Neuropsychopharmacology (2004) 29, 1282–1293

www.neuropsychopharmacology.org

Alpha2A-Adrenoceptors are Important Modulators of the Effects of D-Amphetamine on Startle Reactivity and Brain Monoamines

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Amphetamines are commonly used to treat attention-deficit hyperactivity disorder, but are also widely abused. They are employed in schizophrenia-related animal models as they disrupt the prepulse inhibition (PPI) of the acoustic startle response. The behavioral effects of amphetamines have mainly been attributed to changes in dopamine transmission, but they also involve increases in the synaptic concentrations of norepinephrine (NE). α_2-Adrenoceptors (α_2-ARs) regulate the excitability and transmitter release of brain monoaminergic neurons mainly as inhibitory presynaptic auto- and heteroreceptors. Modulation of acoustic startle and its PPI by the α_{2A}-AR subtype was investigated with mice lacking the α_{2A}-AR (α_{2A}-KO) and their wild-type (WT) controls, without drugs and after administration of the α_2-AR agonist dexmedetomidine or the antagonist atipamezole. The interaction of D-amphetamine (D-amph) and the α_2-AR-noradrenergic neuronal system in modulating startle reactivity and in regulating brain monoamine metabolism was assessed as the behavioral and neurochemical responses to D-amph alone, or to the combination of D-amph and dexmedetomidine or atipamezole. α_{2A}-KO mice were supersensitive to both neurochemical and behavioral effects of D-amph. Brain NE stores of α_{2A}-KO mice were depleted by D-amph, revealing the α_{2A}-AR as essential in modulating the actions of D-amph. Also, increased startle responses and more pronounced disruption of PPI were noted in D-amph-treated α_{2A}-KO mice. α_{2A}-AR also appeared to be responsible for the startle-modulating effects of α_2-AR drugs, since the startle attenuation after the α_2-AR agonist dexmedetomidine was absent in α_{2A}-KO mice, and the α_2-AR antagonist atipamezole had opposite effects on the startle reflex in α_{2A}-KO and WT mice.
Neuropsychopharmacology (2004) **29,** 1282–1293, advance online publication, 24 March 2004; doi:10.1038/sj.npp.1300428

Keywords: receptors, adrenergic, alpha-2; norepinephrine; startle reaction; sensorimotor gating; dextroamphetamine; mice, genetically modified

INTRODUCTION

Amphetamine-like psychostimulants are the best-documented pharmacotherapy for attention-deficit hyperactivity disorder (ADHD) (Arnold *et al*, 1976; Wilens *et al*, 1995; Spencer *et al*, 1996). Amphetamines are also powerful reinforcing drugs, primarily due to their capacity to activate the mesocorticolimbic dopamine (DA) system (Wise, 1996). They are therefore widely abused, with repeated administration increasing susceptibility to drug-induced psychosis (Ujike, 2002). D-Amphetamine (D-Amph) acts by inhibiting neuronal reuptake and by facilitating release of DA, norepinephrine (NE), and serotonin (5-HT). In addition to the established role of DA, there is emerging evidence that also NE contributes to the behavioral effects of D-Amph either through modulation of DA transmission or through independent mechanisms (Segal and Kuczenski, 1994; Xu *et al*, 2000; Weinshenker *et al*, 2002).

Activation of presynaptic and somatodendritic α_2-adrenoceptors (α_2-ARs) inhibits NE release and firing of noradrenergic neurons. The α_{2A}-AR subtype mainly contributes to the inhibition of both neuronal excitability and NE release; the α_{2C}-AR has a similar, but lesser role (Lakhlani *et al*, 1997; Starke, 2001). Moreover, the α_{2A}-AR and to a minor extent the α_{2C}-AR modulate the neurotransmitter release of brain dopaminergic and serotonergic neurons (Yavich *et al*, 1997; Scheibner *et al*, 2001). In rat frontal cortex, the increased NE release following systemic administration of D-amph has been shown to be augmented under blockade of all α_2-AR subtypes with subtype nonselective antagonists (Wortley *et al*, 1999; Geranton *et al*, 2003). In addition, recent evidence from microdialysis

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Received 29 October 2003; revised 27 January 2004; accepted 29 January 2004
Online publication: 6 February 2004 at http://www.acnp.org/citations/Npp02060403498/default.pdf

studies in rats suggests that there are regional differences in the NE-modulating effects of locally administered D-Amph in the CNS (Geranton *et al*, 2003). In the hypothalamus, there was a stronger increase in D-Amph-induced NE efflux in comparison to the frontal cortex; pretreatment with atipamezole, an α_2-AR antagonist, abolished this difference by augmenting NE release in the frontal cortex, but not in the hypothalamus (Geranton *et al*, 2003). Thus, it seems that the NE turnover in the frontal cortex is under stronger inhibitory α_2-autoreceptor control than that in the hypothalamus. Interestingly, the α_{2A}-ARs in mouse frontal cortex were also reported to mediate the beneficial effects of cognitive-enhancing effects of guanfacine, an α_2-AR agonist clinically employed in the treatment of ADHD (Franowicz *et al*, 2002). Still, the extent to which α_2-ARs contribute to modulating the neurochemical and behavioral response to D-Amph-like psychostimulants is not thoroughly understood.

The startle reflex, a fast twitch of the body musculature by a sudden and intense tactile, visual, or acoustic stimulus, is usually classified as a defensive response. The startle reflex is an important behavioral tool to assess the mechanisms of sensorimotor response plasticity. By modulating the experimental settings, the startle reflex can be used, for example, as a model to investigate the neurobiology of anxiety and fear. Prepulse inhibition (PPI) of the startle response is a cross-species measure of sensorimotor gating. In PPI, the startle magnitude is reduced when the startle stimulus is preceded by a low-intensity prepulse (Koch, 1999; Swerdlow *et al*, 2000). In humans, disruption of PPI has been reported in schizophrenia (Braff *et al*, 2001) and ADHD (Hawk *et al*, 2003). In rodents, D-amph is used to pharmacologically disrupt PPI (Mansbach *et al*, 1988; Ralph *et al*, 2001). Drug-induced PPI disruptions are restored by antipsychotic compounds, and because of its predictive validity, the PPI model is used in antipsychotic drug development (Swerdlow *et al*, 2000).

The α_2-AR agonist clonidine attenuates (Kumari *et al*, 1996) and the antagonist yohimbine enhances (Morgan *et al*, 1993) the startle response in humans, but the receptor subtype(s) mediating these effects is unclear. α_2-AR-dependent modulation of PPI has also been reported. In mice, inactivation of the α_{2C}-AR gene resulted in increased startle and decreased PPI, whereas α_{2C}-AR overexpression increased the PPI (Sallinen *et al*, 1998). Here we examined the startle response and its PPI in mice lacking the gene encoding the α_{2A}-AR (α_{2A}-knockout, α_{2A}-KO) (Altman *et al*, 1999). Previous studies have linked genetic α_{2A}-AR inactivation to impaired function of the prefrontal cortex (Franowicz *et al*, 2002) and to increased brain NE turnover (Lähdesmäki *et al*, 2002).

Since D-amph affects NE release and uptake, and because noradrenergic neurotransmission and NE metabolism in the CNS are regulated by α_2-ARs, D-amph was given alone, and in combination with the α_2-AR agonist dexmedetomidine or the antagonist atipamezole. In addition to startle reactivity and PPI, the neurochemical effects of D-amph were monitored by measuring the content of NE, DA, 5-HT, and their metabolites in several monoaminergic brain regions.

MATERIALS AND METHODS

Experimental Animals

A total of 119 female and 104 male mice of 20–35 weeks of age, weighing 21–32 g (females) or 26–36 g (males), were used. The generation of a mouse strain with targeted inactivation of the gene for the α_{2A}-adrenergic receptor has been described previously (Altman *et al*, 1999). Heterozygous α_{2A}-KO mice were bred to wild-type C57Bl/6J for five successive generations to produce a strain of mice with a uniform, predominantly C57Bl/6J genetic background. Age-matched wild-type C57Bl/6J (WT) mice of the same genetic background (Jackson Laboratories, Bar Harbor, ME, USA) were used as control animals. All efforts were made to minimize animal suffering and the number of animals used. The experiments conformed to the International Council for Laboratory Animal Science (ICLAS) guidelines, and the study had approval of the local committee for laboratory animal welfare. The mice were housed under standard laboratory conditions with a 12 : 12-h light/dark cycle (lights on at 0600 and off at 1800). Experiments were conducted between 0800 and 1630 h. In startle experiments, each animal was tested in three or four startle sessions. Animals used in the analysis of brain monoamines were used solely in this experiment.

Drugs

Dexmedetomidine hydrochloride (Dex; Orion Corporation, Orion Pharma, Turku, Finland) and atipamezole hydrochloride (Ati; Orion Pharma) were injected subcutaneously (s.c.). D-amph sulfate (D-amph; Sigma, St Louis, MO) was administered intraperitoneally (i.p.). All drugs were dissolved in distilled water, and the injection volume was either 5 ml/kg (s.c.) or 10 ml/kg (i.p.).

Startle Apparatus

Startle experiments were performed in two identical ventilated and illuminated startle chambers ($39 \times 38 \times 58\,cm^3$ (length $\times$ width $\times$ height)) (SR-LAB system, San Diego Instruments, San Diego, CA). The chambers consisted of a nonrestrictive Plexiglas cylinder (3.9 cm in diameter) resting on a Plexiglas platform. Piezoelectric accelerometers mounted under the cylinders detected and transduced the animal movements. High-frequency speakers (Radio Shack Supertweeter, San Diego, CA), mounted 25 cm above the cylinder, provided all acoustic stimuli. Presentation of the acoustic stimuli and the piezoelectric responses from the accelerometer were controlled and digitized by the SR-LAB software and interface system. The sensitivity of the chambers was adjusted at average readings of 1000 using the standardization unit from San Diego Instruments. Sound levels within each chamber were measured repeatedly using the A weighing scale (Radio Shack Sound Level Meter, Fort Worth, TX) and were found to remain constant.

Startle Experiments

Groups of female α_{2A}-KO ($n = 60$) and female WT ($n = 59$) mice underwent four separate 13-min startle sessions, with

a minimum session interval of 12 days. Prior to startle and PPI measurements, they were treated as follows:

Experiment 1: Startle responses and PPI of 30 α_{2A}-KO and 30 WT mice were evaluated at baseline without treatments. No injections were given in this experiment.

Experiments 2 and 3: 60 α_{2A}-KO and 59 WT mice were divided randomly into four groups to receive the subtype-nonselective α_2-antagonist atipamezole (0.3, 1 or 3 mg/kg) (experiment 2) or the agonist dexmedetomidine (10 or 30 μg/kg) (experiment 3) or vehicle 20 min before the start of the startle session.

Experiment 4: In the final startle session, the effects of high-dose D-amph (10 mg/kg) were explored. Vehicle, Ati 1 mg/kg, or Dex 3 μg/kg were injected 20 min, and D-amph 10 min before the test session.

Design of Startle Sessions

The following session protocol was employed in all experiments: After a 3-min habituation period, the mice were first exposed to 10 PULSE ALONE trials (block 1); then, in a pseudorandom order to 13 PULSE ALONE trials and three types of PREPULSE + PULSE trials, each consisting of eight trials (block 2); and finally, the mice were again exposed to five PULSE ALONE trials (block 3). The duration of a startle session was 13 min. There was an average of 10 s interval (range, 5–30 s) between trials.

A PULSE ALONE trial consisted of a 40-ms broadband 120 dB burst. In PREPULSE + PULSE trials, a 40 ms long 3, 6 or 15 dB stimulus above the 72 dB background preceded the 120 dB pulse by 100 ms. The startle amplitudes from PULSE ALONE and PREPULSE + PULSE trials were determined by averaging 100 readings of 1 ms, each taken from the beginning of the PULSE stimulus onset.

Analysis of Brain Monoamines and Their Metabolites

Male α_{2A}-KO ($n = 52$) and WT ($n = 52$) mice were randomly divided into six groups and received two drug injections 50 and 40 min before decapitation. The first drug injection (referred here as 'Drug') was either vehicle, Dex (3 μg/kg) or Ati (1 mg/kg); the second drug injection (referred here as 'Amph') was either vehicle or D-amph (10 mg/kg). Treatments were vehicle + vehicle, vehicle + D-amph, Dex + D-amph, Ati + D-amph, Dex + vehicle, and Ati + vehicle. Core body temperatures were measured before the first injection and just prior to decapitation, using a rectal probe and a digital thermometer (Ellab, Roedovre, Denmark). After decapitation, the brains were rapidly removed. The cerebral cortex, hippocampus, striatum and the thalamus, and hypothalamus were dissected and placed in preweighed tubes on dry ice.

Biogenic amines (NE, DA, 5-HT), the 5-HT precursor tryptophan (TRP), and the monoamine metabolites were determined from brain homogenates in 0.1 M perchloric acid using electrochemical detection (ESA Coulochem 5011, Bedford, MA) after separation by HPLC on a reversed-phase C18 column (Ultrasphere ODS, 4.6 × 250 mm, Beckman Instruments, Fullerton, CA). The buffer systems described by Mefford (1981) were used, with the minor modifications described elsewhere (MacDonald *et al*, 1988).

Data Analysis

Statistical analyses were conducted using SPSS for Windows release 11.0. (SPSS Inc., Chicago, IL). Genotype comparisons between nontreated or vehicle-treated α_{2A}-KO and WT mice were performed using independent samples *t*-tests. Results from brain biogenic amine determinations were analyzed using three-way analysis of variance (ANOVA) with genotype (α_{2A}-KO/WT), amph (vehicle/D-amph), and drug (vehicle/Dex/Ati) as factors. For brevity, the main effects of drug and the amph × drug interaction are not presented. A more comprehensive analysis of the effects of Dex and Ati on the levels of brain monoamine neurotransmitters in α_{2A}-KO and WT mice has also been reported previously (Lähdesmäki *et al*, 2003). Within genotypes, contrasts were used in the general linear models as *post hoc* tests for the differences between treatments.

The extent of PPI was determined as PPI%, according to the formula {100−[(mean startle amplitude of PREPULSE + PULSE−trials)/(mean startle amplitude of PULSE ALONE−trials) × 100]}. Averaged PULSE ALONE trials across blocks 1–3 were used in the calculation of PPI. Startle responses were analyzed from averaged block 1 PULSE ALONE trials to evaluate the potential genotype differences and drug effects on startle reactivity, while avoiding the confounding effect of habituation (Dulawa *et al*, 2000).

Startle reactivity in experiments 2 and 3 was analyzed using two-way ANOVA, with genotype and drug as factors. A three-way ANOVA, employed similarly as in the analysis of the results of brain biogenic monoamine determinations, was used to analyze startle reactivity and PPI from experiment 4. Since the ANOVA assessing PPI yielded no interaction including all factors in experiment 4, the data were collapsed across prepulse intensities to reduce the number of *post hoc* tests required. To analyze genotype differences in PPI, results from all nontreated (experiment 1) and vehicle-treated (control groups from experiments 2, 3, and 4) mice were pooled and analyzed using repeated-measures ANOVA. Independent samples *t*-tests were used at each prepulse intensity for comparison. Two-way ANOVA for repeated measures was employed to calculate PPI from experiments 2 and 3. Habituation of the startle reflex was calculated as a decrease in startle responses across blocks 1–3 and analyzed with repeated-measures ANOVA. A three-way ANOVA was also used to analyze data from body temperature measurements. ANOVAs were followed by LSD *post hoc* tests, where applicable. Alpha was set at 0.05.

RESULTS

Startle Experiment 1. Baseline Differences of Startle Responses and PPI, and Habituation of the Startle Reflex in Mice with Altered α_{2A}-AR Expression

Lack of α_{2A}-AR was associated with changes in PPI and startle reactivity at baseline, without drug challenge. Prepulses inhibited startle responses more efficiently in α_{2A}-KO mice in comparison with WT animals (PPI × genotype interaction: $F_{(2,147)} = 3.57$; $p = 0.031$) (Figure 1). At the highest prepulse intensity, 15 dB, the

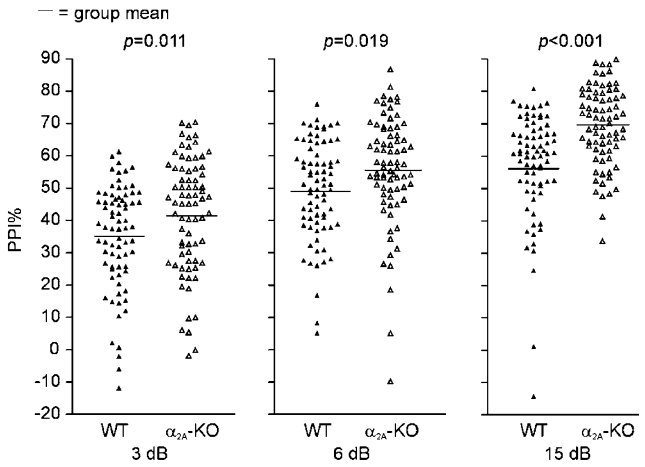

Figure 1 Baseline PPI (individual means) of nontreated or vehicle-treated α_{2A}-KO (open triangles) and WT (closed triangles) mice from all experiments (Exp 1–4) ($n = 75$/group). The PPI values of α_{2A}-KO mice were consistently greater at all prepulse intensities (3, 6, and 15 dB) as compared to WT control mice (independent samples *t*-test). Horizontal lines represent the group means.

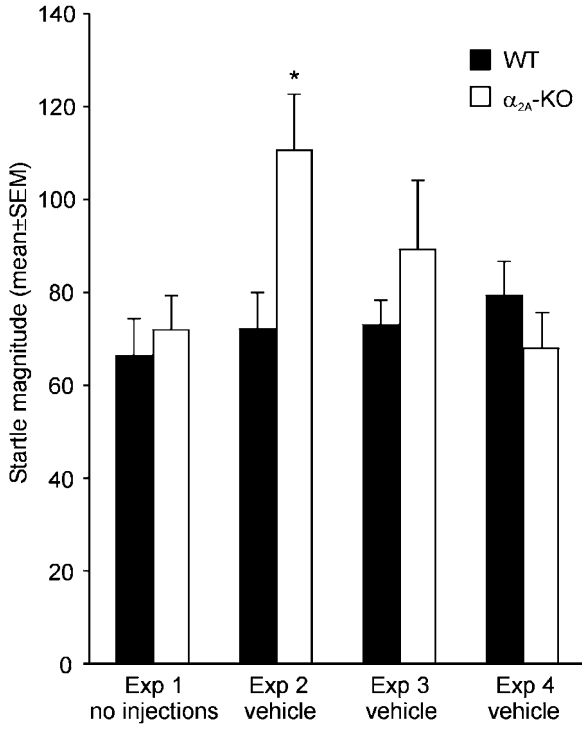

Figure 2 Startle responses of nontreated or vehicle-treated α_{2A}-KO (open bars) and WT (closed bars) mice in each experiment (Exp 1–4). The values are mean startle amplitudes (arbitrary units) ± SEM ($n = 15$–30/group). The first vehicle injection given in experiment 2 increased the startle reactivity of α_{2A}-KO mice compared to control animals (*$p = 0.012$; independent samples *t*-test). No differences were observed in subsequent experiments.

α_{2A}-KO mice had 13% higher PPI levels than WT mice (mean ± SEM PPI%; 57 ± 2 and 70 ± 1 for WT and α_{2A}-KO mice; $t = 5.15$; $p < 0.001$). The α_{2A}-KO and WT mice had similar startle amplitudes to PULSE ALONE stimuli in the first startle experiment, where no injections were given. Vehicle injections in the second startle experiment increased the startle responses of α_{2A}-KO mice (mean increase 54% compared to experiment 1), whereas only a minor, nonsignificant increase (8.6%) was observed in WT mice ($t = 2.67$; $p = 0.012$ for genotype difference in experiment 2) (Figure 2). The difference in startle reactions between the genotypes induced by the first vehicle injection was attenuated in the third experiment and completely abolished in the fourth experiment. As expected, habituation of the startle reflex was observed in all experiments in both genotypes, as revealed by the significant main effects of block on startle reactivity. The values (mean ± SEM, arbitrary units) for the startle reactions across blocks 1–3 in experiment 1 were 72 ± 7, 66 ± 6, and 55 ± 5 for α_{2A}-KO and 66 ± 8, 60 ± 6, and 56 ± 5 for WT animals. There were no interactions between the effects of block and genotype, suggesting that the α_{2A}-KO mice had normal startle habituation.

Startle Experiments 2 and 3: Effects of Atipamezole and Dexmedetomidine

Effects of both Ati and Dex were dissimilar between the α_{2A}-KO and WT mice. A significant genotype × dose interaction was observed in startle amplitudes after treatment with Ati ($F_{(3,111)} = 2.74$; $p = 0.044$) (Figure 3a). In response to the subtype nonselective α_2-antagonist, the startle amplitudes of α_{2A}-KO mice decreased from 110 ± 12 (control) (arbitrary units; mean ± SEM) to 79 ± 12 (3 mg/kg), whereas an opposite trend (increase) was observed in WT mice, ranging from 72 ± 8 to 101 ± 11. Dex attenuated startle amplitudes dose-dependently in WT mice, but had no effect on startle responses of α_{2A}-KO animals ($F_{(3,111)} = 3.14$; $p = 0.028$ for genotype × dose interaction) (Figure 3b). The inhibition of startle responses in WT mice was significant even after the smallest dose (3 μg/kg) of Dex ($p < 0.001$).

The difference in PPI, observed at baseline between the two genotypes, remained after Ati 0.3 and 1 mg/kg (Figure 4a) and Dex 3 μg/kg (Figure 4b), with the α_{2A}-KO mice having slightly higher PPI levels. Dex did not modulate PPI in α_{2A}-KO mice. The PPI of WT mice was markedly attenuated after Dex 10 and 30 μg/kg, but not after 3 μg/kg. However, since these doses cause sedation in normal mice, evidenced here as almost nonexistent startle responses of WT mice (Figure 3b), the relevance of the PPI results after the two highest doses of Dex in WT mice is questionable.

Startle Experiment 4. Effects of D-amph on Startle Responses and PPI, and Modulation of D-amph Responses by Subtype Nonselective α_2-AR Drugs

In startle amplitudes, there were highly significant genotype × amph ($F_{(1,111)} = 13.3$; $p < 0.001$) and genotype × drug ($F_{(2,111)} = 8.28$; $p < 0.001$) interactions, indicating a genotype difference in the response to D-amph alone and in the modulation of D-amph responses by Ati and/or Dex (Figure 5a). D-amph increased startle responses of α_{2A}-KO mice by 80% ($F_{(1,56)} = 9.90$; $p = 0.003$) compared to vehicle-treated controls, but in WT mice the mean startle responses tended to be decreased (−27%) after D-amph. Ati did not alter the effect of D-amph on startle responses of α_{2A}-KO mice, but in WT mice treated with Ati + D-amph the response was increased to the level of α_{2A}-KO mice after

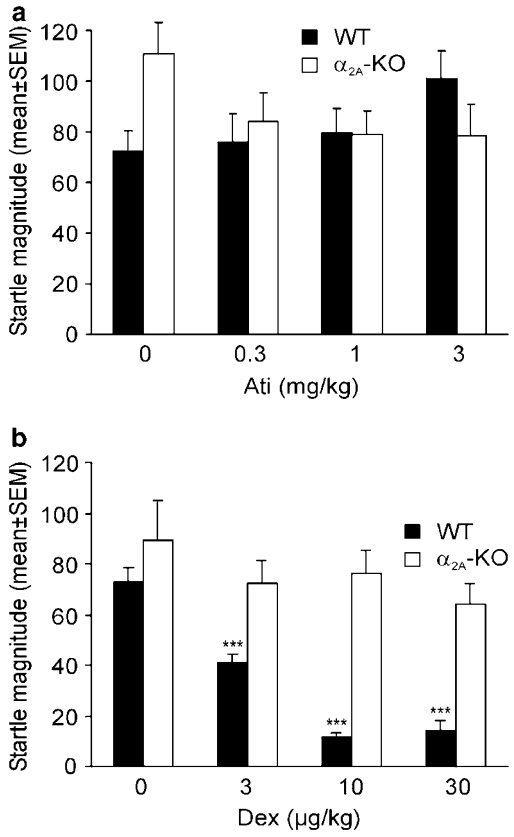

Figure 3 Startle responses of α_{2A}-KO (open bars) and WT (closed bars) mice (mean ± SEM, $n = 14$–15/group). (a) Atipamezole had opposite effects on the startle reactivity of α_{2A}-KO and WT control mice, as revealed by significant genotype × amph interaction in two-way ANOVA ($p = 0.044$). (b) Dexmedetomidine dose-dependently decreased the startle reactions of WT mice, but had no effect in α_{2A}-KO animals ($p = 0.028$ for genotype × dose interaction). ***$p < 0.001$ in comparison to the vehicle control group.

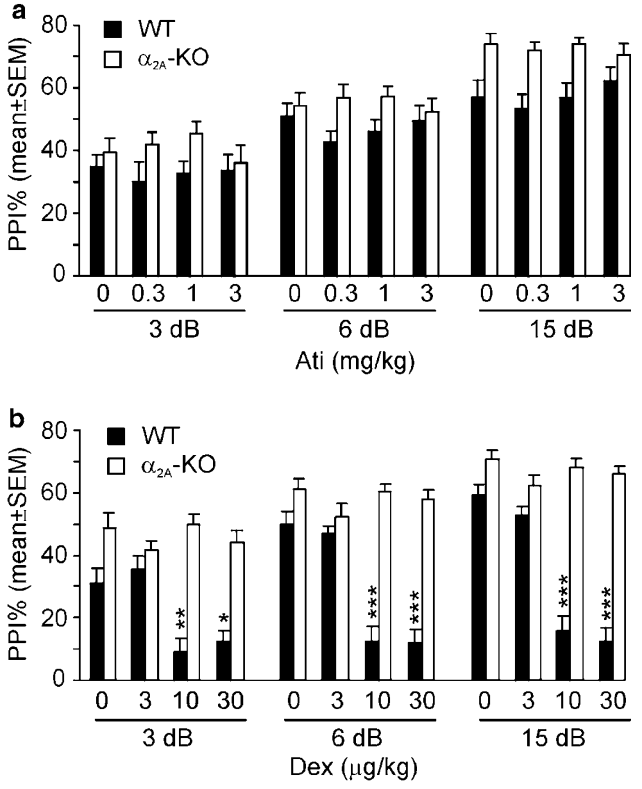

Figure 4 PPI levels of α_{2A}-KO (open bars) and WT (closed bars) mice (mean ± SEM, $n = 14$-15/group) after treatment with atipamezole (a) or dexmedetomidine (b). Atipamezole was ineffective in both genotypes. Dexmedetomidine decreased PPI of WT mice, but the α_{2A}-KO mice were unaffected ($p < 0.001$ for genotype × dose interaction in two-way ANOVA at all prepulse intensities). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in comparison to vehicle control group. It should be noted that the calculated PPI for 10 and 30 μg/kg Dex groups is questionable, since the drug inhibited startle amplitudes *per se*.

D-amph alone ($F_{(1,55)} = 24.2$; $p < 0.001$). Small doses of Dex (3 μg/kg) did not modify the effects of D-amph on startle responses, neither in α_{2A}-KO nor in WT mice.

Significant genotype × amph ($F_{(1,111)} = 6.19$; $p = 0.014$) and genotype × drug ($F_{(2,111)} = 4.49$; $p = 0.013$) interactions were observed also in PPI (Figure 5b). The % PPI decreased from 51 ± 2.4 (vehicle-treated, mean ± SEM) to 40 ± 3.3 (vehicle-D-amph treated) in WT mice ($p = 0.016$) after D-amph. In α_{2A}-KO mice, the D-amph alone treatment caused a more pronounced decrease in % PPI from 57 ± 2.9 (vehicle treated, mean ± SEM) to 30 ± 0.8 (vehicle-D-amph treated) ($p < 0.001$). Dex partially restored the D-amph-disrupted PPI of α_{2A}-KO mice (Figure 5b) ($p = 0.003$ for the difference between vehicle-amph- and Dex-amph-treated α_{2A}-KO mice), but it had no effect in WT mice. Ati did not modulate the effects of D-amph on PPI.

Effects of D-amph on Brain Biogenic Amines and Their Metabolites, and Modulation of D-amph Responses by Subtype Nonselective α_2-AR Drugs

NE and MHPG. Statistically significant main effects of genotype and amph were noted in almost all studied brain regions in the levels of NE and its main metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in the three-way ANOVA. The effects of D-amph on NE and MHPG were different between the α_{2A}-KO and WT mice, as NE levels were markedly reduced in all examined brain regions of α_{2A}-KO mice, but unchanged or only slightly reduced in WT mice, and as MHPG levels were markedly reduced in WT mice, but unchanged or increased in α_{2A}-KO mice (Figure 6). The genotype × amph interaction was statistically significant for NE ($p < 0.001$) in all brain regions, and for MHPG in the cortex ($F_{(1,89)} = 24.78$, $p < 0.001$) and thalamus–hypothalamus ($F_{(1,92)} = 4.23$, $p = 0.042$). In the hippocampus, there was also a similar, nonsignificant trend for the genotype × amph interaction in MHPG ($F_{(1,88)} = 2.95$, $p = 0.090$). The reduction in the NE content by D-amph was 43% in the cortex of α_{2A}-KO mice, compared to only a 9% reduction in WT controls (Figure 6a). Conversely, the MHPG content was not influenced by D-amph in α_{2A}-KO mice (+6% in cortex), whereas a 48% decrease was observed in the cortex of WT mice (Figure 6a).

The D-amph-induced alterations in brain NE metabolism were differently modified in α_{2A}-KO and WT mice by the α_2-AR antagonist Ati. The concurrent administration of Ati

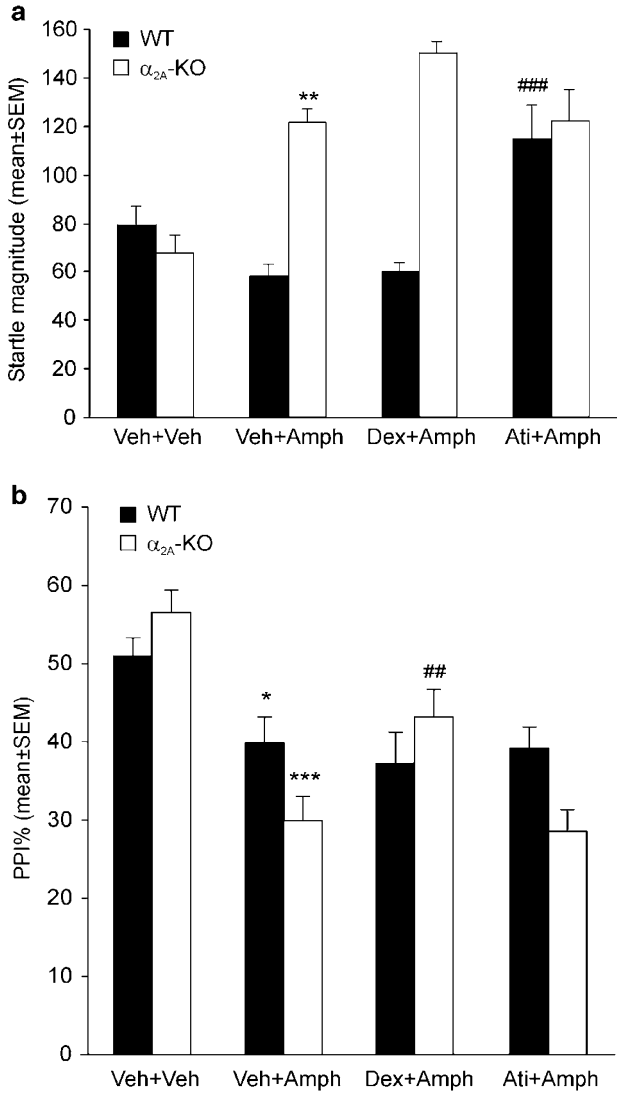

Figure 5 Mean ± SEM ($n = 14$–15/group) startle magnitude (a) and PPI levels (b) of α_{2A}-KO (open bars) and WT (closed bars) mice. PPI is shown collapsed across prepulse intensities. Treatment groups were as follows: vehicle (Veh + Veh), D-amph 10 mg/kg (Veh + Amph), Dex 3 μg/kg and D-amph (Dex + Amph), Ati 1 mg/kg and D-amph (Ati + Amph). Vehicle, Ati, and Dex were injected 20 min, and D-amph 10 min before the start of the test. D-amph had opposite effects on startle responses in α_{2A}-KO and WT mice ($p < 0.001$; genotype × amph interaction). Atipamezole increased the D-amph modulated startle responses of WT mice to the level of α_{2A}-KO mice after D-amph alone. PPI was more effectively disrupted by D-amph in α_{2A}-KO mice ($p = 0.014$ for genotype × amph interaction). Dexmedetomidine partly counteracted the D-amph-induced PPI disruption in α_{2A}-KO mice, whereas it had no effect in WT control mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to vehicle group of the same genotype. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ in comparison to Veh + Amph group of the same genotype.

(1 mg/kg) with D-amph produced marked effects in WT, but not in α_{2A}-KO mice. Consequently, the genotype differences in the responses to D-amph alone in the content of NE and MHPG were abolished after Ati (Figure 6). There was also a tendency for augmentation of the D-amph-induced NE depletion and MHPG increase after administration of Ati to α_{2A}-KO mice, but this effect reached statistical significance only for NE in cortex ($p = 0.007$). The α_{2A}-AR agonist Dex (3 μg/kg), administered together with D-amph, had no modulatory effects on NE or MHPG responses in any brain region in comparison to the effects of D-amph alone in either genotype (Figure 6).

As reported earlier (Lähdesmäki *et al*, 2002), the MHPG levels of vehicle-treated α_{2A}-KO mice were greater than those of WT controls. The differences were statistically significant in the cortex ($t = 3.34$; $p = 0.004$) and the thalamus–hypothalamus ($t = 3.68$; $p = 0.002$), and a similar, nonsignificant trend was also seen in the hippocampus (Figure 6).

DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). In response to D-amph, the most prominent changes in DA metabolism were observed in the thalamus and hypothalamus, where the main effect of amph was significant for the parent amine DA ($F_{(1,92)} = 67.05$, $p < 0.001$), and for the metabolites DOPAC ($F_{(1,92)} = 210.9$, $p < 0.001$) and HVA ($F_{(1,92)} = 56.97$, $p < 0.001$). A main effect of genotype was only observed for DOPAC in the cortex ($F_{(1,92)} = 6.135$, $p = 0.015$). The D-amph injections had opposing effects on the levels of DA in α_{2A}-KO and WT mice. In α_{2A}-KO mice, D-amph tended to increase the brain DA content (except in the thalamus–hypothalamus), whereas slight decreases were noted in all brain regions of WT animals. However, the genotype × amph interaction for DA was only significant in the striatum ($F_{(1,92)} = 5.174$, $p = 0.025$), where D-amph caused a 17% increase in α_{2A}-KO mice, compared to a 8% decrease in WT mice. For the DA metabolites DOPAC and HVA, a significant genotype × amph interaction was observed in the striatum for DOPAC ($F_{(1,92)} = 5.843$, $p = 0.018$), and in all brain regions for HVA (eg $F_{(1,87)} = 16.81$, $p < 0.001$ in the hippocampus) (Figure 7). The levels of DOPAC were reduced or unaffected by D-amph depending on the brain region, with no major differences observed between the genotypes. The two regions having the highest DA content, the striatum and the thalamus and hypothalamus, were subject to the most marked effects of D-amph. In the striatum, there were 59% (α_{2A}-KO) and 64% (WT) reductions in DOPAC concentrations in response to D-amph. On the contrary, the levels of HVA were differently changed after D-amph in the α_{2A}-KO and WT mice (Figure 7; results not shown for DA and DOPAC). In the cortex, hippocampus, and striatum of WT mice, the levels of HVA were unaltered or slightly decreased after D-amph, while statistically significant increases occurred in the cortex, hippocampus, and thalamus–hypothalamus of α_{2A}-KO mice.

The modulation of D-amph-elicited changes in the metabolism of DA by Dex and Ati was clearly less prominent than the drugs' effects on NE metabolism. Yet, the genotype × amph × drug interaction was statistically significant for HVA in the hippocampus ($F_{(2,88)} = 3.700$, $p = 0.029$) and in the thalamus and hypothalamus ($F_{(2,92)} = 4.927$, $p = 0.009$) (Figure 7). The administration of Dex together with D-amph did not cause additional effects on DA metabolism in α_{2A}-KO or WT mice, compared to D-amph alone. However, the combination of Ati and D-amph consistently elevated the levels of HVA in WT mice

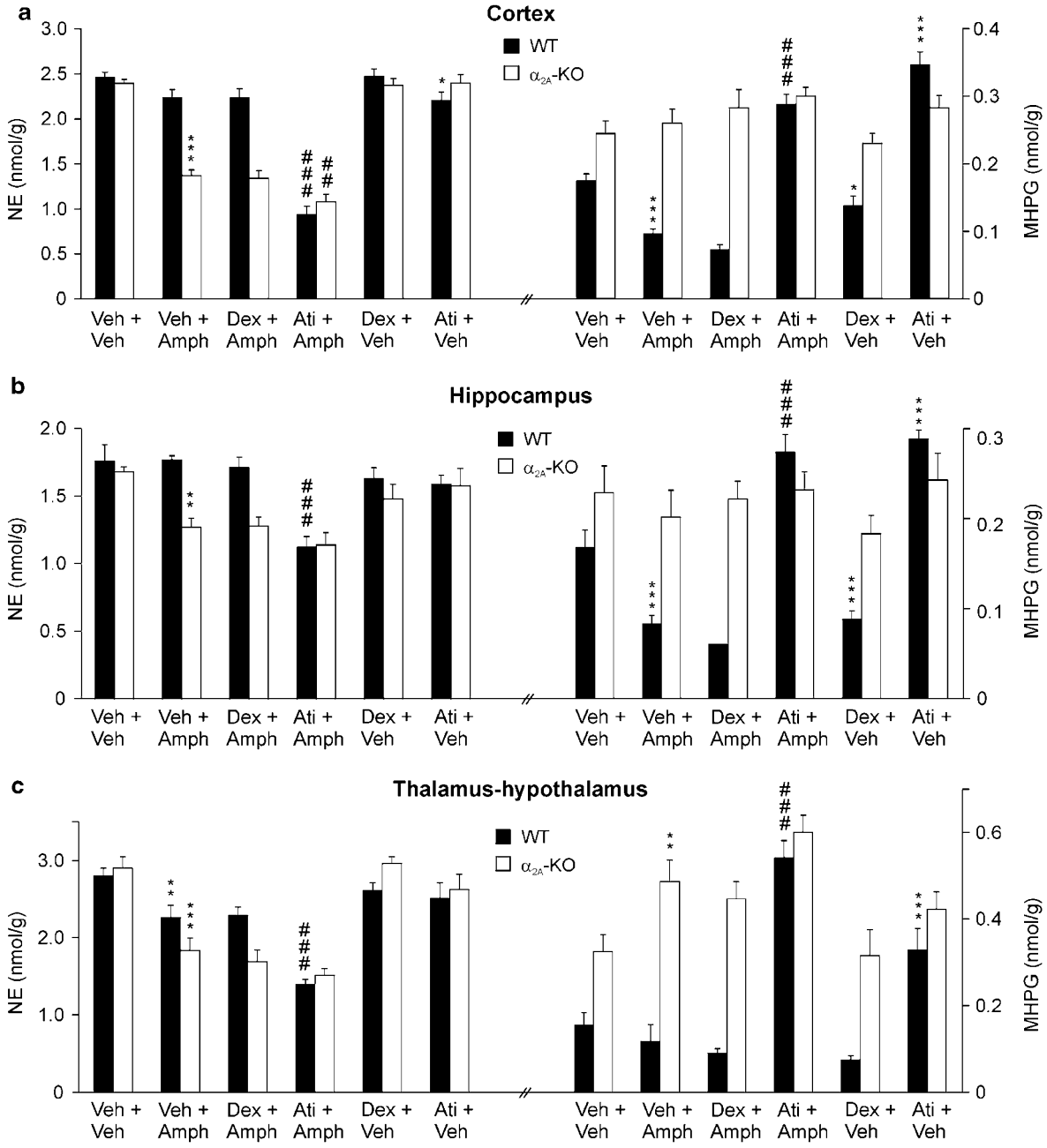

Figure 6 Concentrations of NE and MHPG of α_{2A}-KO (open bars) and WT (closed bars) mice measured from the cortex (a), hippocampus (b), and thalamus–hypothalamus (c). Treatment groups were vehicle (Veh + Veh), D-amph 10 mg/kg (Veh + Amph), Dex 3 μg/kg and D-amph (Dex + Amph), Ati 1 mg/kg and D-amph (Ati + Amph), Dex 3 μg/kg (Dex + Veh), and Ati 1 mg/kg (Ati + Veh). Values are nmol/g tissue (mean ± SEM) ($n = 8$–10). The first injection was given 50 min and the second injection 40 min before the mice were killed. In α_{2A}-KO mice, the NE content of all brain regions was depleted by D-amph, but in WT animals a significant reduction occurred only in the thalamus–hypothalamus. Conversely, MHPG levels of α_{2A}-KO mice were unaffected or increased (thalamus–hypothalamus) by D-amph, whereas marked reductions were noted in the cortex and hippocampus of WT mice. α_2-AR blockade by Ati, administered together with D-amph, abolished the genotype differences seen after D-amph alone. $^{**}p<0.01$, $^{***}p<0.001$ in comparison to vehicle group of the same genotype. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ in comparison to Veh + Amph group of the same genotype.

with statistically significant changes noted in the hippocampus (+71% compared to D-amph alone) and the thalamus and hypothalamus (+28% compared to D-amph alone) (Figure 7), thus abolishing the genotype differences after D-amph alone. In α_{2A}-KO mice, Ati failed to modify the D-amph effects on DA and its metabolites.

TRP, 5-HT, and 5-HIAA. The D-amph injections strongly influenced 5-HT metabolism throughout the brain. In all brain regions, the main effect of amph was significant for the 5-HT precursor TRP, the parent amine 5-HT, and the metabolite 5-hydroxyindoleacetic acid (5-HIAA). The content of TRP was increased more in α_{2A}-KO than in WT mice

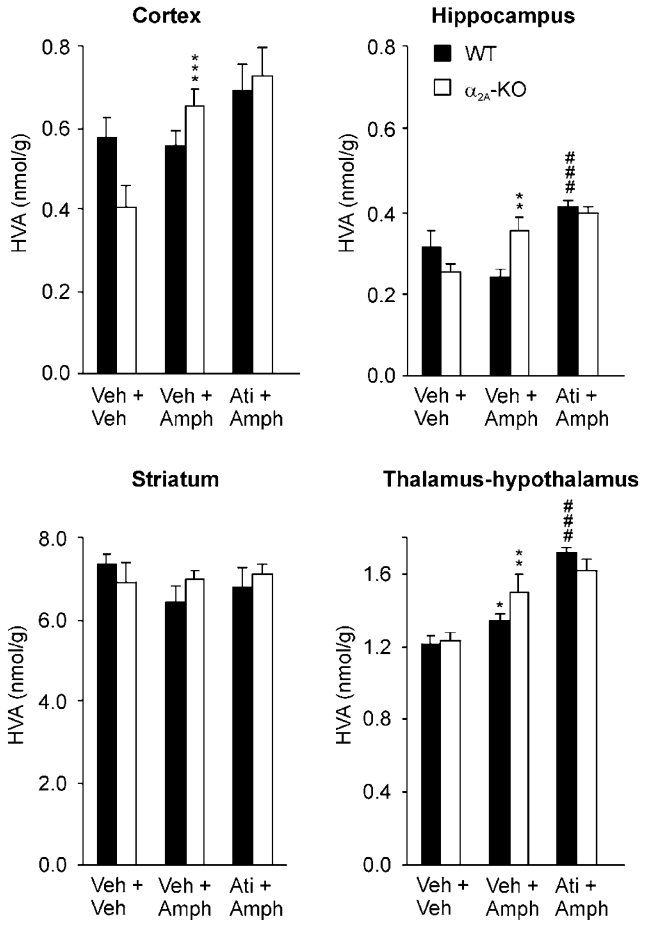

Figure 7 Effects of D-amph on the brain concentrations of the DA metabolite HVA in α_{2A}-KO (open bars) and WT (closed bars) mice (as nmol/g, mean ± SEM) ($n = 8$–10). Treatment abbreviations as in Figure 6. HVA levels of α_{2A}-KO mice were increased by D-amph, but in WT mice only minor changes were observed (genotype × amph interaction significant in all brain regions, for example, $F_{(1,87)} = 16.81$, $p < 0.001$ in the hippocampus). Ati abolished the genotype difference by increasing the HVA levels of WT mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to vehicle group of the same genotype. $^{\#\#\#}p < 0.001$ in comparison to Veh + Amph group of the same genotype.

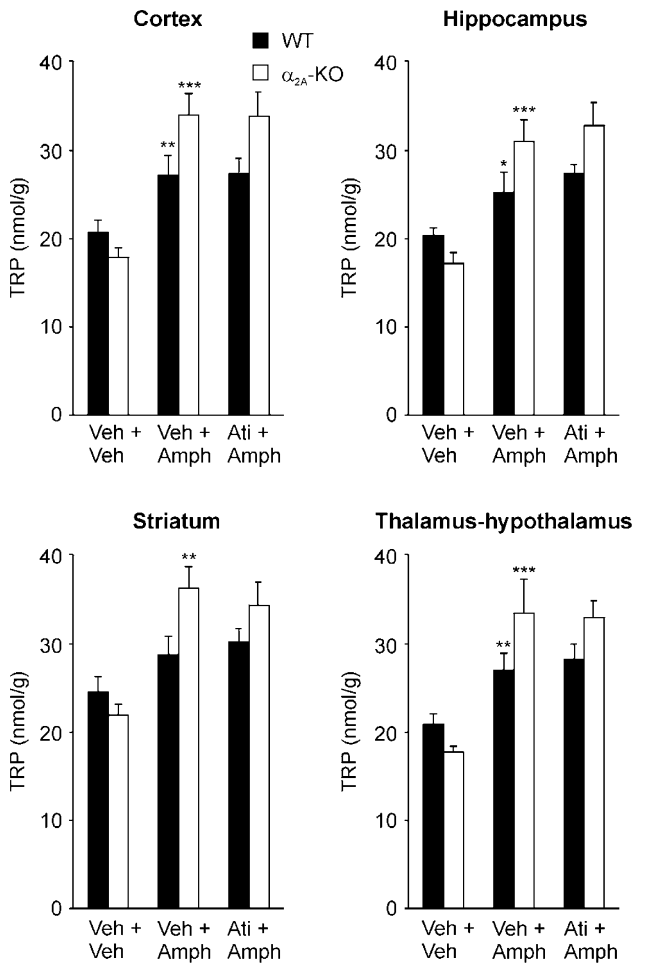

Figure 8 Effects of *d*-amph on the brain concentrations of the 5-HT precursor, TRP in α_{2A}-KO (open bars) and WT (closed bars) mice (as nmol/g, mean ± SEM) ($n = 8$–10). Treatment abbreviations as in Figure 6. D-amph increased TRP concentrations more in α_{2A}-KO mice than in WT controls in all brain regions (eg $F_{(1,88)} = 10.68$, $p = 0.002$ for genotype × amph interaction in cortex). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to vehicle group of the same genotype.

by D-amph, that is, there was a significant genotype × amph interaction in all brain regions (eg $F_{(1,88)} = 10.68$, $p = 0.002$ in the cortex) (Figure 8). After D-amph, the level of TRP in the cortex of α_{2A}-KO mice was increased by 90% compared to a 33% increase in WT control animals. In both genotypes, D-amph increased the concentrations of 5-HT in the cortex, the hippocampus, and the thalamus–hypothalamus, but had no effect in the striatum. D-amph also similarly decreased the levels of 5-HIAA in both genotypes, with a significant effect of amph observed in each brain region (results not shown). The modulation of D-amph responses by the concomitant administration of Dex or Ati was restricted to a minor increase in the cortical 5-HT content in α_{2A}-KO mice with Ati (results not shown).

Body temperature. Body temperature before drug administration was similar in α_{2A}-KO (39.1 ± 0.1°C; mean ± SEM across all groups) and WT (39.2 ± 0.1°C) mice. In the second measurement, prior to decapitation, there was slight reduction in vehicle-treated α_{2A}-KO (0.5 ± 0.2°C) and WT (0.3 ± 0.1°C) mice. Treatment with D-amph slightly increased the body temperature of α_{2A}-KO mice (+0.6 ± 0.3°C) ($F_{(1,46)} = 10.02$, $p = 0.003$), but no change (+0.1 ± 0.3°C) ($F_{(1,46)} = 1.94$, $p = 0.17$) was observed in WT mice after D-amph.

DISCUSSION

In these experiments, genetically altered mice and pharmacological manipulations were used to clarify the α_2-AR subtypes involved in the modulation of the acoustic startle response and its PPI. Additionally, the involvement of the α_{2A}-AR in the neurochemical and startle-modulating effects of the psychostimulant D-amph was evaluated. The major finding of the current study was that α_{2A}-KO mice were

supersensitive to D-amph, revealed as markedly depleted brain NE stores, increased startle amplitudes, and accentuated PPI disruption. The blockade of all α_2-AR subtypes in WT mice by the subtype-nonselective antagonist atipamezole abolished the D-amph-induced genotype differences in NE levels and startle responses, confirming the α_2-AR dependence of these effects.

The formation of MHPG in brains of WT mice was decreased by D-amph, in agreement with an earlier study in mice that suggested that the MHPG reduction resulted primarily from NE reuptake inhibition by D-amph, and thereby reduced intraneuronal MHPG formation (Heal *et al*, 1989). The lack of α_{2A}-AR expression prevented the D-amph-induced MHPG decline. Together, the brain NE and MHPG results suggest that the released NE after D-amph administration normally activates presynaptic α_{2A}-AR to inhibit further NE release. In the absence of α_{2A}-AR, or during pharmacological blockade of all α_2-AR subtypes with an antagonist, the NE stores in noradrenergic neurons are susceptible to depletion by D-amph. This corroborates the evidence from α_2-AR subtype-deficient mice revealing the principal role of the α_{2A}-AR in regulating the stimulation-induced NE release in cortical and hippocampal slices *in vitro* (Hein *et al*, 1999; Trendelenburg *et al*, 1999, 2001; 2003; Scheibner *et al*, 2001; Bücheler *et al*, 2002) and in the prefrontal cortex *in vivo* (Ihalainen and Tanila, 2002). Furthermore, the observed contribution of the α_2-AR-noradrenergic system to modulation of the effects of D-amph is in good agreement with two earlier extensive studies on genetically modified mice, reporting increased sensitivity to psychostimulants when the brain NE homeostasis is disturbed, that is, in mice lacking the NE transporter (Xu *et al*, 2000) or incapable of synthesizing NE (Weinshenker *et al*, 2002).

Recent studies on gene-targeted mice have confirmed that non-α_{2A}-AR subtypes also are involved in the autoreceptor function (for a review, see Philipp *et al*, 2002). This was also supported by the current study showing that, although statistically significant only in the cortex, D-amph supplementation with Ati tended to cause an additional NE decrease and an MHPG increase in α_{2A}-KO mice, too. The regional differences in the effects of D-amph alone were not marked. Yet, the decline in the NE levels of WT mice was restricted to the thalamus–hypothalamus, in accordance with a microdialysis study in rats, which reported an enhanced NE release by D-amph in hypothalamus in comparison to the frontal cortex (Geranton *et al*, 2003). In addition to the dependence on the brain region, the α_2-AR modulation of D-amph-induced NE responses has also been shown to depend on the dose used. Low doses of D-amph have been proposed to elevate extracellular NE mainly by inhibiting its reuptake; this impulse-flow-dependent effect is modulated by presynaptic α_2-autoreceptors (Florin *et al*, 1994; Geranton *et al*, 2003). On the contrary, the NE release after high doses of D-amph, suggested to be mediated by an impulse-flow-independent mechanism, was not significantly modulated by the administration of an α_2-AR agonist or antagonist (Florin *et al*, 1994; Geranton *et al*, 2003). In our study, however, regardless of the high D-amph dose employed, lack of α_{2A}-AR expression or blockade of α_2-ARs in WT mice with Ati resulted in more efficient depletion of NE stores by D-amph. These results suggest that, at least in mice, the lack or pharmacological blockade of inhibitory α_{2A}-ARs is able to significantly augment the NE response also to a high dose of D-amph. On the contrary, activation of α_2-ARs with Dex did not affect the D-amph-induced NE or MHPG changes, probably because of the already maximal activation of the inhibitory α_2-AR subtype(s) by endogenous NE.

As expected, D-amph had marked effects also on the metabolism of DA, but, compared to its effects on NE, the α_{2A}-KO and control mice did not show such clear-cut differences. The concentration of DA was reduced by D-amph in the thalamus–hypothalamus (by 18% in α_{2A}-KO and by 22% in WT mice). DOPAC was decreased similarly in both genotypes. Yet, the effects of D-amph on HVA differed in all brain regions between the genotypes, with increases in α_{2A}-KO mice, in contrast to only minor effects in WT mice. Considering the importance of noradrenergic transmission in regulating the DA-related neurochemical and behavioral effects of psychostimulants (Pan *et al*, 1996; Darracq *et al*, 1998; Drouin *et al*, 2002; Ventura *et al*, 2003), and the disturbed basal NE metabolism in the brains of α_{2A}-KO mice (Lähdesmäki *et al*, 2002), the increased HVA formation in D-amph-treated α_{2A}-KO mice was not surprising. Decreased DOPAC levels after D-amph administration, observed in both genotypes, corroborate results in rats from regions representing dopaminergic projection areas (Kuczenski, 1980; Nicolaou, 1980; Elverfors and Nissbrandt, 1992; Karoum *et al*, 1994). The decreased DOPAC formation, reflecting reduced intraneuronal metabolism of DA resulting from reuptake blockade of DA by D-amph, thus, is not affected by α_{2A}-AR. On the contrary, the formation of HVA occurs mainly extracellularly, and the elevated HVA concentrations of α_{2A}-KO mice may therefore represent increased DA release in the absence of α_{2A}-AR heteroreceptor-mediated inhibition (Bücheler *et al*, 2002). Supporting this view, the HVA levels of WT mice after combined Ati-D-amph treatment were elevated to the level observed in α_{2A}-KO mice after D-amph alone, except in the striatum, where the α_{2C}-AR subtype predominates.

D-amph increased the concentrations of the 5-HT precursor, TRP. In α_{2A}-KO mice, the TRP increase by D-amph was augmented throughout the brain (eg a 90% increase in cortex compared to a 33% increase in WT animals). Recently, pharmacological activation of both β_2- and β_3-adrenergic receptors was reported to markedly increase brain TRP levels (Lenard *et al*, 2003). Lack of sympathetic inhibition in α_{2A}-KO mice (Brede *et al*, 2002) after stimulation with D-amph, leading to enhanced β-adrenergic activation, probably explains this TRP difference. Independent of the genotype and the brain region, D-amph increased 5-HT and decreased 5-HIAA levels.

Baseline startle reactivity, in the absence of injections, was similar in α_{2A}-KO and WT mice. The first vehicle injections significantly increased the startle responses of α_{2A}-KO mice compared to baseline, but WT mice were not similarly influenced. The increased startle of α_{2A}-KO mice after the first injections may be attributed to elevated stress caused by the injections and/or the somewhat aversive test situation, in agreement with the increased sensitivity to injections previously reported in α_{2A}-KO mice in the open-field test and the light–dark paradigm (Schramm *et al*, 2001). Moreover, the neurobehavioral phenotype of α_{2A}-KO

mice is characterized by increased anxiety-related behaviors (Lähdesmäki *et al*, 2002). Increased startle has been consistently reported after the α_2-AR antagonists yohimbine, idazoxan, and RS-79948-197 in rats (White and Birkle, 2001), and after yohimbine in humans (Morgan *et al*, 1993, 1995). Interestingly, Ati had opposite effects on startle in α_{2A}-KO and WT mice, indicating that blockade of α_{2A}-AR mediates the startle-enhancing, anxiogenic-like effects of α_2-AR antagonists. Considering the altered startle reflex and cortical arousal in mice lacking the α_{2C}-AR (Sallinen *et al*, 1998; Puoliväli *et al*, 2002), it is possible that the slight startle reduction by Ati in α_{2A}-KO mice is based on α_{2C}-AR blockade.

Dex decreased startle dose-dependently in WT mice, whereas the α_{2A}-KO animals were unaffected. Earlier studies have shown that the α_2-AR agonist clonidine decreases startle after systemic administration in humans (Kumari *et al*, 1996) and rats (Davis *et al*, 1977), and after spinal (Davis and Astrachan, 1981) or intra-amygdaloid (Schulz *et al*, 2002) administration in rats. The almost totally abolished startle reflex of WT mice after Dex 10 and especially 30 μg/kg was likely due to its sedative effect. The sedation elicited by α_2-AR agonists is mainly mediated through activation of α_{2A}-AR, and has previously been shown to be attenuated, but not totally absent in mice lacking functional α_{2A}-AR (Hunter *et al*, 1997; Lakhlani *et al*, 1997; Lähdesmäki *et al*, 2003). The smallest Dex dose, 3 μg/kg, is not, however, sedative in mice (Hunter *et al*, 1997), but still effectively reduced startle in WT mice. This suggests that the α_2-AR agonist-mediated startle attenuation is also α_{2A}-AR dependent.

Consistent with the accentuated D-amph response in brain NE metabolism, increased sensitivity of α_{2A}-KO mice to the effects of D-amph was also observed in startle reactivity. In WT mice, D-amph mildly (statistically non-significantly) decreased startle amplitudes, as reported earlier in C57Bl/6 mice (Ralph *et al*, 2001; Varty *et al*, 2001). It seems evident that the 80% increase of startle in α_{2A}-KO mice induced by D-amph was, indeed, due to the lack of α_{2A}-AR expression, since the startle responses of WT mice after supplementation with Ati were increased to the level of α_{2A}-KO mice treated with D-amph alone. This suggests that the synaptic NE, increased after D-amph administration, activates α_{2A}-AR in WT mice, thereby inhibiting further NE release and also preventing the startle enhancement.

The PPI levels of α_{2A}-KO mice were slightly elevated compared to WT mice in control groups of all experiments, regardless of whether the control groups were (experiments 2, 3 and 4) or were not (experiment 1) given vehicle injections. Recently, several genetically altered mouse lines with modified expression of neurotransmitter receptors or transporters have been examined to investigate the potential genetic basis of sensorimotor gating (Geyer *et al*, 2002). Mouse strains reported to have increased basal PPI have included $5HT_{1B}$-KO (Dulawa *et al*, 2000) and α_{2C}-over-expressing mice (Sallinen *et al*, 1998). It can be speculated that endogenous NE tonically reduces the level of PPI via α_{2A}-AR, and inactivation of the α_{2A}-AR gene thus results in increased PPI. However, in spite of the baseline PPI difference between the genotypes, no significant modulation of PPI was observed after Ati or nonsedative doses of Dex. In line with the neurochemistry and startle results, D-amph again had more pronounced effects on PPI in α_{2A}-KO mice. D-amph caused clear PPI disruption across all prepulse intensities in α_{2A}-KO mice, compared to a less clear-cut decrease in WT mice only at the 3 dB intensity (data not shown for individual prepulse intensity levels). The PPI disruption in α_{2A}-KO mice was also partly opposed by Dex, suggesting that other α_2-AR subtypes, possibly α_{2C}-AR (Sallinen *et al*, 1998), modulate PPI during D-amph challenge.

The current results show that the perturbation in brain NE homeostasis of α_{2A}-KO mice is drastically enhanced when the mice are challenged with D-amph. The supersensitivity of α_{2A}-KO mice to the behavioral and neurochemical effects of D-amph indicates a crucial involvement of α_{2A}-AR in the modulation of the actions of the psychostimulant. It is possible that the therapeutic effects of D-amph in ADHD, as well as the stimulant effects of abused D-amph, are significantly regulated by brain α_2-ARs. In the absence of the inhibitory control normally attributable to α_{2A}-AR, D-amph administration results in increased startle reactivity and more pronounced impairment of sensorimotor gating, which are probably caused by concomitant changes in either NE or DA release. These results provide evidence for participation of α_{2A}-AR in neurobiological processes related to disturbed attentional regulation or impaired sensorimotor information processing, such as ADHD or schizophrenia. The results also suggest that a potentially harmful drug interaction may exist between amphetamine-like psychostimulants and α_2-AR antagonists.

ACKNOWLEDGEMENTS

Brian Kobilka and John Altman are acknowledged for providing the α_{2A}-KO mice. The expertise of Lauri Sillanmäki in statistical analyses is also acknowledged. In addition, the help of Veronica Fagerholm has been invaluable. The work was supported by the Academy of Finland and Orion Corporation.

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