hindbrain regions. Genotype influenced c-Fos responses to arm exposures in 6 of the 51 activated regions: the cingulate cortex, barrel field of the primary somatosensory cortex, nucleus accumbens, dorsal lateral septum, amygdala and lateral periaqueductal gray. These differences in neuronal activity responses to the novel environments were more pronounced after OA than after CA exposure. Mice lacking Y2 receptors exhibited reduced neuronal activation when compared to WT animals in response to the emotional stressors. Reduced neuronal excitability in the identified brain areas relevant to the processing of motivated, explorative as well as anxiety-related behaviors is suggested to contribute to the reduced anxiety-related behavior observed in $Y2^{-/-}$ mice. **Synapse 63:236–246, 2009.**

INTRODUCTION

Neuropeptide Y (NPY), a highly conserved 36 amino acid peptide, is abundantly expressed in the central and peripheral nervous systems (Tatemoto et al., 1982). The biological actions of NPY are mediated by the activation of at least five receptors known as the Y1, Y2, Y4, Y5, and Y6 receptor (for review, see Michel et al., 1998; Pedrazzini et al., 2003). In the brain Y1 and Y2 receptors are the most abundant, with high expression of Y1 receptors in the cortex, hippocampus, and thalamic nuclei and of Y2 receptors in the hippocampal formation, lateral septum, amygdala, and locus coeruleus (Dumont et al., 1996, 1998; Kopp et al., 2002; Stanic et al., 2006). However, while Y1 receptors are mainly localized on somata and dentrites, Y2 receptors mostly appear to function as presynaptic receptors (Kopp et al., 2002; Stanic et al., 2006; Wahlestedt et al., 1986, 1993; for review, see Pedrazzini et al., 2003).

NPY has been shown to serve important roles in a number of physiological functions including ingestive behavior, energy homeostasis, cardiovascular regulation, stress, memory, and seizures (Michalkiewicz

N.K.N. and S.B.S. contributed equally to this work.

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*Correspondence to: Nicolas Singewald, Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Innsbruck, Peter-Mayr-Str. 1, A-6020 Innsbruck, Austria. E-mail: nicolas.singewald@uibk.ac.at

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et al., 2001; Pedrazzini et al., 1998; Sainsbury et al., 2002; Sweerts et al., 2001; for review, see Pedrazzini et al., 2003). Furthermore, NPY has a critical role in the regulation of anxiety-like responses as results from behavioral experiments indicate that central administration of NPY induces anxiolytic-like effects in various animal paradigms including the elevated plus maze (EPM), light/dark test, Montgomery's and Vogel's conflict tests (Heilig et al., 1989, 1993; Karlsson et al., 2005; Kask et al., 1998; Nakajima et al., 1998; Pich et al., 1993). Accumulating evidence indicates that the anxiolytic-like response of NPY is mediated via Y1 receptors. For example, intracerebroventricular injection of antisense oligodeoxynucleotide targeting the Y1 receptor mRNA, resulting in decreased density of Y1 receptors, is associated with a reduction of anxiolytic effects of intra-amygdalar injection of NPY (Heilig, 1995). Additionally, injection of the Y1 receptor antagonist BIBO3304 into the basolateral amygdala results in the blockade of the anxiolytic-like effects of NPY in the social interaction test (Sajdyk et al., 1999). Interestingly, the genetic deletion of the Y1 receptor in mice induces an anxiogenic-like effect in the light/dark test box, but an anxiolytic-like effect in the open field and EPM, which is explained by the influence of additional factors such as circadian rhythm (Karl et al., 2006; Karlsson et al., 2008).

On the other hand, there is also growing evidence for an involvement of Y2 receptors in anxiety. The administration of Y2 receptor agonists has demonstrated anxiogenic effects in the social interaction test and in the EPM (Nakajima et al., 1998; Sajdyk et al., 2002), whereas a selective Y2 receptor antagonist has demonstrated an anxiolytic-like profile in the EPM (Bacchi et al., 2006). Studies on Y2 knockout ($Y2^{-/-}$) mice have confirmed that the deletion of Y2 receptors suppresses anxiety-related behaviors in the EPM, light/dark test, and open field paradigms (Painsipp et al., 2008; Redrobe et al., 2003; Tschenett et al., 2003), which may contribute to their enhanced impulsivity (Greco and Carli, 2006).

Until now, no studies have been conducted in order to investigate neuronal activation patterns associated with the altered behavioral reactivity to novelty of $Y2^{-/-}$ mice. Indeed, the anxiolytic effect of Y2 gene disruption on the EPM as revealed in independent studies (Painsipp et al., 2008; Redrobe et al., 2003; Tschenett et al., 2003) offers an opportunity to investigate neuronal correlates underlying their behavioral responses by using c-Fos immunohistochemistry as a marker of neuronal activation (for review, see Hoffman and Lyo, 2002; Singewald, 2007). Based on the results of the EPM studies (see above), we hypothesized that $Y2^{-/-}$ mice perceive the open arm (OA) of an EPM as less anxiogenic than wildtype (WT) mice. Consequently, less neuronal activation in critical anxiety-related brain regions should indicate neuronal populations contributing to or mediating the altered neurobehavioral reactivity to novelty of $Y2^{-/-}$ mice vs. WT mice.

MATERIALS AND METHODS

Animals

Naïve age-matched (22-weeks-old), male mice were used in all experiments. The generation of germline $Y2^{-/-}$ mice was described previously (Sainsbury et al., 2002). Noninduced conditional $Y2^{lox/lox}$ mice that do not differ from WT mice in terms of Y2 receptor binding, body weight, or plasma corticosterone levels (Sainsbury et al., 2002) were used as controls and are termed hereafter as WT mice. Germline $Y2^{-/-}$ and conditional $Y2^{lox/lox}$ mice were generated from the same founders on a 129Sv × C57BL/6 background (Sainsbury et al., 2002). All mice were housed in groups of 3–5 mice per cage on a 12:12 h light/dark schedule (lights on at 07.00) with food and water available ad libitum. Forty-eight hours before the experiment, animals were single housed, taken in their home cages from the animal facility to the experimental rooms, and allowed to habituate. Behavioral experiments were carried out during the light phase of the cycle from 9.00 to 14.00. The experimental studies described here were designed to minimize animal suffering and number of animals used. They were approved by the Ethical Committee on Animal Care of the Austrian Ministry for Education, Science and Art and are in compliance with international laws and policies.

Experiment 1: c-Fos induction in response to open arm exposure

Mice (WT: $n = 9$; $Y2^{-/-}$: $n = 9$) were placed in the middle part of the OA (30 × 5 cm, rims 2 mm, elevation 73 cm) of an EPM for 10 min, where the access to the neutral zone and the closed arms (CAs) of the maze was prevented by a bar (Salome et al., 2004, 2006). The light intensity on the OA was about 300 lux. During the 10-min arm exposure, the distance the animals traveled on the OA was tracked by an automated system (videomot, TSE, Bad Homburg, Germany). Immediately after OA exposure, animals were returned to their home cages. The OA was cleaned thoroughly with water before each trial. Animals assigned to the basal group (WT: $n = 5$; $Y2^{-/-}$: $n = 5$) remained undisturbed in their home cages.

Experiment 2: c-Fos induction in response to open vs. closed arm exposure

Stimulated by studies showing that rodents show stronger emotional responses to a forced OA vs. a CA exposure of an EPM (Holmes and Rodgers, 1999; Pellow et al., 1985), we decided to include another control condition. Therefore, mice were subjected either

to the OA (WT: $n = 4$; $Y2^{-/-}$: $n = 4$) or the CA (WT: $n = 7$; $Y2^{-/-}$: $n = 6$). The CA was identical to the OA, but was enclosed by 14-cm high, nontransparent walls. Settings and procedure for both OA and CA exposure were as in Experiment 1.

c-Fos immunohistochemistry

Given that stress-induced c-Fos protein expression has been shown to reach its maximum after 2 h (e.g., Zangenehpour and Chaudhuri, 2002), we used this time frame also in the present study. Accordingly, 2 h after onset of OA or CA exposure, animals were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 20 mL of 0.9% saline followed by 20 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffered solution (PBS, pH 7.4). Animals not exposed to the OA were treated identically immediately after removal from their home cages. Brains were then removed and postfixed in 4% paraformaldehyde in PBS at 4°C overnight. Coronal sections (100 μm) were cut with a vibratome (Ted Pella, Redding, CA) and collected freely floating in PBS.

The sections were processed for c-Fos immunoreactivity as described previously (Singewald et al., 2003). They were incubated for 48 h in a polyclonal primary antibody (s.c.-52, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:20,000 in immunobuffer (pH 7.4) comprising 0.1 mol/L NaCl, 5 mmol/L KCl, 8 mmol/L Na_2HPO_4, 15 mmol/L NaH_2PO_4, 10 mmol/L Tris-HCl, 0.3% Triton X-100, and 0.04% thimerosal. The rabbit primary antibody was raised against a peptide in the amino terminus of human c-Fos p62 identical to the corresponding mouse sequence and does not cross-react with FosB, Fra-1, or Fra-2. The sections were then rinsed and incubated with a biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 24 h. An avidin-biotin-horseradish peroxidase procedure with 3,3′-diaminobenzidine as the chromogen was used to visualize the immunoreactivity. Cells containing a nuclear brown-black reaction product were considered positive for c-Fos-immunoreactivity and are referred to hereafter as c-Fos-positive cells. The anatomical localization of c-Fos-positive cells was aided by using the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997). For quantitative analysis, the number of c-Fos-positive cells was counted bilaterally in a tissue area of 0.01 mm^2 in 54 areas with the exception of specific cortical areas where tissue areas of 0.04 mm^2 were evaluated (Fig. 1).

Data and statistical analysis

Results are expressed as means ± SEM. Data analysis of the number of c-Fos-positive cells per brain region examined as well as of the distance traveled on the respective arms was performed using a two-way ANOVA for genotype and stressor or arm in Experiments 1 and 2, respectively. Two group comparisons were made by Bonferroni test or by independent *t*-test. P values less than 0.05 were considered to be significant, while P values less than 0.09 were used to indicate a trend toward significance.

RESULTS

Locomotion of WT and $Y2^{-/-}$ mice during OA and CA exposure

When placed on a particular arm of an EPM, mice irrespective of genotype explored the OA and the CA with similar interest as the distance traveled did not differ between the two arm types. During the 10-min arm exposure, locomotor activity was comparable between WT and $Y2^{-/-}$ mice in Experiment 1 as well as in Experiment 2 (Table I).

Experiment 1: c-Fos induction in response to open arm exposure

Mean numbers ± SEM of cells expressing c-Fos in all quantified brain regions are shown in Table II. Two-way ANOVA (stress × genotype) revealed that exposure to the OA for 10 min induced increased c-Fos expression in 51 of the 54 investigated brain areas, including parts of the medial prefrontal cortex (cingulate, prelimbic, infralimbic cortex), piriform cortex, bed nucleus of the stria terminalis, lateral septum, habenula, various amygdaloid and hypothalamic nuclei, the parts of the periaqueductal gray and locus coeruleus. Genotype significantly affected the number of c-Fos-positive cells induced by OA exposure in the prelimbic cortex ($F_{1,27} = 9.14$; $P = 0.006$; Figs. 1 and 2), barrel field of the primary somatosensory cortex ($F_{1,27} = 16.34$; $P < 0.001$; Figs. 1, 2, and 3A), nucleus accumbens core region ($F_{1,26} = 7.49$; $P = 0.012$; Figs. 1, 2, and 3A), dorsal part of the lateral septum ($F_{1,26} = 6.14$; $P = 0.021$; Figs. 1, 2, and 3A), granular layer of the dentate gyrus ($F_{1,27} = 21.13$; $P < 0.001$), CA3 pyramidal cell layer of the hippocampus ($F_{1,27} = 6.22$; $P = 0.020$), posteroventral part of the medial amygdala ($F_{1,26} = 4.53$; $P = 0.044$; Figs. 1, 2, and 3A), lateral periaqueductal gray ($F_{1,27} = 5.38$; $P = 0.029$), and central nucleus of the inferior colliculus ($F_{1,27} = 7.47$; $P = 0.012$). Furthermore, a trend toward a significant genotype effect was found in the cingulate cortex ($F_{1,27} = 3.68$; $P = 0.067$; Figs. 1, 2, and 3A), claustrum ($F_{1,27} = 3.72$; $P = 0.066$), zona incerta ($F_{1,27} = 3.77$; $P = 0.064$), and ventral part of the lateral septum ($F_{1,26} = 4.25$; $P = 0.051$). Stress × genotype interactions were detected in the prelimbic cortex ($F_{1,27} = 6.01$; $P = 0.022$), barrel field of the somatosensory cortex ($F_{1,27} = 12.38$; $P = 0.002$), nucleus accumbens core region ($F_{1,22} = 7.14$; $P = 0.014$), granular layer of the dentate gyrus ($F_{1,27} = 7.66$; $P = 0.011$), and CA3 pyramidal cell layer of the hippocam-

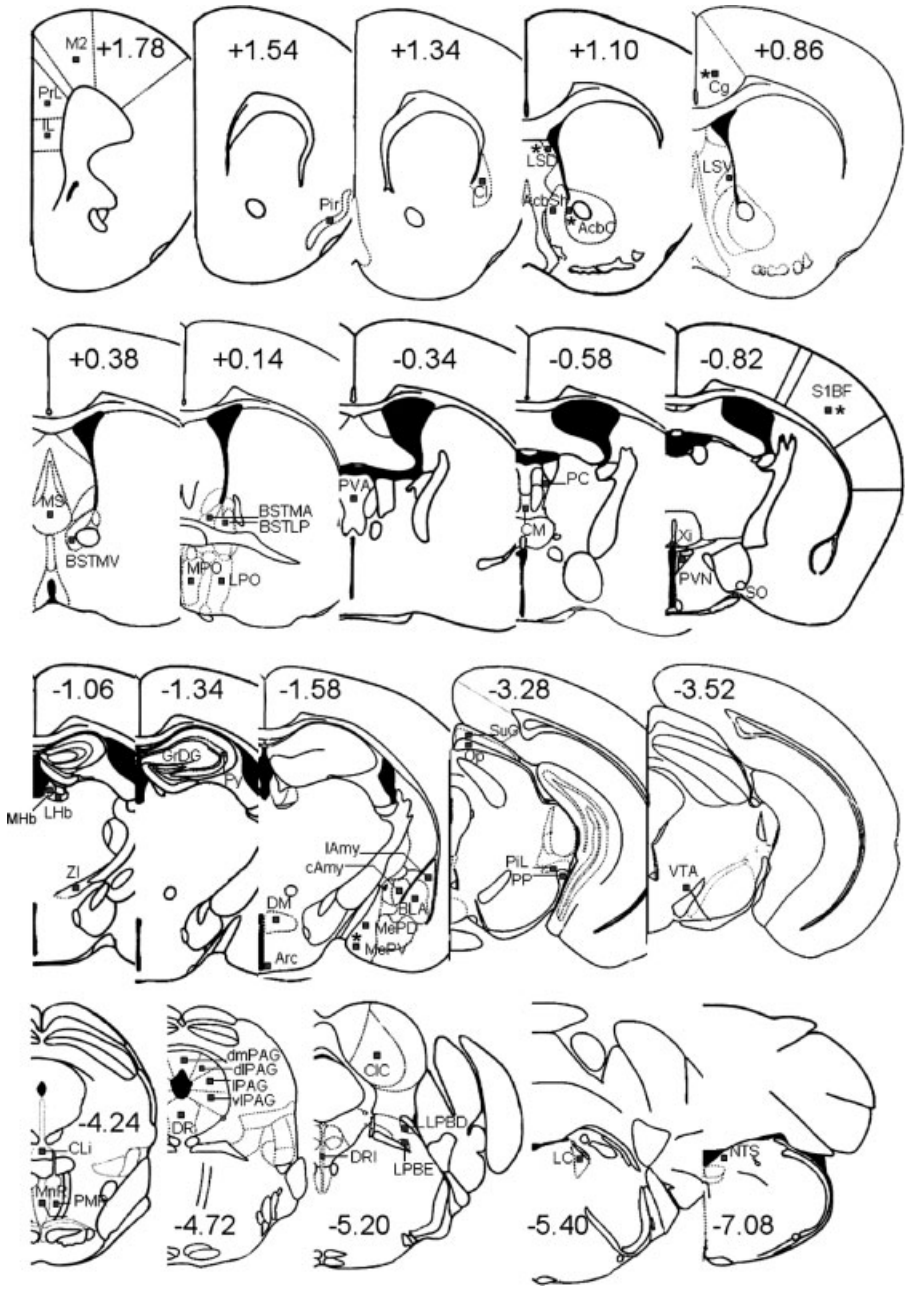

Fig. 1. Schematic diagrams adapted from the mouse brain atlas (Franklin and Paxinos, 1997), showing the 54 brain regions in which c-Fos expression was evaluated. The squares indicate the placement of grids for counting of c-Fos positive cells. Asterisks indicate the regions in which genotype ($Y2^{-/-}$ vs. WT mice) influenced c-Fos induction to open or closed arm exposure in Experiments 1 and 2. The number in each slide indicates the Bregma level of brain section. Abbreviations in alphabetical order: AcbC, accumbens nucleus, core; AcbSh, accumbens nucleus, shell; Arc, arcuate hypothalamic nucleus; BLA, basolateral amygdaloid nucleus; BSTLP, bed nucleus of the stria terminalis, lateral division, posterior part; BSTMA: bed nucleus of the stria terminalis, medial division, anterior part; BSTMV, bed nucleus of the stria terminalis, medial division, ventral part; cAmy, central nucleus of the amydala; Cg, cingulate cortex; CIC, central nucleus of the inferior colliculus; Cl, claustrum; Cli, caudal linear nucleus of the raphe; CM, central medial thalamic nucleus; dlPAG, dorsolateral periaqueductal gray; DM, dorsomedial hypothalamic nucleus; dmPAG, dorsomedial periaqueductal gray; DR, dorsal raphe nucleus; DRI, dorsal raphe nucleus, inferior part; GrDG, granular layer of the dentate gyrus; IL, infralimbic cortex; lAmy, lateral amygdaloid nucleus; LC, locus coeruleus; LHb, lateral habenular nucleus; lPAG, lateral periaqueductal gray; LPBD, lateral parabrachial nucleus, dorsal part; LPBE, lateral parabrachial nucleus, external part; LPO, lateral preoptic area; LSD, lateral septal nucleus, dorsal part; LSV, lateral septal nucleus, ventral part; M2, secondary motor cortex; MePD, medial amygdaloid nucleus, posterodorsal part; MePV, medial amygdaloid nucleus, posteroventral part; MHb, medial habenular nucleus; MnR, median raphe nucleus; MPO, medial preoptic nucleus; MS, medial septal nucleus; NTS, nucleus of the solitary tract; Op, optic nerve layer of the superior colliculus; PC, paracentral thalamic nucleus; PIL, posterior intralaminar thalamic nucleus; Pir, piriform cortex; PMR, paramedian raphe nucleus; PP, peripeduncular nucleus; PrL, prelimbic cortex; PVA, paraventricular thalamic nucleus, anterior part; PVN, paraventricular hypothalamic nucleus; Py, pyramidal cell layer of the hippocampus; S1BF, primary somatosensory cortex, barrel field; SO, supraoptic nucleus; SuG, superficial gray layer of the superior colliculus; vlPAG, ventrolateral periaqueductal gray; VTA, ventral tegmental area; Xi, xiphoid thalamic nucleus; ZI, zona incerta.

TABLE I. Locomotor activity of wildtype (WT) and Y2 knockout ($Y2^{-/-}$) mice during 10-min exposure to either the open (WT: n = 4 and 9; $Y2^{-/-}$: n = 4 and 9) or closed arm (WT: n = 5; $Y2^{-/-}$: n = 6) of an elevated plus maze

	Distance traveled (cm)		
Experiment	WT	$Y2^{-/-}$	
Experiment 1			
Open arm	354 ± 19	379 ± 54	$P = 0.667$
Experiment 2			
Open arm	390 ± 103	562 ± 54	$F_{1,17} = 2.49$, $P = 0.137$ (genotype)
Closed arm	546 ± 75	701 ± 112	$F_{1,17} = 2.04$, $P = 0.175$ (arm)

The distance travelled is given in cm as mean ± SEM. Statistical analysis was performed using a *t*-test in Experiment 1 and a two-way ANOVA in Experiment 2, respectively.

pus ($F_{1,27} = 5.50$; $P = 0.028$), and there was a trend toward significance in the piriform cortex ($F_{1,27} = 3.15$; $P = 0.088$), posteroventral part of the medial amygdala ($F_{1,26} = 3.69$; $P = 0.067$), and the locus coeruelus ($F_{1,27} = 3.41$; $P = 0.076$). Specifically, while basal c-Fos expression was generally low and comparable between the two genotypes, $Y2^{-/-}$ relative to WT mice displayed attenuated stress-induced c-Fos expression in the prelimbic, cingulate and primary somatosensory (barrel field) cortices, nucleus accumbens core region, dorsal and ventral lateral septum, granular layer of the dentate gyrus, CA3 pyramidal cell layer of the hippocampus, posteroventral part of the medial amygdala, and locus coeruleus (Table II; Fig. 3A).

Experiment 2: c-Fos induction in response to open vs. closed arm exposure

Two-way ANOVA (genotype × arm) revealed a significant effect of genotype in c-Fos responses to arm exposure in the cingulate cortex (Fig. 3B), motor cortex, barrel field of the primary somatosensory cortex (Fig. 3B), nucleus accumbens core region (Fig. 3B), dorsal part of the lateral septum (Fig. 3B), and posteroventral part of the medial amygdala (Fig. 3B) which are summarized in Table III. Genotype additionally affected c-Fos induction in the lateral periaqueductal gray ($F_{1,19} = 4.82$; $P = 0.043$), ventral ($F_{1,19} = 11.37$; $P = 0.004$), and inferior ($F_{1,20} = 5.65$; $P = 0.030$) dorsal raphe and external lateral parabrachial nucleus ($F_{1,26} = 4.79$; $P = 0.043$). Trends toward a significant genotype effect were detected in the dorsomedial periaqueductal gray ($F_{1,19} = 3.50$; $P = 0.080$), median raphe ($F_{1,19} = 4.16$; $P = 0.058$), and dorsal lateral parabrachial nucleus ($F_{1,26} = 3.28$; $P = 0.089$). Moreover, an effect of arm type was observed in the cingulate cortex ($F_{1,17} = 4.97$; $P = 0.043$), ventral dorsal raphe ($F_{1,19} = 4.89$; $P = 0.042$), and lateral periaqueductal gray ($F_{1,19} = 6.14$; $P = 0.025$) as well as a trend toward significance in the dorsal part of the lateral septum ($F_{1,19} = 4.42$; $P = 0.052$). There was a significant genotype × arm interaction in the lateral periaqueductal gray ($F_{1,19} = 6.14$; $P = 0.025$) with WT mice displaying reduced c-Fos expression after CA vs. OA exposure ($P = 0.018$), and in the paraventricular hypothalamic nucleus (magnucellular part) ($F_{1,19} = 5.67$; $P = 0.030$). Further, trends toward a significant genotype × arm interaction were detected in the central amygdala ($F_{1,19} = 3.86$; $P = 0.067$) and xiphoid nucleus ($F_{1,17} = 4.11$; $P = 0.082$).

Differences in the numbers of c-Fos-positive cells between WT and $Y2^{-/-}$ mice were observed in several brain areas. Compared to WT mice, $Y2^{-/-}$ had lower numbers of c-Fos-positive cells in the cingulate cortex after both OA ($P = 0.003$) and CA ($P = 0.005$) exposure (Fig. 3B). Furthermore, a significantly reduced neuronal activation was observed in knockout vs. WT animals in the posteroventral part of the medial amygdala ($P = 0.035$; Fig. 3B) and lateral periaqueductal gray ($P = 0.049$) after OA exposure as well as in the motor cortex ($P = 0.018$) following CA arm exposure.

DISCUSSION

Our present experiments demonstrate (1) a pronounced over-expression of the neuronal activity marker c-Fos in brain areas related to the processing of emotions in response to novel environments with proposed high and low anxiogenic potentials, respectively (Pellow et al., 1985), and (2) that genotype significantly affects stress-induced c-Fos expression in a specific subset of brain areas, namely the cingulate cortex, barrel field of the somatosensory cortex, nucleus accumbens core region, dorsal lateral septum, posteroventral part of the medial amygdala, and lateral periaqueductal gray. Specifically, this effect is attenuated in mice lacking the Y2 receptor that display an anxiolytic phenotype also as neurobehavioral response to novelty (Painsipp et al., 2008; Redrobe et al., 2003; Tschenett et al., 2003), and it seems to be more pronounced after OA then CA exposure.

Enhanced c-Fos expression by OA and CA exposure

In the present study, c-Fos expression was triggered in the majority of the analyzed regions following OA exposure (Tables II and III). This activation pattern is in accordance with previous studies using OA exposure as a challenge (Nguyen et al., 2006; Salome et al., 2004; Viltart et al., 2006). Similarly, the CA also induced a pronounced c-Fos expression which did not differ from that after OA exposure in all of the brain areas investigated with the exception of the cingulate cortex, dorsal lateral septum, ventral dorsal raphe, and lateral periaqueductal gray. So far, only one study (Mairesse et al., 2007) compared neuronal

TABLE II. c-Fos expression in wildtype (WT) and Y2 knockout ($Y2^{-/-}$) mice in different brain areas under basal conditions (WT: n = 5; $Y2^{-/-}$: n = 5) or after open arm exposure (WT: n = 9; $Y2^{-/-}$: n = 9)

	Basal		Open arm exposure		Stress	
Brain area	WT	$Y2^{-/-}$	WT	$Y2^{-/-}$	*F*	*P*
Forebrain						
Cortical areas						
Infralimbic cortex (§)	13.8 ± 1.7	10.9 ± 1.7	48.9 ± 2.4	48.5 ± 2.7	188.69	<0.001
Prelimbic cortex (§)	7.7 ± 1.6	6.1 ± 1.5	**54.2 ± 2.3**	**38.8 ± 3.2**$^{\bullet\bullet\bullet}$	199.95	<0.001
Motor cortex (§)	0.1 ± 0.1	0.2 ± 0.2	6.8 ± 0.7	6.1 ± 1.0	57.43	<0.001
Piriform cortex	1.9 ± 0.6	2.9 ± 0.9	28.6 ± 1.4	24.6 ± 1.6	255.90	<0.001
Claustrum	3.0 ± 1.4	2.0 ± 0.5	13.7 ± 0.9	10.6 ± 1.0	82.18	<0.001
Cingulate cortex (§)	7.1 ± 1.9	6.4 ± 2.2	**53.8 ± 1.3**	**40.9 ± 4.8**$^{\bullet}$	130.24	<0.001
Somatosensory 1, barrel field (§)	1.8 ± 1.4	0.6 ± 0.4	**30.6 ± 2.9**	**13.3 ± 1.6**$^{\bullet\bullet\bullet}$	81.87	<0.001
Nucleus accumbens, core	1.9 ± 0.6	1.8 ± 0.4	**16.9 ± 0.7**	**10.6 ± 1.4**$^{\bullet}$	82.91	<0.001
Nucleus accumbens, shell	1.7 ± 0.4	1.7 ± 0.3	9.8 ± 0.5	8.8 ± 1.0	86.52	<0.001
Lateral septal nucleus, dorsal	4.1 ± 1.4	2.2 ± 0.5	17.0 ± 0.6	13.4 ± 1.3^{b}	115.95	<0.001
Lateral septal nucleus, ventral	5.8 ± 2.5	3.9 ± 1.3	39.6 ± 1.8	33.2 ± 1.7^{b}	249.76	<0.001
Medial septal nucleus	0.1 ± 0.1	0.5 ± 0.3	3.6 ± 0.4	2.8 ± 0.4	59.71	<0.001
Bed nucleus, stria terminalis, ventral	2.0 ± 0.2	1.7 ± 0.3	9.3 ± 1.3	7.6 ± 0.6	46.76	<0.001
Bed nucleus, stria terminalis, anterior	2.3 ± 0.5	2.3 ± 0.4	9.6 ± 0.7	9.8 ± 0.8	104.30	<0.001
Bed nucleus, stria terminalis, lateral division, posterior	2.3 ± 0.6	3.2 ± 0.5	6.7 ± 0.7	7.6 ± 0.9	29.40	<0.001
Lateral preoptic area	4.6 ± 0.6	4.4 ± 0.4	10.4 ± 0.8	9.2 ± 0.7	48.07	<0.001
Medial preoptic area	2.9 ± 0.6	2.7 ± 0.3	19.0 ± 1.6	18.7 ± 1.7	101.31	<0.001
Hippocampal formation						
Granular layer, dentate gyrus	6.9 ± 1.3	5.3 ± 1.0	**17.2 ± 0.4**	**10.8 ± 0.9**$^{\bullet\bullet\bullet}$	81.53	<0.001
Pyramidal cell layer, CA3	1.0 ± 0.3	0.9 ± 0.2	**7.7 ± 0.4**	**4.4 ± 0.9**$^{\bullet\bullet}$	58.78	<0.001
Amygdala						
Lateral nucleus	2.0 ± 0.5	2.7 ± 0.7	7.7 ± 0.5	7.4 ± 0.5	87.46	<0.001
Central nucleus	3.0 ± 1.4	3.5 ± 0.4	4.2 ± 0.7	7.7 ± 1.6	4.00	0.058
Medial nucleus, posterodorsal	2.7 ± 0.8	2.4 ± 1.1	15.6 ± 2.0	12.2 ± 1.4	45.31	<0.001
Medial nucleus, posteroventral	1.7 ± 0.3	1.5 ± 0.4	**14.8 ± 0.7**	**10.8 ± 1.2**$^{\bullet}$	133.89	<0.001
Basolateral nucleus	2.4 ± 1.1	2.9 ± 0.9	14.4 ± 0.8	12.6 ± 0.9	131.55	<0.001
Diencephalon						
Thalamus						
Paraventricular nucleus, anterior	13.4 ± 1.1	12.0 ± 1.3	24.2 ± 1.9	25.7 ± 2.1	37.56	<0.001
Paracentral nucleus	5.6 ± 1.3	5.8 ± 1.8	19.8 ± 2.1	20.1 ± 3.0	29.17	<0.001
Central medial nucleus	6.0 ± 1.4	6.1 ± 0.9	16.8 ± 1.8	18.7 ± 1.3	55.49	<0.001
Xiphoid nucleus	4.8 ± 2.6	3.4 ± 1.3	17.2 ± 1.9	13.2 ± 1.3	334.26	<0.001
Lateral habenular nucleus	1.2 ± 0.8	3.1 ± 0.4	14.3 ± 2.1	11.6 ± 1.6	38.69	<0.001
Medial habenular nucleus	0.0 ± 0.0	0.60 ± 0.5	0.75 ± 0.4	0.89 ± 0.5	1.50	0.234
Zona incerta	14.6 ± 1.7	12.3 ± 1.3	25.8 ± 2.2	19.6 ± 2.1	17.65	<0.001
Posterior intralaminar nucleus	4.3 ± 0.4	4.5 ± 0.8	11.6 ± 0.7	9.8 ± 0.9	59.94	<0.001
Peripeduncular nucleus	3.6 ± 0.8	4.2 ± 0.8	8.4 ± 0.9	7.7 ± 1.3	12.29	0.003
Hypothalamus						
Dorsomedial nucleus	6.4 ± 1.8	6.9 ± 2.6	29.4 ± 2.0	23.0 ± 2.3	67.84	<0.001
Paraventricular nucleus	2.5 ± 1.0	3.1 ± 1.0	22.8 ± 3.6	29.7 ± 6.2	22.87	<0.001
Supraoptic nucleus	2.2 ± 0.5	2.4 ± 1.1	8.7 ± 0.9	7.1 ± 0.7	38.13	<0.001
Arcuate nucleus	1.6 ± 0.6	1.2 ± 0.5	7.0 ± 1.1	7.3 ± 1.0	31.27	<0.001
Midbrain, pons, hindbrain						
Superficial gray, superior colliculus	9.4 ± 1.6	6.7 ± 1.7	23.0 ± 1.5	18.4 ± 2.6	32.94	<0.001
Optic nerve layer of the superior colliculus	8.4 ± 1.9	7.7 ± 2.5	20.4 ± 1.0	17.3 ± 1.3	46.56	<0.001
Ventral tegmental area	0.1 ± 0.1	0.5 ± 0.4	2.2 ± 0.4	1.9 ± 0.2	28.96	<0.001
Raphe nuclei						
Caudal linear nucleus	1.9 ± 0.9	2.9 ± 0.7	8.8 ± 1.5	7.0 ± 0.9	19.05	<0.001
Paramedian nucleus	0.9 ± 0.3	1.2 ± 0.1	6.9 ± 0.8	5.1 ± 0.5	54.09	<0.001
Median nucleus	1.3 ± 0.5	1.3 ± 0.4	19.2 ± 1.0	17.2 ± 2.0	122.15	<0.001
Dorsal	2.0 ± 0.6	2.3 ± 0.4	8.9 ± 0.7	9.7 ± 0.6	101.90	<0.001
Dorsal nucleus, inferior	2.3 ± 0.7	1.9 ± 0.8	12.2 ± 2.1	13.1 ± 1.0	43.18	<0.001
Periaqueductal gray						
Lateral	4.3 ± 1.1	2.6 ± 0.4	10.8 ± 0.6	9.3 ± 0.5	88.51	<0.001
Ventrolateral	2.6 ± 0.6	3.5 ± 0.3	10.4 ± 0.4	9.9 ± 0.7	145.21	<0.001
Dorsolateral	2.2 ± 0.6	2.7 ± 0.9	8.9 ± 0.6	7.6 ± 0.7	63.39	<0.001
Dorsomedial	2.7 ± 0.5	3.4 ± 0.8	8.9 ± 0.4	7.2 ± 0.8	46.97	<0.001
Lateral parabrachial nucleus, dorsal	6.2 ± 1.3	5.7 ± 1.5	14.2 ± 1.2	13.1 ± 2.1	18.07	<0.001
Lateral parabrachial nucleus, external	3.1 ± 0.6	2.1 ± 0.4	8.8 ± 1.2	7.2 ± 1.0	25.34	<0.001
Central nucleus, inferior colliculus	4.8 ± 0.3	3.6 ± 0.6	5.5 ± 0.2	4.5 ± 0.4	3.95	0.058
Locus coeruleus	3.6 ± 0.4	3.8 ± 0.5	**19.2 ± 1.4**	**14.6 ± 1.2**$^{\bullet}$	105.71	<0.001
Nucleus of the solitary tract	1.4 ± 0.6	0.8 ± 0.4	5.6 ± 1.4	8.3 ± 1.3	18.43	<0.001

The number of c-Fos-positive cells/0.01 mm^2 and /0.04 mm^2 (regions marked with §), respectively, is given as mean ± SEM. Brain areas where a significant difference between WT and $Y2^{-/-}$ was observed are highlighted by bold letters. Statistical analysis was performed using two-way ANOVA and post Bonferroni test: $^{b}P < 0.09$, $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet\bullet}P < 0.001$ vs. respective WT group.

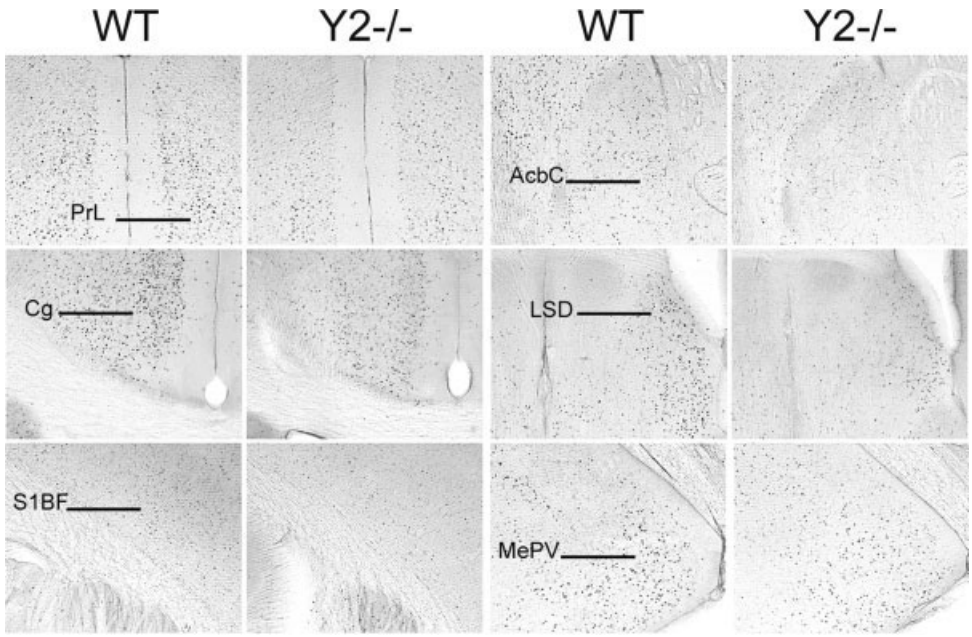

Fig. 2. Representative bright-field photomicrographs showing decreased Fos expression in Y2 knockout ($Y2^{-/-}$) compared to wildtype (WT) mice in response to open arm exposure. Cortical areas: prelimbic cortex (PrL), cingulate cortex (Cg), primary somatosensory cortex (S1BF); nucleus accumbens core (AcbC); dorsal lateral septum (LSD) and medial amygdala, posteroventral part (MePV). Scale bar = 200 μm.

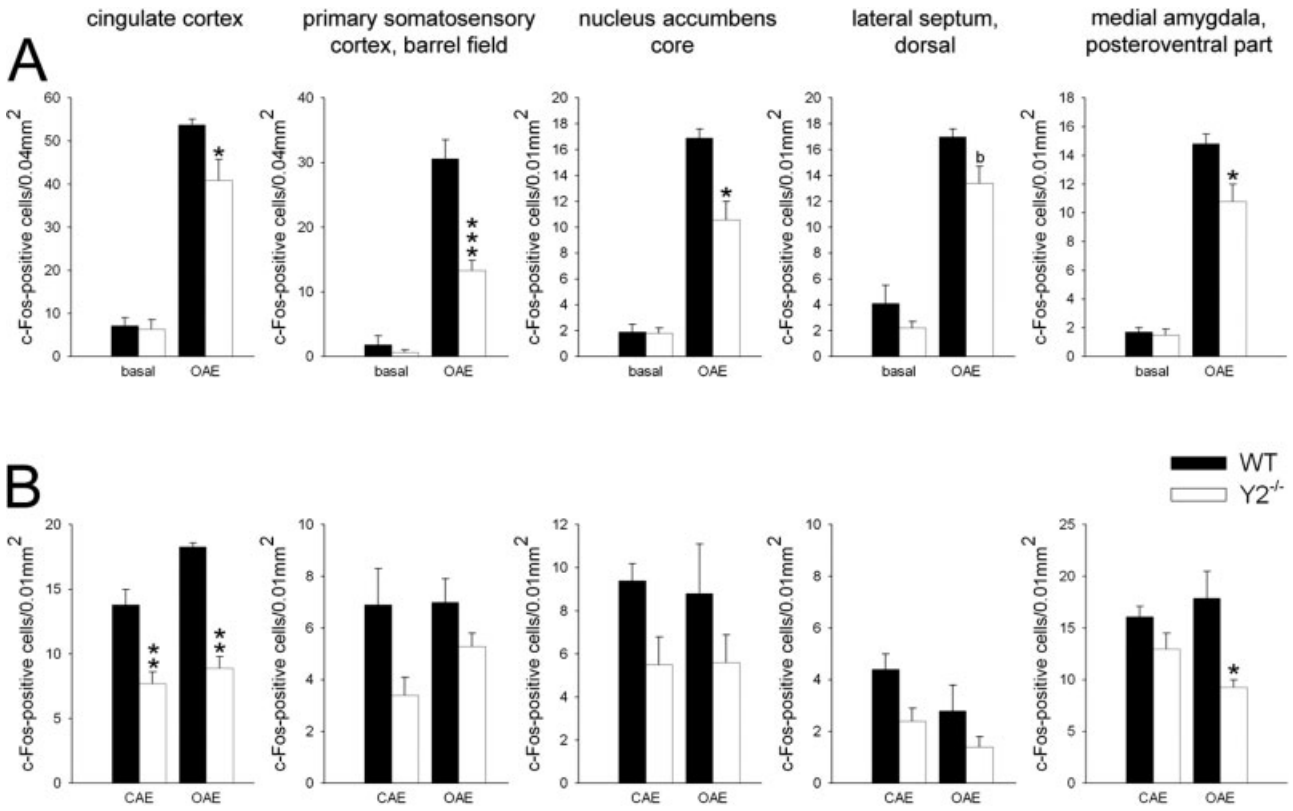

Fig. 3. Quantitative analysis of c-Fos-like immunoreactivity in Y2 knockout ($Y2^{-/-}$) and wildtype (WT) mice under basal conditions (**A**), after open arm (OAE; A and **B**) and closed arm (CAE; B) exposure. Only those brain areas are graphically presented in which the difference in stress-induced c-Fos expression between the two genotypes was statistically significant and consistent in both Experiments 1 (A) and 2 (B). Each column indicates the mean ± SEM number of c-Fos-positive cells in a tissue area of 0.01 or 0.04 mm^2. n = 4–9 per experimental group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ $Y2^{-/-}$ vs. WT mice.

TABLE III. c-Fos expression in wildtype (WT) and Y2 knockout ($Y2^{-/-}$) mice in different brain areas after open arm exposure (WT: n = 4; $Y2^{-/-}$: n = 4) and closed arm exposure (WT: n = 7; $Y2^{-/-}$: n = 6)

	Open arm exposure		Closed arm exposure		Genotype	
Brain area	WT	$Y2^{-/-}$	WT	$Y2^{-/-}$	*F*	*P*
Prelimbic cortex	5.4 ± 1.4	4.1 ± 1.1	5.0 ± 0.3	3.5 ± 0.6	2.71	0.121
Cingulate cortex	18.3 ± 0.3	8.9 ± 0.9●●	13.8 ± 1.2	7.7 ± 0.9●●	37.32	<0.001
Secondary motor cortex	9.5 ± 2.0	5.4 ± 0.8	9.2 ± 0.5	4.8 ± 0.9●	18.70	<0.001
Somatosensory 1, barrel field	7.0 ± 0.9	5.3 ± 0.5	6.9 ± 1.4	3.4 ± 0.7	4.87	0.042
Nucleus accumbens, core	8.8 ± 2.3	5.6 ± 1.3	9.4 ± 0.8	5.5 ± 1.3	8.92	0.008
Lateral septal nucleus, dorsal	2.8 ± 1.0	1.4 ± 0.4	4.4 ± 0.6	2.4 ± 0.5	6.87	0.019
Lateral septal nucleus, ventral	15.0 ± 1.4	15.1 ± 1.4	19.4 ± 2.5	15.5 ± 1.1	0.83	0.376
Granular layer, dentate gyrus	10.3 ± 1.6	11.3 ± 1.6	10.0 ± 1.2	10.2 ± 0.7	0.21	0.656
Pyramidal cell layer, CA3	5.4 ± 0.9	3.6 ± 0.6	5.4 ± 0.6	4.8 ± 0.7	2.86	0.109
Medial amygdala, posteroventral	17.9 ± 2.6	9.3 ± 0.7●	16.1 ± 1.0	13.0 ± 1.5	12.20	0.003
Locus coeruleus	19.0 ± 4.3	16.6 ± 2.8	17.0 ± 1.1	18.6 ± 2.6	0.02	0.881

The number of Fos-positive cells/0.01 mm^2 is given as mean ± SEM. Statistical analysis was performed using two-way ANOVA and post Bonferroni test: ●$P < 0.05$, ●●$P < 0.01$ vs. respective WT group.

activity in limbic regions in response to OA vs. CA exposure and reported reduced effects after CA exposure in the infralimbic cortex, paraventricular hypothalamus, hippocampus, and amygdala which are not supported by the present findings. These discrepancies in OA- vs. CA-induced neuronal activity patterns between the present and previous study may be due to some methodological differences including exposure times (5 vs. 10 min arm exposure).

Here, we show that exposure to either the OA or CA of an EPM that both elicit behavioral activity evokes c-Fos expression in numerous brain regions. Of these, the motor, cingulate and piriform cortices, nucleus accumbens, and locus coeruleus are associated with the elaboration of motivated behaviors induced by novelty (Stone et al., 2006) (for review, see Sewards and Sewards, 2003) or exploration of a novel environment (Handa et al., 1993; Staiger et al., 2000; Uslaner et al., 2001, 2003). Many of these brain areas express motoric α1-adrenoreceptors (Stone et al., 2004), suggesting that they could be activated in the murine brain upon traveling in the arms. However, both the OA and CA are thought to be associated with very low locomotor activation. Interestingly, in the EPM, which the OA test is related to, motor activity is only one factor, with the strongest measure being anxiety (File, 2001). In line with that, many of the activated regions such as amygdala, bed nucleus of the stria terminalis, paraventricular hypothalamic nucleus, lateral septum, and locus coeruleus are thought to be also involved in anxiety-related processing (Charney et al., 1998). Given the influence of arm type on c-Fos responses in the cingulate cortex, dorsal lateral septum, ventral dorsal raphe, and lateral periaqueductal gray, it is suggested that these identified brain areas primarily mediate the more anxiogenic potential of the OA vs. the CA (Pellow et al., 1985). Thus, our results provide further evidence that OA exposure represents a valuable challenge to study central mechanisms underlying behavioral reactivity to novelty including anxiety-related behaviors.

Effect of genotype on challenge-induced c-Fos responses

The genetic deletion of Y2 receptors reproducibly influenced neuronal activity in response to a novel environment in the cingulate cortex, barrel field of the somatosensory cortex, nucleus accumbens, dorsal lateral septum, medial amygdala, and lateral periaqueductal gray. Specifically, the c-Fos response in $Y2^{-/-}$ mice was attenuated in these aforementioned brain regions after OA exposure, while in the remaining areas similar activation was observed in $Y2^{-/-}$ and WT Mice.

All of the above brain areas are thought to mediate motivational behavior during novelty (Handa et al., 1993; Staiger et al., 2000; Stone et al., 2006; Uslaner et al., 2001, 2003; for review, see Sewards and Sewards, 2003), as well as stress-associated responses and to be part of proposed fear/anxiety circuitries (Charney et al., 1998). Accordingly, the administration of anxiogenic drugs has been shown to elevate c-Fos expression in the medial prefrontal cortex, amygdala, periaqueductal gray and the lateral septum (Singewald and Sharp, 2000; Singewald et al., 2003). Conversely, administration of anxiolytic drugs such as benzodiazepines or NK1 receptor antagonists reduces stressor-induced c-Fos expression in prelimbic cortex, cingulate cortex, medial amygdala, and nucleus accumbens as well as the dorsomedial hypothalamic nucleus, hippocampus, and locus coeruleus (Beck and Fibiger, 1995; Hahn and Bannon, 1999; McGregor et al., 2004). Hence, the effect of genotype and, in particular, the attenuation of the OA- and CA-induced c-Fos expression in these same regions observed in $Y2^{-/-}$ as compared to WT mice is suggested to contribute to the altered neurobehavioral reactivity to novelty of these animals, possibly resulting in their anxiolytic phenotype (Painsipp et al., 2008; Redrobe et al., 2003; Tschenett et al., 2003). However, it is unlikely that the differences in the activation patterns between $Y2^{-/-}$ and WT mice are due to altered explorative

behavior, given that the two genotypes did not differ in locomotion in both the OA and the CA of the EPM.

Possible mechanism(s) underlying the altered, challenge-induced neuronal excitability of $Y2^{-/-}$ mice

In situ hybridization techniques (Naveilhan et al., 1998; Parker and Herzog, 1999), binding studies (Dumont et al., 1996, 1998; Gackenheimer et al., 2001), and immunohistochemical studies (Fetissov et al., 2004; Stanic et al., 2006) confirm the localization of Y2 receptors in each of the six identified areas showing altered challenge-induced neuronal activation in $Y2^{-/-}$ mice.

Y2 receptors are thought to be mainly presynaptic receptors mediating an inhibition of transmitter release by inhibiting Ca^{2+} influx through N-type channels as demonstrated in the hippocampus in vitro and in vivo (El Bahh et al., 2005). Depending on the nerve terminal on which they are located, they may reduce GABA, NPY, glutamate, or noradrenaline release (Chen and van den Pol, 1996; Greber et al., 1994; Klapstein and Colmers, 1992; Martire et al., 1993; Sun et al., 2001). The anxiolytic phenotype of $Y2^{-/-}$ mice, thus, could be mediated by augmented release of NPY and/or of GABA, for example, in the amygdala due to loss of presynaptic Y2-mediated inhibition.

During stressful conditions, the release of NPY and GABA is enhanced (Cook, 2004; Husum et al., 2002) presumably resulting in an attenuation of the stress response by stimulating postsynaptic, inhibitory $GABA_A$, and Y1 receptors (Heilig et al., 1989; Wahlestedt et al., 1993), respectively. $GABA_A$ receptors mediate a hyperpolarization of postsynaptic neurons by opening their Cl^--channels and a Cl^- influx. Stimulation of Y1 receptors by NPY also causes inhibition of postsynaptic cells by voltage-dependent inhibition of Ca^{2+} currents and/or activation of inwardly rectifying K^+ currents (McQuiston et al., 1996; Sun and Miller, 1999). At the same time, presynaptically located Y2 receptors may physiologically dampen down the release of NPY and GABA enhanced by stress. Since this presynaptic regulatory mechanism is impaired in $Y2^{-/-}$ mice, a disinhibited release of NPY and GABA is suggested to result in a higher stimulation of postsynaptic inhibitory Y1 (Sun et al., 2001) and GABA receptors, respectively, both of which may then attenuate stress-induced c-Fos expression in the postsynaptic cells. Indeed, it has been shown that pharmacological blockade of Y2 receptors enhances stress-induced NPY release (King et al., 2000).

Interestingly, we did not observe any differences in c-Fos expression in the arcuate nucleus of the hypothalamus in $Y2^{-/-}$ mice compared to WT following OA and CA exposure. From the arcuate nucleus of the hypothalamus, neurons expressing NPY mRNA send projections to various regions such as the nucleus accumbens, lateral septum (dorsal and ventral), and amygdala (for review, see Chronwall, 1985; Heilig, 2004). In all these mentioned target areas, altered neuronal activity has been observed in $Y2^{-/-}$ mice indicating that Y2 autoreceptors on nerve terminals primarily determine the putatively enhanced NPY release in $Y2^{-/-}$ mice. In addition to a local effect of enhanced NPY on c-Fos expression, effects may also be mediated indirectly via the well-known interconnections within the fear-anxiety circuitry (for review, see Gray and McNaughton, 2000).

The hypothesis that enhanced NPY release in specific anxiety-related regions may be involved in the anxiolytic phenotype of $Y2^{-/-}$ mice is consistent with previous observations. For example, it has been shown that intracerebroventricular application of NPY elicits an anxiolytic effect (Heilig et al., 1989; Karlsson et al., 2005; Nakajima et al., 1998). Moreover, reduced anxiety-related behavior was found in the EPM in rats with upregulated NPY levels in the amygdala (by injecting viral vector encoding NPY), as compared to rats with downregulated NPY release (by injecting NPY antisense) (Primeaux et al., 2005). Hence, it is likely that attenuated neuronal activation in the amygdala observed in the present study is crucially implicated in the anxiolytic phenotype of $Y2^{-/-}$ mice. Indeed, it has been most recently demonstrated that NPY in the amygdala induces resilience to stress-induced reductions in social responses (Sajdyk et al., 2008). Whether local modulation of NPY transmission in (dorsal) lateral septum can also alter anxiety-related behavior remains to be shown.

In summary, exposure to either the OA or CA of an EPM was used as stimuli with putatively different anxiogenic potentials for activating neurons in regions. By evaluating subsequent c-Fos expression patterns, we found that $Y2^{-/-}$ mice show altered neuronal activity (hyperexcitability) in defined regions, namely the cingulate cortex, amygdala, nucleus accumbens, dorsal lateral septum, barrel field of the primary somatosensory cortex and lateral periaqueductal gray. These brain regions are well known to be associated with diverse behavioral reactivity to novelty including motivated and explorative as well as anxiety-related responses. Therefore, our results suggest that the altered neuronal activation patterns in anxiety-relevant substrates may mediate or contribute to the anxiolytic-like phenotype observed in $Y2^{-/-}$ mice.

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