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The effect of angiotensin AT_{1A} inactivation on innate and learned fear responses in mice and its relationship to blood pressure

Kwok H.C. Choy, Carolina A. Chavez, Jing Yu, Dmitry N. Mayorov*

Dept. of Pharmacology, University of Melbourne, Victoria, Australia

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ABSTRACT

Angiotensin AT_1 receptors are implicated in behavioral and physiological processes associated with fear and stress. However, the precise role of AT_1 receptors in modulating fear-related behavior and its relation to their physiological effects remains unclear. Here, we examined innate and learned fear responses and their relationship to cardiovascular arousal in AT_{1A} receptor knockout ($AT_{1A}^{-/-}$) mice. Using synchronized video and blood pressure telemetry, we found that, in a novel test environment, $AT_{1A}^{-/-}$ mice showed reduced neophobia but a similar rise in blood pressure, as compared to $AT_{1A}^{+/+}$ mice. In response to a discrete threat, footshock, both flight behavior and cardiovascular arousal were decreased in $AT_{1A}^{-/-}$ mice. Reduced flight behavior was also observed in $AT_{1A}^{-/-}$ mice in the elevated T-maze test. During fear conditioning, the immediate freezing response to the first shock, but not the rate of freezing acquisition was decreased in $AT_{1A}^{-/-}$ mice. Likewise, $AT_{1A}^{-/-}$ mice showed reduced freezing and pressor responses to the first re-exposure, but normal rate of freezing extinction over subsequent trials. Similarly, in the elevated T-maze, the rates of avoidance acquisition and escape learning remained unchanged in $AT_{1A}^{-/-}$ mice. Finally, after re-exposure, $AT_{1A}^{-/-}$ mice displayed altered c-Fos expression, compared to $AT_{1A}^{+/+}$ mice, in the hypothalamus and periaqueductal gray but not in fear-related limbic-cortical areas, nor in medullary nuclei that convey visceral afferent information. We conclude that AT_{1A} receptor knockout reduces innate fear responses, without affecting learning efficiency in mice. These effects are dissociable from cardiovascular effects and likely reflect altered neurotransmission in hypothalamic-midbrain defense regions.

1. Introduction

Angiotensin AT_1 receptors are increasingly implicated in the regulation of various behavioral processes, including defensive/aversive responses (Wright et al., 2008). Numerous experimental studies have reported that AT_1 receptor inactivation (either through pharmacological inhibition or genetic manipulations) reduces measures of innate anxiety/fear in rodent models (Barnes et al., 1990; Kulakowska et al., 1996; Shekhar et al., 2006; Wang et al., 2016). Clinical research, although limited, has also demonstrated that AT_1 receptor blockers (ARBs) decrease depression, anxiety and post-traumatic stress disorder (PTSD) symptoms (Khoury et al., 2012; Nylocks et al., 2015; Pavlatou et al., 2008), whereas AT_1 receptor gene variation is associated with depression in elderly subjects (Taylor et al., 2012). However, not all animal studies support the importance of AT_1 receptors in innate fear regulation (Braszko, 2005), while their role in fear learning appears even more complex, with both positive and negative effects being reported (Barnes et al., 1990; DeNoble et al., 1991; Kulakowska et al., 1996; Lazaroni et al., 2016; Marvar et al., 2014; Tota et al., 2009).

AT_1 receptors are also known to influence a variety of physiological functions, including the regulation of blood pressure (BP) and cardiovascular stress reactions (Watanabe et al., 1998). In respect to the latter, multiple animal studies have shown that pharmacological blockade of AT_1 receptors reduces autonomic and neuroendocrine responses to intense aversive stimuli, e.g. restraint or immersion in shallow cold water (for references, see Mayorov, 2011). Likewise, in human studies, ARBs decrease the pressor response to noxious cold-pressor test (Israel et al., 2006; Vase et al., 2008), whereas genetic variation in the AT_1 receptor gene correlates with this response (Wang et al., 2010). However, there is some controversy regarding the role of AT_1 receptors in physiological reactivity to mild stressors, as ARBs were found to have little effect on pressor responses to mental arithmetic or computer-based tasks (Arosio et al., 2005; Heusser et al., 2003). Whether these discrepancies indicate that AT_1 receptors are involved in

* Corresponding author. Current address at: School of Physiology, Pharmacology and Neuroscience, Medical Sciences Building, University of Bristol Bristol, BS8 1TD, UK.
E-mail addresses: dmitry.mayorov@bristol.ac.uk, dmitry.n.mayorov@gmail.com (D.N. Mayorov).

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responding to only certain types of aversive stimuli, or that their functional role depends on the level/imminence of threat, remains elusive.

Another key question that remains unanswered concerns the role of visceral afferent feedback in the anxiolytic effect of AT_1 receptor inactivation. Indeed, substantial evidence indicates that emotional regulation can be profoundly influenced by ascending, "bottom-up" inputs from visceral and somatic afferents (Berntson et al., 2003). Given the well-established role of AT_1 receptors in cardiovascular control, it is possible that ARB-induced changes in the circulatory homeostasis and/or reactivity and thereby in afferent feedback to the brain are responsible, at least in part, for their anxiolytic effects.

To address these questions, we examined innate and learned fear responses and their relation to cardiovascular arousal in mice lacking the AT_{1A} receptor, the predominant isoform of the AT_1 receptor in rodent brain (Davisson et al., 2000). We first used a fear conditioning/extinction paradigm to assess the influence of AT_{1A} receptors on the major types of defensive responding, namely risk assessment, freezing and flight-fight (Fanselow, 1994). Using this paradigm, we also compared the effects of AT_{1A} receptor knockout ($AT_{1A}^{-/-}$) on neuronal c-Fos responses in forebrain-midbrain nuclei involved in fear learning and expression, and in hindbrain nuclei relaying visceral afferent information. Finally, we investigated defensive behavior of $AT_{1A}^{-/-}$ mice in a pain-unrelated task, the elevated T-maze.

2. Materials and methods

2.1. Animals

The experiments were performed using 2–3 month old male $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the University of Melbourne Animal Ethics Committee. $AT_{1A}^{-/-}$ mice and their own control strain were bred in the animal facilities of the University of Melbourne. The generation of these mice has been described earlier (Ito et al., 1995). Genotypes were determined by PCR, as described (von Bohlen et al., 2001).

2.2. Synchronized behavioral and cardiovascular monitoring

Under isoflurane anesthesia, mice were implanted with TA11PA-C10 telemetry transmitters (Data Sciences International (DSI), St. Paul, MS) with catheters placed in the ascending aorta via the left carotid artery (Davern et al., 2009). The mice were then housed individually and allowed 10 days of recovery. Body weight, food and water consumption were routinely checked during the recovery period. Each mouse received a subcutaneous injection of carprofen (5 mg/kg) before surgery and 24 h after surgery to alleviate any pain or discomfort.

Mice were maintained on a 12:12-h light-dark cycle (lights off at 6 p.m.) with water and food ad libitum. All tests were conducted between 10 a.m. and 5 p.m. During each test, data from the telemetry device were radio-transmitted, and BP, heart rate (HR) and locomotor activity were recorded using the DSI acquisition system. Animal behavior was video-recorded using a digital camera (AXIS 221, 640 × 480 resolution, 30 frames per s) and the video signal was synchronized with telemetry data using synchronization software (DSI). Video recordings were analyzed using ANY-maze video tracking system (Stoelting, Wood Dale, IL). Freezing was defined as the absence of all movement, except respiration, for at least 1 s (Anagnostaras et al., 2010), and was scored automatically using the ANY-maze. Scanning was defined as side-to-side head movements, with unchanged posture for at least 1 s, and was assessed using simultaneous tracking of the animal's nose- and center-points (Choy et al., 2012). In addition, two commonly used indices of anxiety, defecation and stretched-attend posture (SAP), were determined in each animal. Given that freezing, defined as the absence of movement, may be confounded with defensive quiescence distinguishable from freezing only by the body postures adopted, e.g. SAP (Gray and McNaughton, 2000), the occurrence of SAP in fear-conditioning mice was also assessed.

2.3. Contextual fear conditioning and extinction

Telemetry-implanted mice (+/+, n = 13; −/−, n = 13) were pre-exposed for 5 min to the context (the conditioning chamber with the open top, 20 × 22 × 33 cm, L × W × H, Med Associates, Albans, VT) 28, 24 and 3 h before conditioning and their spontaneous behavior was recorded by a video camera positioned 1 m above the chamber. A telemetry receiver (RPC-1, 32.8 × 22.7 × 3.3 cm, DSI) was placed underneath the chamber, ~1 cm below the footshock grid. Mice were then subjected to the 5-min conditioning session (CS+) which consisted of four footshocks (1.0 mA, 3.5 s) delivered at an average interval of 60 s (55–65 s). The response to shock was measured as activity burst velocity (Anagnostaras et al., 2000). Mice were re-exposed for 5 min to the same context 4, 24, 48 and 96 h after conditioning. A time-control (CS−) group of mice (+/+, n = 6; −/−, n = 6) was implanted with telemetry devices and subjected to 8 preconditioning sessions separated by the same time intervals as the CS+ group.

2.4. Effect of diazepam on anxiety-like behavior

Mice (+/+, n = 21; −/−, n = 20) underwent sham operation in which the neck incision was performed and the carotid artery was isolated and ligated with a silk suture. Mice were then allowed to recover and were subjected to three pre-exposures, as described above. Sixty min before the second session, mice were injected with diazepam (1 and 2 mg/kg, i.p.) or vehicle (10% DMSO in saline).

2.5. Flinch-jump threshold test

Sham-operated mice (+/+, n = 8; −/−, n = 5) were individually placed into the conditioning chamber and, following 10-min habituation period, were given footshocks of increasing amplitude (ranged from 0 to 1 mA, 0.1 mA step, 1 s). Response to shock was video recorded and graded in ascending order: 0 = no response, 1 = flinch (a startle response different from normal activity), 2 = jump/run without vocalization, and 3 = jump/run and vocalization (Wittmann et al., 2009).

2.6. c-Fos immunohistochemistry

Sham-operated mice were subjected to the conditioning protocol, as described above, except only two first re-exposures were performed (CS + group: +/+, n = 5; −/−, n = 7; CS− group: +/+, n = 5; −/−, n = 6). Ninety min after the 24-h re-exposure, mice were perfused and brains were processed for c-Fos immunohistochemistry, as described in Supplementary Information.

2.7. Elevated T-maze (ETM)

Sham-operated mice (+/+, n = 10; −/−, n = 9) were tested in the ETM model of anxiety and panic, as described in Supplementary Information.

2.8. Tail suspension test (TST)

Depression-related behavior of sham-operated mice (+/+, n = 5; -/-, n = 5) was assessed in the TST, as described in Supplementary Information.

2.9. Statistical analysis

All values are expressed as mean ± SEM. Data analysis was performed with Prism 7 software (GraphPad) using two-way ANOVA

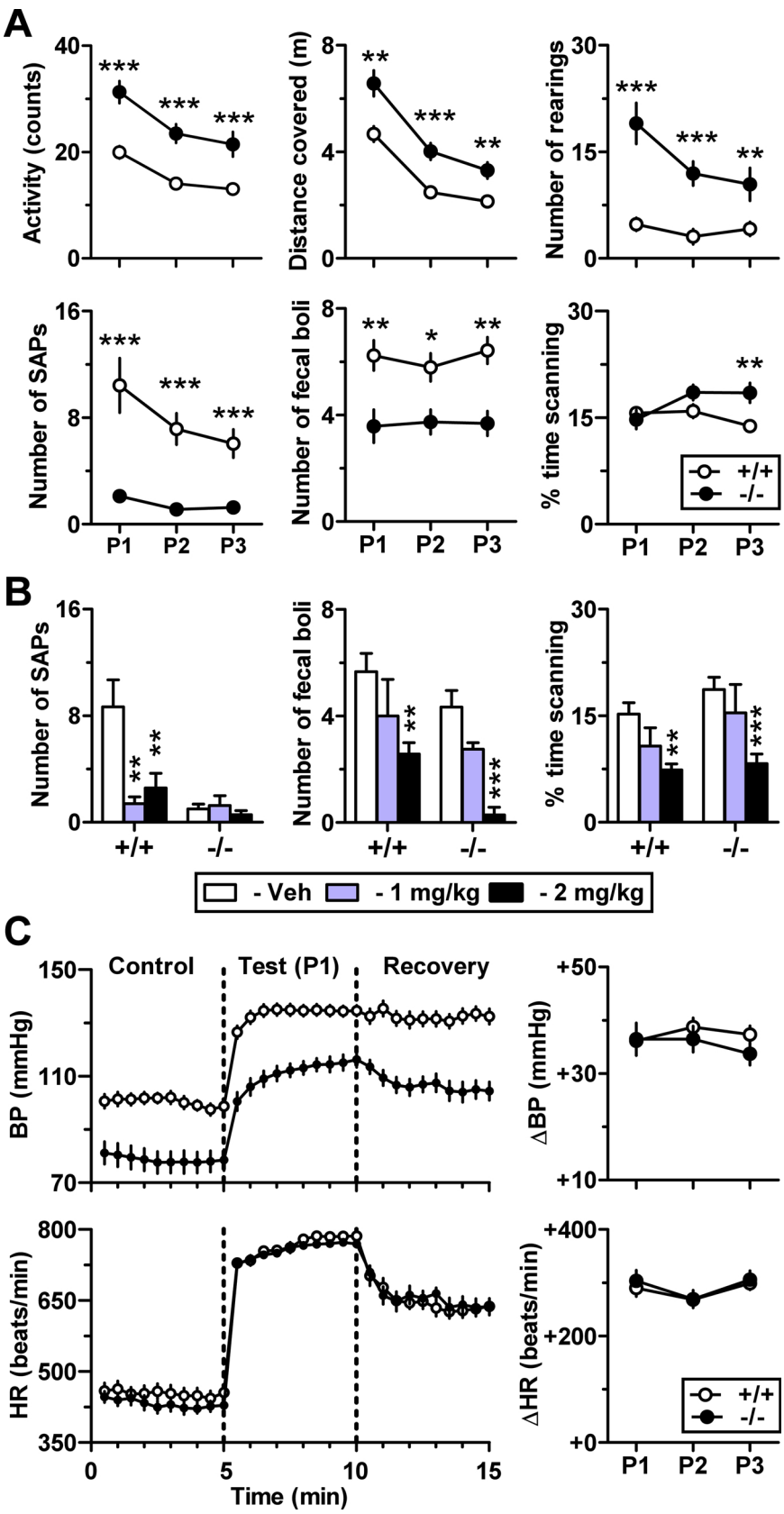

Fig. 1. Increased activity, reduced neophobia and unchanged cardiovascular arousal in $AT_{1A}^{-/-}$ mice during pre-exposure. **(A)** Locomotor and anxiety measures across three pre-exposure (P) sessions (+/+, n = 19; −/−, n = 19). Because motor and cardiovascular responses to pre-exposure were similar between the CS + and CS- groups of the same genotype, data were pooled over corresponding sessions. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between genotypes. **(B)** Validation of SAP, scanning and defecation, as anxiety measures. Mice received an i.p. injection of vehicle (+/+, n = 9; −/−, n = 9), diazepam 1 mg/kg (+/+, n = 5; −/−, n = 4), or 2 mg/kg (+/+, n = 7; −/−, n = 7) 60 min before the second pre-exposure. $^{**}p < 0.01$, $^{***}p < 0.001$ diazepam vs. vehicle. **(C)** Minute-to-minute values of blood pressure (BP) and heart rate (HR) before, during and after the first pre-exposure (left) and their mean change (right) during pre-exposure sessions (+/+, n = 14; −/−, n = 14).

followed by Bonferroni's post-hoc test. The Brown-Forsythe test was used to test for homogeneity of variance, and, when this requirement was not satisfied, data were subjected to square root transformation (activity, distance, rearing, SAP and freezing). Pairwise comparisons were performed using two-tailed Student's *t*-test (with Welch's correction where appropriate). Statistical significance was set at a value of $p < 0.05$.

3. Results

3.1. Pre-exposure to the context

In a novel test environment, $AT_{1A}^{-/-}$ mice showed increased locomotor activity, distance traveled and rearing relative to $AT_{1A}^{+/+}$ mice (ANOVA, effect of genotype: all $F(1,72) > 15.4$, $p < 0.001$; Fig. 1A). These differences reflected more active exploratory behavior in $AT_{1A}^{-/-}$ mice, which persistently inspected the enclosure and this gradually diminished with repeated testing (ANOVA, effect of time: all $F(2,72) > 18.7$, $p < 0.001$). Conversely, when the mice were tested under basal conditions in home cages, no difference in 24-h levels of locomotor activity was observed between genotypes (Supplementary Fig. S1A).

Two commonly used indices of anxiety, SAP and defecation, were reduced in $AT_{1A}^{-/-}$ mice during pre-exposure (both $F(1,72) > 23.1$, $p < 0.001$), while scanning behavior was fairly similar between genotypes (Fig. 1A). No difference in 24-h defecation levels was found between genotypes under basal conditions in home cages (Supplementary Fig. S1B). To confirm the validity of above parameters, as measures of anxiety, a separate set of mice were injected with diazepam before the second pre-exposure. Diazepam dose-dependently decreased defecation and scanning duration in both genotypes, and reduced SAP in $AT_{1A}^{+/+}$ mice to levels observed in untreated $AT_{1A}^{-/-}$ mice (all $F(2,35) > 4.9$, $p = 0.01$; Fig. 1B). These changes likely reflect an anxiolytic drug effect rather than motor impairment or sedation, as diazepam did not alter measures of gross or fine motor activity, such as distance covered, rearing and scanning (nose-point) speed (all $F(2,35) < 1$, $p > 0.44$, not shown).

Upon pre-exposure, $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ showed rapid increases in BP and HR of similar magnitudes (both $F(1,52) < 1$, $p > 0.26$), which did not diminish with repeated testing (Fig. 1C). Because baseline BP was lower in $AT_{1A}^{-/-}$ than $AT_{1A}^{+/+}$ mice (Fig. 1D and Supplementary Fig. S1A), the relationship between individual differences in pre-test BP and anxiety measures was examined, but little or no correlation was found (Supplementary Fig. S1B).

3.2. Footshock

In response to footshock, both genotypes displayed short activity bursts, which did not vary in magnitude with repeated shock presentations ($F(3,72) = 1.1$, $p = 0.36$; Fig. 2A). These reactions were reduced in $AT_{1A}^{-/-}$ mice, as assessed by the distance covered ($F(1,72) = 9.3$, $p < 0.01$) and by activity burst velocity (Fig. 2A). With repeated shock presentations, both genotypes showed the acquisition of freezing behavior ($F(3,72) = 25.3$, $p < 0.001$; Fig. 2B), with freezing scores being substantially lower in $AT_{1A}^{-/-}$ mice ($F(1,72) = 14.4$, $p < 0.001$), as was the defecation score over the testing period (Fig. 2B). The rate of freezing acquisition, however, was not different between groups, as ANOVA revealed no significant interaction between the effects of genotype and shock number on freezing ($F(3,72) < 1$, $p = 0.61$). This phenomenon was also reflected by similar slopes of linear regression lines relating freezing and shock number in each genotype ($F(1,100) < 1$, $p = 0.44$; Fig. 2B).

Upon exposure to the conditioning chamber, BP and HR increased in both CS + and CS− groups (Fig. 2C). ANOVA revealed significant effects of genotype ($F(1,24) = 6.2$, $p = 0.02$) and conditioning ($F(1,24) = 9.2$, $p < 0.01$) on the BP response. Post-hoc analysis showed that this response was reduced in $AT_{1A}^{-/-}$ mice in comparison with $AT_{1A}^{+/+}$ mice, and with $AT_{1A}^{-/-}$ mice of the CS− group (Fig. 2C). Conversely, the HR response was not different between $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice of the CS+ group ($F(1,24) < 1$, $p = 0.39$).

To test whether the reduced freezing response in $AT_{1A}^{-/-}$ mice was due to altered sensorimotor function, flinch, jump and vocalization thresholds were examined in a follow-up experiment. Repeated measures ANOVA found a significant effect of shock intensity ($F(2,22) = 45.1$, $p < 0.001$), but not genotype ($F(1,22) = 3.1$, $p = 0.11$) on these thresholds (Fig. 2D).

3.3. Re-exposure to the context

Upon re-exposure, both genotypes showed increased freezing behavior in comparison to the corresponding CS- groups (Fig. 3A). This increase was however less pronounced in $AT_{1A}^{-/-}$ than $AT_{1A}^{+/+}$ mice ($F(1,72) = 8.6$, $p < 0.01$). With repeated testing, freezing decreased in both genotypes ($F(3,72) = 9.1$, $p < 0.001$), with its levels remaining consistently lower in $AT_{1A}^{-/-}$ mice (Fig. 3A). Remarkably, the rate of freezing extinction was similar between $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice, as reflected by the lack of genotype × time interaction ($F(3,72) < 1$, $p = 0.82$) and by similar slopes of linear regressions between freezing and trial number ($F(1,100) < 1$, $p = 0.65$). Along with freezing extinction, $AT_{1A}^{-/-}$ mice showed a gradual increase in locomotion, while it remained low in $AT_{1A}^{+/+}$ mice, indicating persistent behavioral inhibition (genotype × time interaction for distance covered: $F(3,72) = 4.0$, $p = 0.01$). $AT_{1A}^{-/-}$ mice exhibited higher scanning activity than $AT_{1A}^{+/+}$ mice, although in both genotypes this parameter remained unchanged across trials ($F(3,72) = 1.8$, $p = 0.15$; Fig. 3A). The number of SAP was low and similar in $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice (1.3 ± 0.3 and 2.1 ± 0.3, respectively) and was unchanged across trials ($F(3,72) = 1.9$, $p = 0.13$; data not shown).

The pressor response to re-exposure was reduced in $AT_{1A}^{-/-}$ mice compared to $AT_{1A}^{+/+}$ mice ($F(1,57) = 18.3$, $p < 0.001$; Fig. 3B), and was thus similar to that observed during conditioning. Fear extinction in $AT_{1A}^{-/-}$ mice was followed by a restoration of BP reactivity which by the fourth re-exposure was no longer different from that in $AT_{1A}^{+/+}$ mice. This rebound could not be ascribed to increased locomotor activity, as it remained low compared to pre-exposure and showed no correlation with BP reactivity (Supplementary Fig. S2A). The HR response to re-exposure did not vary across trials ($F(3,57) < 1$, $p = 0.86$) and was similar between genotypes ($F(1,57) < 1$, $p = 0.70$; Fig. 3B). There was little or no correlation between pre-test BP and freezing in both genotypes (Supplementary Fig. S2B).

3.4. c-Fos expression following re-exposure

There were significant main or interaction effects of conditioning and genotype on expression of c-Fos, a marker of neuronal activation, in 11 out of 25 of the brain areas studied, including the bed nucleus of stria terminalis (BNST), periaqueductal grey (PAG), and medial preoptic (MPO), dorsomedial (DMH), paraventricular (PVN) and lateral hypothalamic (LH) regions (Supplementary Table S1). Specifically, conditioned $AT_{1A}^{-/-}$ mice showed reduced c-Fos levels relative to $AT_{1A}^{+/+}$ mice in the PAG, PVN and perifornical area encompassing the dorsal DMH (DMD) and LH (Fig. 3D), three regions that are critical for expressing, respectively, behavioral (freezing), neuroendocrine and pressor responses to conditioned fear (Furlong and Carrive, 2007; LeDoux et al., 1988). Conversely, conditioned $AT_{1A}^{-/-}$ mice showed increased c-Fos levels in the BNST, MPO and ventral and rostral DMH (DMV and DM, respectively; Fig. 3D and Supplementary Fig. S3). There was no difference between genotypes in c-Fos expression in the suprahypothalamic structures examined, including the prefrontal cortex and the CA1/CA2 region of the hippocampus, or in brainstem nuclei known to convey visceral afferent information, such as the nucleus tractus solitarius and A1/C1 region (Supplementary Table S1).

3.5. ETM

When evaluated by the latency to leave the closed arm of the ETM, both genotypes showed passive avoidance acquisition ($F(3,51) = 18.1$, $p < 0.001$), with the latency being lower in $AT_{1A}^{-/-}$ mice ($F(1,51) = 4.5$, $p < 0.05$), as was the defecation score over the testing period (Fig. 4A). Conversely, the rate of avoidance acquisition was not

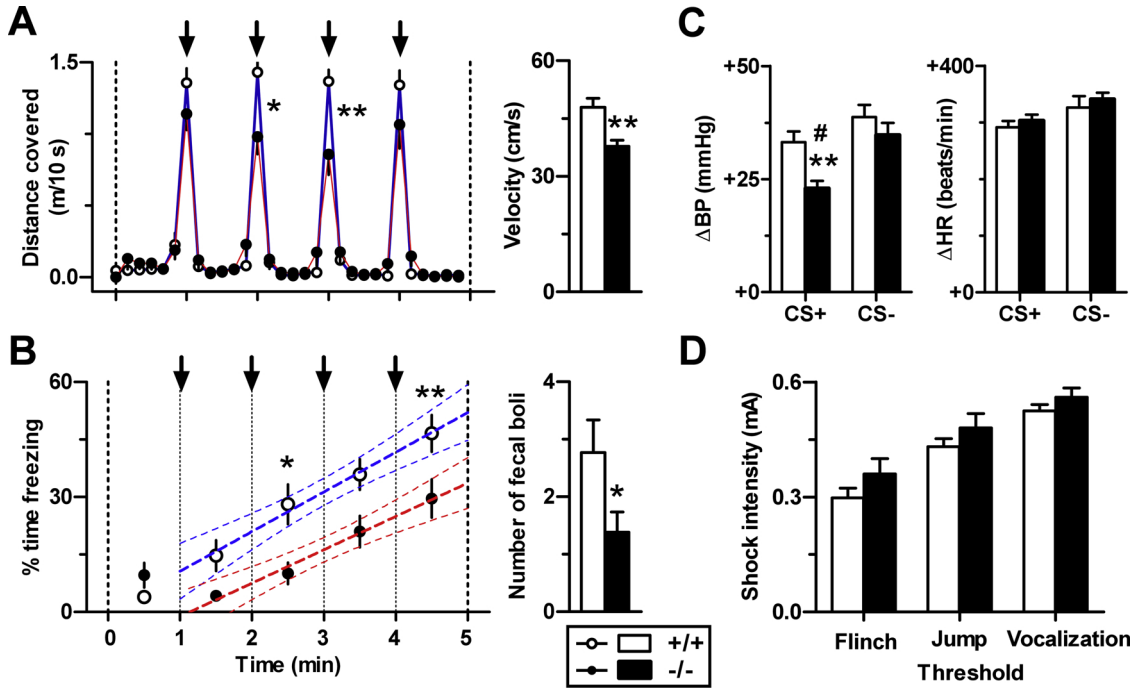

Fig. 2. Reduced flight, freezing and cardiovascular responses to footshock in $AT_{1A}^{-/-}$ mice. **(A)** Distance covered per 10-s interval (left) and activity burst velocity (right) during the session. Arrows represent individual shocks. **(B, left)** Freezing acquisition in response to four consecutive shocks. Dashed lines represent linear regression fits (with 95% confidence bands) between freezing and shock number for each genotype (goodness of fit: $r^2 > 0.36$, $F(1,50) > 28.6$, $p < 0.001$). **(B, right)** Defecation score over the testing period. $^{*}p < 0.05$, $^{**}p < 0.01$ between genotypes (+/+, n=13; −/−, n=13). **(C)** Changes in BP and HR during the session. $^{**}p < 0.01$ between genotypes; $^{\#}p < 0.05$ between CS+ and CS− groups (CS+ group: +/+, n=11; −/−, n=10; CS− group: +/+, n=3; −/−, n=4). **(D)** Flinch, jump and vocalization thresholds to footshocks of increasing amplitude are similar in $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice (+/+, n = 8; −/−, n = 5).

different between genotypes, as ANOVA found no interaction between the effects of genotype and time ($F(3,51) < 1$, $p = 0.86$). In the open arm, both groups showed escape learning ($F(3,21) = 5.0$, $p < 0.01$), with similar latencies to leave the open arm ($F(1,21) = 2.2$, $p = 0.18$; Fig. 4B). These latency periods, however, reflected different escape-oriented strategies in individual mice, ranging from a slow flat-back movement ($AT_{1A}^{+/+}$ mice only) to fast run to the closed arm, i.e. flight. The occurrence of flight-like responses was similar between genotypes, while the flight velocity was lower in $AT_{1A}^{-/-}$ mice (Fig. 4C).

3.6. TST

In a depression-relevant paradigm, the TST, $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ mice showed similar immobility scores (71 ± 8 and 89 ± 21 s, respectively, $p = 0.45$). These data suggest that changes in depression-related behavior are unlikely to underlie reduced anxiety-like behavior in $AT_{1A}^{-/-}$ mice.

4. Discussion

Here, we demonstrate that AT_{1A} receptor knockout in mice reduces both innate and learned fear responses and that these effects are unrelated to changes in resting BP or cardiovascular reactivity measures. We also show that the reduction in learned fear is driven by changes in baseline responding rather than the rate of learning, and that this phenomenon is associated with a selective effect of AT_{1A} receptor knockout on neuronal activity in the hypothalamic-midbrain defense regions.

4.1. Innate fear responses

Ethological and human studies suggest that distinct levels of defensive reactions occur depending on the imminence of threating events, a concept termed the “threat imminence continuum” (Blanchard and Blanchard, 1988; Blanchard et al., 2001a, b; Fanselow, 1994; Mobbs et al., 2009). When a threat is uncertain, like in a novel environment, exploratory/risk assessment activity is elicited. When a tangible threat is identified, freezing occurs to impair detection. Finally, when physical contact with the threat is inevitable, flight or fight ensues (Fanselow, 1994). Given that these defensive modes can also be assessed in a well-controlled conditioning paradigm (Fanselow, 1994), we employed this paradigm first and then validated our findings in a pain-unrelated behavioral task, the ETM (Graeff et al., 1998, 1993).

In response to environmental novelty (pre-exposure), $AT_{1A}^{-/-}$ mice showed increased exploratory activity, along with reduced ethological and autonomic indices of fear, SAP and defecation. These differences could not be ascribed to variation in baseline (24-h home-cage) levels and persisted with repeated testing. Furthermore, these differences were not attributable to attentional deficits in $AT_{1A}^{-/-}$ mice, as vigilant scanning during pre-exposure and the flinch response to shock were similar between genotypes. Thus, the observed differences likely reflect reduced emotional reactivity, or fearfulness, of $AT_{1A}^{-/-}$ mice in potentially threatening situations associated with novelty. This possibility is consistent with previous findings that ARBs produce anxiolytic effects in several rodent models of anxiety including the open field, elevated plus-maze and social interaction tests (Kulakowska et al., 1996; Saavedra et al., 2006; Shekhar et al., 2006), and that hypothalamic AT_{1A} receptor gene expression correlates with anxiety measures in the elevated plus-maze in mice (Golding et al., 2011).

In response to a discrete aversive stimulus, footshock, $AT_{1A}^{-/-}$ mice showed a reduced flight reaction. This could not be ascribed to altered nociceptive sensory inputs, as pain thresholds remained unchanged in $AT_{1A}^{-/-}$ mice, and these animals showed reduced flight behavior in a pain-unrelated paradigm, the ETM. These data are in line with previous findings that intrahypothalamic microinjection of ARBs decreases panic-like responses (escape-oriented locomotion) in panic-prone rats (Shekhar et al., 2006). Thus, in addition to anxiolytic action, AT_1 receptor inactivation can reduce innate defensive responses

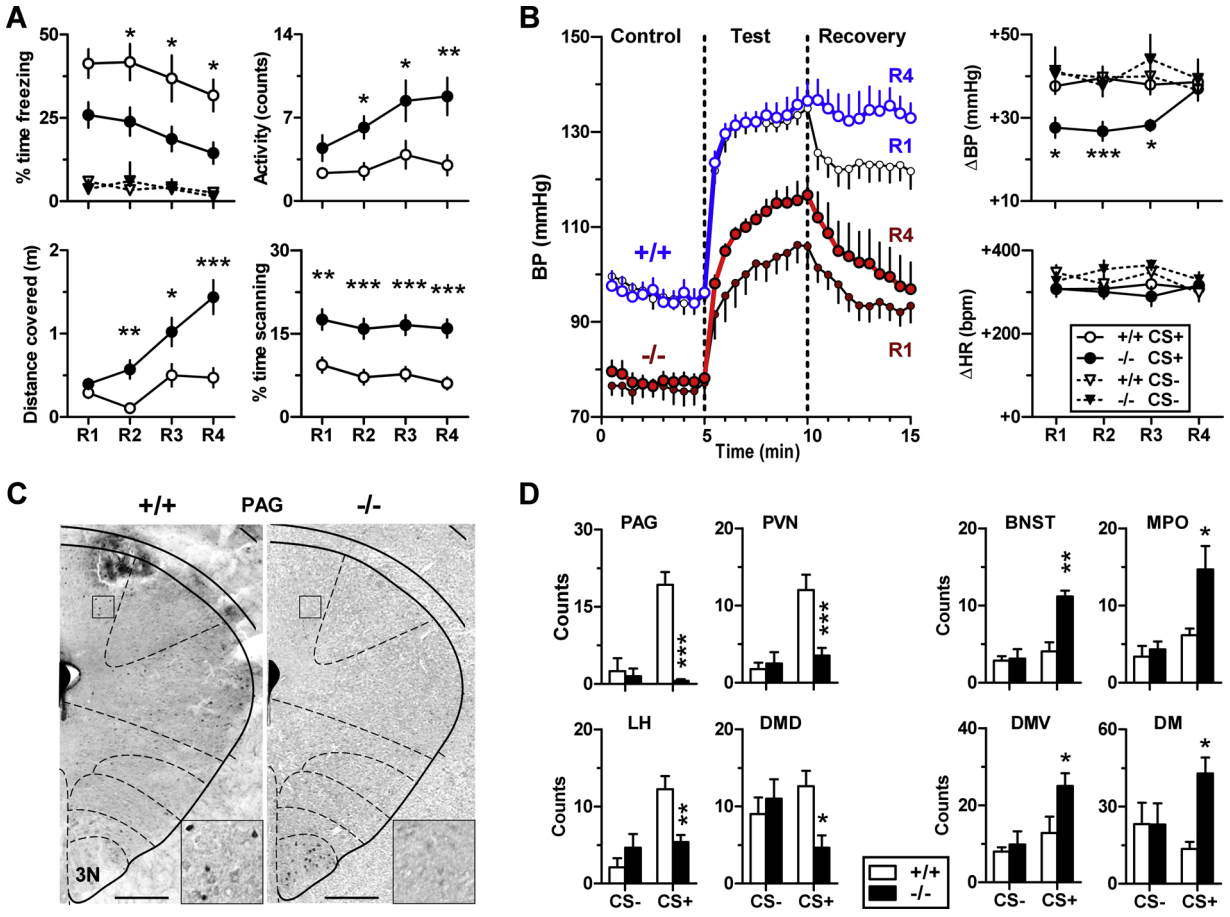

Fig. 3. Reduced freezing, cardiovascular and c-Fos responses to re-exposure in $AT_{1A}^{-/-}$ mice. **(A)** Freezing and motor behaviors across four re-exposure (R) trials (CS + group: +/+, n = 13; −/−, n = 13; CS− group: +/+, n = 6; −/−, n = 6). **(B, left)** Minute-to-minute values of BP in the CS+ groups during the first (R1) and the last (R4) re-exposures. Minute-to-minute values of the corresponding CS− groups are omitted for clarity; **(B, right)** Mean values of BP and HR during the trials (CS+ group: +/+, n = 11; −/−, n = 10; CS− group: +/+, n = 3; −/−, n = 4). **(C)** Representative photomicrographs illustrating c-Fos expression within the PAG of an $AT_{1A}^{+/+}$ mouse and $AT_{1A}^{-/-}$ mouse of the CS+ group after 24-h re-exposure. Line drawings were imported from the mouse brain atlas (Franklin and Paxinos, 2008) and overlaid on photographs of coronal sections (Bregma −4.16 mm). Note little c-Fos expression in the PAG, but robust expression in the oculomotor nucleus (3 N) of the $AT_{1A}^{-/-}$ mouse. *Scale bars*, 200 µm. **(D)** The average counts of c-Fos-positive cells per section in selected fear-related brain regions. CS+ group: +/+, n = 4–5; −/−, n = 4–7; CS− group: +/+, n = 4–5; −/−, n = 4–6. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between genotypes.

associated with intense fear, such as undirected and directed flight, indicating a panicolytic effect. Given this broad action, therapeutic targeting of AT_1 receptors may provide an advantage over other classes of anxiolytic drugs, including benzodiazepines, 5-HT_{1A} agonists and 5-HT_2 antagonists, which have little effect on panic-like behavior (Graeff, 2002; Perkins et al., 2009).

4.2. Learned fear responses

In contrast to innate fear responses, the rates of freezing acquisition during conditioning, and of avoidance and escape acquisition in the ETM remained unaltered in $AT_{1A}^{-/-}$ mice. Moreover, reduced levels of freezing in $AT_{1A}^{-/-}$ mice across re-exposures were mainly due to changes in the initial level of conditioning, and not the rate of freezing extinction. Together, these data suggest that, although AT_{1A} receptors strongly influence performance of fear learning tasks, this is driven by changes in initial levels rather than the efficiency of learning processes, i.e. rate of learning (Rescorla, 2002). Consistent with this notion, conditioned $AT_{1A}^{-/-}$ mice showed altered c-Fos levels in several brain regions important for fear expression, such as in the BNST, LH/DMH and PAG, but not in limbic-cortical areas regulating fear learning and plasticity (Furlong and Carrive, 2007; LeDoux et al., 1988; Walker and Davis, 1997; Wilensky et al., 2000). This heterogeneity may relate to the CNS distribution of AT_1 receptors, with high densities of these receptors localized mainly in the hypothalamus and brainstem and low densities in cortical and limbic structures (Allen et al., 1998).

The results of our fear conditioning experiments are in general agreement with a recent study by Marvar et al. (2014) who showed that treatment with the ARB losartan impaired retention of freezing behavior in mice in a cued fear conditioning paradigm, although no statistically significant effects on freezing acquisition were found. Interestingly, treatment with losartan immediately after each of the five daily retention sessions (to interfere with consolidation of extinction rather than acquisition of extinction) had no effect on contextual freezing response in mice (Lazaroni et al., 2016). Taken together, our and these findings strongly suggest that AT_1 receptor inactivation, either through pharmacological or genetic manipulations, enhances extinction of behavioral reactions associated with intense fear, such as freezing, and that this enhancement is not due to reduced consolidation of extinction learning. To our knowledge, these are the only data currently available that address the role of AT_1 receptors in classical (Pavlovian) conditioning paradigms.

In contrast, numerous operant conditioning studies have reported that ARBs either enhance or have no effect on retention of avoidance behavior (Barnes et al., 1990; DeNoble et al., 1991; Tota et al., 2009). Although differences in animal models and experimental conditions may account for the distinct roles of AT_1 receptors in the Pavlovian and avoidance studies, this seems unlikely given the number and diversity

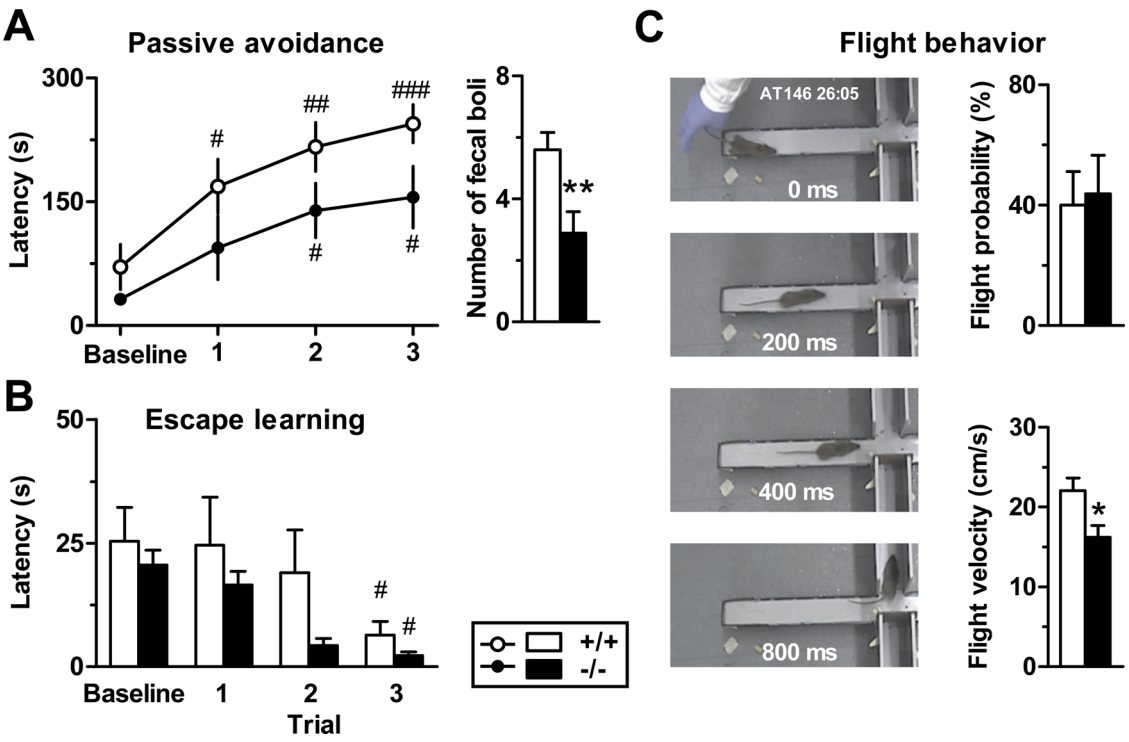

Fig. 4. Reduced passive avoidance acquisition and flight behavior, and unaltered escape learning and retention in $AT_{1A}^{-/-}$ mice in the ETM. **(A)** Latency to leave the closed arm (left) and defecation (right) in the passive avoidance task (+/+, n = 10; −/−, n = 9). **(B)** Latency to leave the open arm in the escape learning and retention task. Retention test (Trial 3) was performed 24 h after initial trials (+/+, n = 5; −/−, n = 4). **(C)** An example of the flight-like escape from the open arm (left) and the velocity and probability of flight response (right) in the escape task. $^{*}p < 0.05$ between genotypes; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus baseline latency.

of latter works. Moreover, in our study, the dissociation between retention of Pavlovian (freezing) and operant (escape in the ETM) responses was observed in the same mouse model, and thus was not attributable to the above factors. Alternatively, the ability of AT_1 receptors to affect differentially Pavlovian and operant conditioning may reflect their distinct roles in regulating neural networks underlying each kind of learning. In this regard, several studies have shown that the entorhinal and parietal cortices are important in avoidance learning, but not Pavlovian conditioning; conversely, the amygdala is critical for Pavlovian conditioning, but may be less crucial for retention of avoidance learning (Wilensky et al., 2000) and references therein). It is thus possible that AT_1 receptor inactivation impairs amygdala-dependent memory of highly emotional events, behaviorally expressed as freezing, without impairing cortex/hippocampus-dependent memory, manifested by avoidance behavior. If confirmed by further studies, these properties of AT_1 receptors may be of clinical relevance, as the inability to selectively extinguish intense fear memories is an important problem in psychiatric disorders, such as PTSD.

4.3. Relationship between emotional and cardiovascular reactivity

In the present study, $AT_{1A}^{-/-}$ mice displayed reduced emotional reactivity to all aversive stimuli tested, regardless of their nature or intensity. This reduction, however, was accompanied by reduced cardiovascular arousal, only in situations that were associated with imminent, tangible threats and that elicited distinct flight or freezing reactions. Previous pharmacological and molecular genetic studies employing intense aversive stimuli of different modalities (footshock, restraint, air-jet, cage oscillation and immersion in shallow cold water) also reported reduction in BP reactivity after central blockade of AT_1 receptors, although behavior was not assessed in these studies (for references, see Mayorov, 2011). Likewise, in our earlier work, $AT_{1A}^{-/-}$ mice displayed reduced BP responses to intense stressors, such as restraint or placement in a cage previously occupied by another male mouse (Chen et al., 2009; Davern et al., 2009). In contrast, cardiovascular arousal was not altered in $AT_{1A}^{-/-}$ mice in situations that posed potential/ambiguous threats and elicited anxiety-like responses. This dissociation was observed during both pre-exposure and late re-exposure and thus was not attributable to the innate or learned origin of threatening stimuli. Consistent with this, we previously found that $AT_{1A}^{-/-}$ mice showed normal cardiovascular arousal in other low-threat situations, such as placement in a novel home cage or presentation of a novel food in the well-habituated cage (Chen et al., 2009, 2010).

Together, these data suggest that the dissociation between emotional and cardiovascular reactivity in $AT_{1A}^{-/-}$ mice primarily relates to the intensity/imminence of threat, and not the type of threat. Our observation that both decrease and rebound of BP reactivity in conditioned $AT_{1A}^{-/-}$ mice occurred in the same test environment, where the type of threat (footshock) remained unchanged but its imminence diminished with time, further supports this possibility. It is thus plausible that the impact of AT_1 receptor inactivation on cardiovascular arousal increases along with the threat level, becoming more pronounced during responding to severe and tangible threats. This type of responding is known to be regulated by the flight-fight-freeze system (McNaughton and Corr, 2004), and with respect to the conditioned BP response, by the LH/DMD area (Furlong and Carrive, 2007; LeDoux et al., 1988). Hence, the decreased BP response in $AT_{1A}^{-/-}$ mice to re-exposure may reflect reduced neuronal activity in this hypothalamic area. Our c-Fos data are consistent with this possibility and also suggest that this decrease may be partly due to increased inhibition from the BNST, MPO, DMV and DM, brain regions that exert a predominantly inhibitory influence on stress-induced hypothalamic activation (Radley et al., 2009; Ulrich-Lai and Herman, 2009).

The observed dissociation between emotional and cardiovascular reactivity in $AT_{1A}^{-/-}$ mice has another important implication. It

suggests that "bottom-up" feedback from cardiovascular afferents is unlikely to underlie the effect of AT_{1A} receptor knockout on defensive responses. Our findings that behavioral activation preceded the restoration of cardiovascular reactivity in conditioned $AT_{1A}^{-/-}$ mice and that c-Fos expression in medullary nuclei known to relay visceral afferent information was unchanged in these animals further support this notion. Alternatively, reduced emotional reactivity in $AT_{1A}^{-/-}$ mice could be due to changes in afferent feedback to the brain caused by low resting BP. Indeed, it is known that large deviations from normal BP can affect cerebral circulation and produce psychosomatic complications (Duschek and Schandry, 2007). This, however, is unlikely to be the case here, as no correlation was found between resting BP and emotional reactivity measures in individual $AT_{1A}^{-/-}$ mice. Moreover, in both humans and animals, hypotension is typically associated with passive emotional coping, increased anxiety and impaired spatial learning (for references, see Hartman et al., 2012), a phenotype that is distinct from that of $AT_{1A}^{-/-}$ mice.

4.4. Limitations

One important issue not addressed in the present study concerns possible alterations in the hypothalamic-pituitary-adrenal axis (HPA) in $AT_{1A}^{-/-}$ mice that may influence stress reactions. Previous studies have shown, however, that basal corticosterone level and its response to dehydration is not altered in $AT_{1A}^{-/-}$ mice (Rocha et al., 2005). In addition, the PVN-selective deletion of AT_{1A} receptors in mice does not affect basal corticosterone and ACTH levels, or the corticosterone response to restraint stress (de Kloet et al., 2013; Wang et al., 2016), nor does chronic systemic treatment with the ARB losartan (Marvar et al., 2014). These data suggest that $AT_{1A}^{-/-}$ receptor inactivation in mice does not cause endocrine abnormalities involving the HPA axis.

5. Conclusion

Our results suggest that AT_{1A} receptor knockout reduces innate and learned fear responses in mice, regardless of threat level, and thus is capable of producing both anxiolytic- and panicolytic-like effects. These effects are unrelated to cardiovascular baseline and reactivity measures and likely reflect central processes. The inhibitory effect of AT_{1A} receptor inactivation on learned fear is mediated by changes in baseline reactivity, rather than learning efficiency, and is limited to highly emotional memories, expressed as freezing. This selectivity may be due to the greater impact of AT_{1A} receptor deficiency on neurotransmission in hypothalamic-midbrain defense regions than in limbic-cortical areas involved in associative learning. With respect to the circulation, the effect of AT_{1A} receptor knockout is most pronounced during the flight–freeze response. Thus, therapeutic targeting of AT_1 receptors may be particularly useful for the combined treatment of emotional and physical symptoms of severe anxiety and panic, with little or no adverse effects on general learning abilities.

Conflict of interest

The authors have no commercial associations or other relationships that might lead to a conflict of interests in connection with submitted article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.05.004.

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