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Effects of genetic background and null mutation of 5-HT_{1A} receptors on basal and stress-induced temperature: Modulation by serotonergic and $GABA_A$ergic drugs

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Investigation of the role of 5-HT_{1A} receptors in affective disorders resulted in the development of a 5-HT_{1A} receptor knockout mice (1AKO), created on three different background strains. Changes in sensitivity to the anxiolytic like effects of diazepam were found, but they appear to be dependent on the genetic background. We compared drug sensitivity to $GABA_A$-ergic and serotonergic drugs of 1AKO mice and the interaction with genetic background in the stress-induced hyperthermia (SIH) procedure. 1AKO and wildtype mice of three background strains (129Sv/Ev, C57BL/6, Swiss Webster) were tested. In SIH, drug effects on basal (T_1) and stress-induced body temperature (T_2) were measured. Anxiolytic-like drug effect results in reduced ΔT (T_2-T_1). $GABA_A$-ergic drugs that have an anxiolytic profile and stimulate α_2 and α_3 subunit containing $GABA_A$ receptors, including diazepam and L838,417, resulted in reduced ΔT. Stimulation of $\alpha 1$ subunit containing $GABA_A$ receptors by zolpidem resulted in hypothermia. In addition, stimulation of 5-HT_{1A} receptors by buspirone in wildtype but not 1AKO mice reduced ΔT, while stimulation of 5-HT_7 receptors by 5-CT primarily resulted in hypothermia. Null mutation of the 5-HT_{1A} receptor resulted in significant alterations in drug-sensitivity that was further modulated by the genetic background. In particular, the null mutation on SW and C57BL/6 backgrounds resulted in differentiation between diazepam/ L838,417 and 5-CT responses, respectively. This indicates an interaction between the 5-HT_{1A} receptor and genetic background and demonstrates the importance of selecting the background strain in a receptor knockout model.

Introduction

Genetic background differences are frequently observed in sensitivity to drug effects on $GABA_A$ receptors and serotonergic receptors (Griebel et al., 2000; Moser, 1991; Rodgers et al., 2002b). Not only pharmacological differences between genetic backgrounds have been described, gene-targeted mice display different pharmacological sensitivities compared to their wildtypes(Guscott et al., 2003; Kralic et al., 2003). Sibille and co-workers (2000) demonstrated that 5-HT_{1A} receptor knockout (1AKO) mice on a Swiss Webster (SW) background display a reduced sensitivity to the anxiolytic and sedative effects of benzodiazepines. Down-regulation of α_1 and α_2 subunits of $GABA_A$ receptors in amygdala and cortex was suggested to be responsible for this reduced sensitivity (Sibille et al., 2000). Normal sensitivity to benzodiazepines was observed in 1AKO mice on the 129Sv/Ev (129S6) and C57BL/6 (B6) backgrounds (Bailey and Toth, 2004; Pattij et al., 2002b), implying that the genetic background strongly affects the behavioural and pharmacological sensitivity for certain drugs (Rodgers et al., 2002a).

The present study is the first to examine modulation of serotonergic and $GABA_A$-ergic drug effects by the interaction between the 5-HT_{1A} receptor and genetic background, in 1AKO mice on three genetic background strains (129S6, B6 and SW). The stress-induced hyperthermia (SIH) paradigm was used to assess the anxiolytic-like activity of drugs. In the SIH, a first rectal temperature measurement is used both for recording basal body temperature (T_1) and for inducing hyperthermia. A second rectal temperature measurement (T_2) is taken 10 min later and the difference in body temperature, ΔT (T_2-T_1) is the SIH (Olivier et al., 2003; van der Heyden et al., 1997; Zethof et al., 1995). The pre-optic area and anterior hypothalamus are considered primary areas involved in homeostatic temperature regulation and conservation of body temperature (Nagashima et al., 2000; Simon et al., 1986). SIH is probably mediated by extra-hypothalamic mechanisms (i.e. limbic) involved in modulation of stress and anxiety (Olivier et al., 2002; Veening et al., 2004). Drugs acting on $GABA_A$ receptors are known to affect both T_1 and ΔT (Olivier et al., 2003; van der Heyden et al., 1997; Veening et al., 2004; Zethof et al., 1995), although these effects can be differentiated.

In the present experiments, we tested various drugs acting on $GABA_A$ receptors including diazepam, a non-subunit selective benzodiazepine receptor agonist stimulating $GABA_A$ receptors containing α_1, α_2, α_3 and α_5 subunits (Mohler et al., 2002; Sieghart, 1995; Wafford et al., 2004), zolpidem a selective agonist of $GABA_A$ receptors containing α_1 subunits and L838,417 that has partial agonistic activity at $GABA_A$ receptors containing α_2, α_3 and α_5 subunits (McKernan et al., 2000; Rowlett et al., 2005). Flumazenil, a non-subunit selective benzodiazepine antagonist was tested to investigate whether changes in the functioning of $GABA_A$ receptors (i.e. in SW 1AKO mice) lead to a change in the intrinsic activity from flumazenil. In addition, serotonergic drugs were tested on their anxiolytic-like and thermoregulatory effects including the partial 5-HT_{1A} receptor agonist buspirone and the 5-HT_7 receptor agonist 5-CT (Guscott et al., 2003; Hedlund et al., 2003; 2004). Here we compared drug sensitivity in various 1AKO mouse strains and the interaction of these drugs with the genetic background on temperature regulation and stress-induced hyperthermia.

Material and Methods

Animals

Groups of 12 male homozygote 1AKO and wildtype mice of 129S6, B6 and SW strains were bred within the laboratory animal facilities of Utrecht University (GDL, Utrecht, The Netherlands). The breeding founders of the 129S6 strain were originally obtained from Dr. R. Hen (Columbia University, New York, USA) and of the B6 and SW strains from Dr. M. Toth (Cornell University, New York, USA). The breeding founders were initially crossbred with commercially available mice (Taconic, M&B, Denmark) from the same background. This crossbreeding resulted in a heterozygote F1 generation, which were used to breed a homozygote 1AKO and wildtype generation (F2). This F2 generation was then used to breed homozygote mice for the present experiment. At the start of the experiments mice were 10-12 weeks of age.

Animals were housed individually the afternoon prior to testing days and were returned to their group-housed cages at the end of each experimental day. Mice were socially housed in same-genotype, same-strain groups with 3-5 animals per cage enriched with bedding and nesting material and with free access to food-pellets and tap water. Animals were housed under a 12-h light/12-h dark cycle (lights on from 0600-1800) at controlled room temperature (20±2°C) and relative humidity (40-60%). Animals were tested during the animals' light phase and experiments were carried out with approval of the ethical committee of the Faculties of Pharmaceutical Sciences, Chemistry and Biology, Utrecht University, The Netherlands (DEC DGK/FSB).

Drugs

5-carboxamido-tryptamine maleate (5-CT; 0/0.5/1/2 mg/kg), buspirone HCl (0/1/2/4 mg/kg) and zolpidem tartrate (0/3/10/30) were obtained from Sigma-Aldrich chemie B.V (Zwijndrecht, The Netherlands). Diazepam base (0/1/2/4 mg/kg) was obtained from Brunschwig Chemie B.V. (Amsterdam, The Netherlands). L838,417 (7-tert-Butyl-3-(2,5-difluoro-phenyl)-6-(2-methyl-2H-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine; 0/3/10/30 mg/kg) was synthesized according to the methods in WO 98/04559 and provided by Roche (Roche, Palo Alto). Flumazenil (0/3/10/30 mg/kg) was obtained from Roche Nederland (Mijdrecht, The Netherlands).

Buspirone HCl (0/1/2/4 mg/kg) and 5-CT were dissolved in 0.9% saline (vehicle), diazepam (0/1/2/4 mg/kg), Zolpidem (0/3/10/30 mg/kg), L838,417 (0/3/10/30 mg/kg) and flumazenil were suspended in 0.5% gelatin/5% mannitol (vehicle). Buspirone, diazepam and 5-CT were injected intra-peritoneal. Zolpidem, L838,417 and flumazenil were administered orally. All drugs were freshly prepared each test day and injected in a volume of 10 ml/kg.

Stress-Induced Hyperthermia – SIH

On the afternoon before the testing day, animals were housed individually and placed randomly in the room in which the experiments were conducted the next day. On the experimental day, mice were injected with either drug or vehicle 60 minutes before the first rectal temperature measurement (stressor). Body temperature was measured by manual fixation of the animals, inserting a thermistor probe of 2 cm in length into

the rectum (Digital Thermometer, Type 971A, Tegam Inc., Geneva Ohio, USA). The probe was dipped in silicon oil before insertion into the rectum until stable temperature readout was obtained for at least 10 seconds, producing the basal body temperature (T_1). This rectal temperature measurement acted furthermore as a stressor, resulting in a rise of body temperature of 1-2 °C. Body temperature was measured again 10 minutes later resulting in T_2. The stress-induced hyperthermia was calculated as the difference between these two temperatures ($\Delta T = T_2 - T_1$). All animals received all doses of all drugs. Mice were tested twice a week (Tuesdays and Fridays) and the different doses of drugs were counterbalanced across and within genotype and strain. Half of the animals were tested in the morning (900-1200 hours), while the other half was tested in the afternoon (1300-1600 hours). In between different drugs, a washout period of at least one week was used.

Statistics

Differences in vehicle conditions between strains and genotypes were analysed using univariate ANOVA with strain and genotype as 'fixed factors'. The effects of 5-CT, buspirone, diazepam, zolpidem, L838,417 and flumazenil on SIH (ΔT) and basal body temperature (T_1) were analysed using repeated measures ANOVA with dose as 'within subject' factor and strain and genotype as 'between subject' factor. In the result section, significant two- or three-way interaction effects ($P<0.05$) between strain, genotype and/or dose are shown. If no interaction effect is mentioned, no significance was found. All statistical analysis were performed using SPSS for Windows version 11.0 (SPSS, Chicago, IL).

Results

Drug effects on basal body temperature, T_1

First, basal body temperature was compared after vehicle injections. Genotype differences between 1AKO and wildtype mice were found only in the 129S6 strain; 1AKO mice having lower basal body temperature (129S6:$F[1,22]=9.8$, $p=0.005$; B6:$F[1,23]=0.05$, N.S.; SW:$F[1,21]=0.03$, NS).

Diazepam reduced body temperature dose dependently in all groups of animals (both wildtype and 1AKO on all three backgrounds), shown in figure 1a ($F[6,126]=3.16$, $p=0.006$). Analysing the background strains individually, we found that basal body temperature in all strains and genotypes was significantly decreased by 2 and 4 mg/kg of diazepam (129S6: $F[3,20]=16.6$, $p<0.001$; B6: $F[3,20]=3.6$, $p=0.03$; SW: $F[3,20]=9.62$, $p<0.001$), with the most prominent reduction in T_1 in the 129S6 strain.

Zolpidem also decreased T_1 in all strains, shown in figure 1b, but differences between the three background strains were seen (dose×strain interaction: $F[6,118]=3.8$, $p=0.002$). Responses were similar in wildtype and 1AKO mice, both 129S6 and SW mice were less sensitive to zolpidem with significant decreases at 10 and 30 mg/kg (129S6: $F[3,18]=22.0$, $p<0.001$; SW: $F[3,17]=25.6$, $p<0.001$), while B6 mice showed a reduction in baseline temperature with as little as 3 mg/kg zolpidem ($F[3,19]=36.6$, $p<0.001$).

L838,417 (fig.1c), flumazenil (fig. 1d) and buspirone (fig. 1e) did not affect the basal

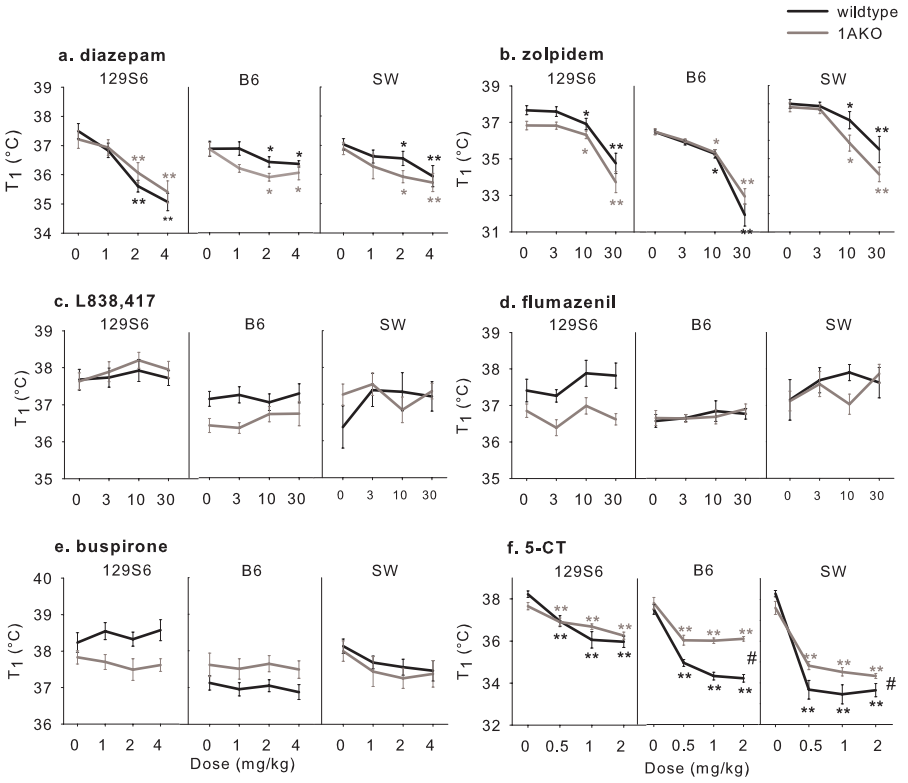

Figure 1: Drug effects on basal body temperature, T_1

Effects on basal body temperature (T_1) of diazepam (a), zolpidem (b), L838,417 (c), flumazenil (d), buspirone (e) and 5-CT (F) using the stress-induced hyperthermia procedure in 5-HT_{1A} receptor knockout and wildtype mice on three different background strains (129S6, B6, SW). * p<0,05 negative difference in T_1 from vehicle condition, ** p<0.05 negative difference in T1 from vehicle condition. # p<0.05 genotype´dose interaction effect

body temperature in any background strain and genotype compared to vehicle condition (L838,417: F[3,62]=2.22, NS; flumazenil: F[3,60]=2.0, NS; buspirone: F[3,60]=1.45, NS). 5-CT (fig. 1f) had an effect on T_1 in all strains, genotypes and all doses, with interactions between dose×strain (F[6,122]=7.1, p<0.001) and dose×genotype (F[3,60]=11.4, p<0.001).

Although a similar decrease after 5-CT was observed in 129S6 1AKO mice, (F[3,18]=26.8, p<0.001), the other strains showed a dose×genotype interaction with 1AKO mice being less sensitive to 5-CT (interaction SW: F[3,18]=3.6, p=0.03; B6: F[3,20]=7.3, p=0.02). Both SW and B6 mice displayed hypothermic effects following all doses (SW WT: F[3,7]=77.9, p,0.001; SW 1AKO: F[3,7]=95.7, p<0.001; B6 WT: F[3,9]=49.5, p<0.001; B6 1AKO:F[3,9]=15.2, p=0.001).

Drug effects on stress-induced hyperthermia, ⊿T

Next to the T_1 measurement, T_2 was measured 10 minutes later and the effect of various drugs on ΔT was analysed in the two genotypes and three backgrounds. No habituation was observed in ΔT over time in vehicle response in any strain or genotype (129S6: F[5,16]=2.0, NS; B6: F[5,6]=2.66, NS; SW: F[5,15]=0.88, NS). No differences between the three background strains in ΔT response after vehicle injection were found either (F[2,68]=1.2, N.S.). No genotype differences between 1AKO and wildtype mice in any strain were observed under vehicle conditions (129S6: F[2,22]=0.98, NS; B6: F[2,23]=0.09, N.S.; SW: F[2,21]=0.57, NS).

Effects of diazepam on ΔT are shown in fig 2a. A dose×strain interaction was observed in ΔT following diazepam (F[6,64]=2.4, p=0.04) with 129S6 being the most and B6 the least sensitive to the effects of diazepam. Although in 129S6 and B6 strains decreases in ΔT were observed in both wildtype and 1AKO mice in response to diazepam (129S6: F[3,20]=21.4, p<0.001; B6: F[3,18]=12.8, p<0.001), there was a difference between wildtype and the 1AKO mice in the SW strain (F[3,20]=5.2, p=0.008). 1AKO mice on a SW background showed no anxiolytic-like response to diazepam (F[3,9]=0.56, N.S.), while wildtype mice on this background showed a significant dose-dependent reduction in ΔT, significant at 2 and 4 mg/kg diazepam (F[3,9]=10.6, p=0.003).

Zolpidem (fig. 2b) shows a tendency towards a dose×strain interaction in ΔT (F[6,118]=2.1, p=0.05) and strains were analysed separately. 129S6 mice, either wildtype or 1AKO, showed no significant reduction in ΔT (F[3,18]=2.9, NS). In contrast, B6 and SW mice, both wildtype and 1AKO, showed a reduced ΔT after zolpidem, in B6 at 10 and 30 mg/kg (F[3,19]=12.4, p<0.001) and in SW at 30 mg/kg (F[3,17]=10.9, p<0,001).

A strain×genotype×dose interaction was observed after the administration of L838,417 (fig. 2c, F[6,126]=3.0, p=0.008). ΔT was reduced in both wildtype and 1AKO 129S6 mice at all doses (F[3,20]=19.6, p<0.001). Similarly, wildtype and 1AKO mice on B6 background showed a decrease in ΔT, but only at 10 and 30 mg/kg (F[3,18]=15.5, p<0.001). In contrast, there was a genotype×dose interaction in the SW strain (F[3,20]=6.8, p=0.003), with wildtype mice showing decreases in ΔT at all doses while 1AKOs demonstrated no response at all (wildtype: F[3,9]=3.9, p=0.001; 1AKO: F[3,9]=0.49, NS). No significant main effects on ΔT of flumazenil (fig. 2d) were found

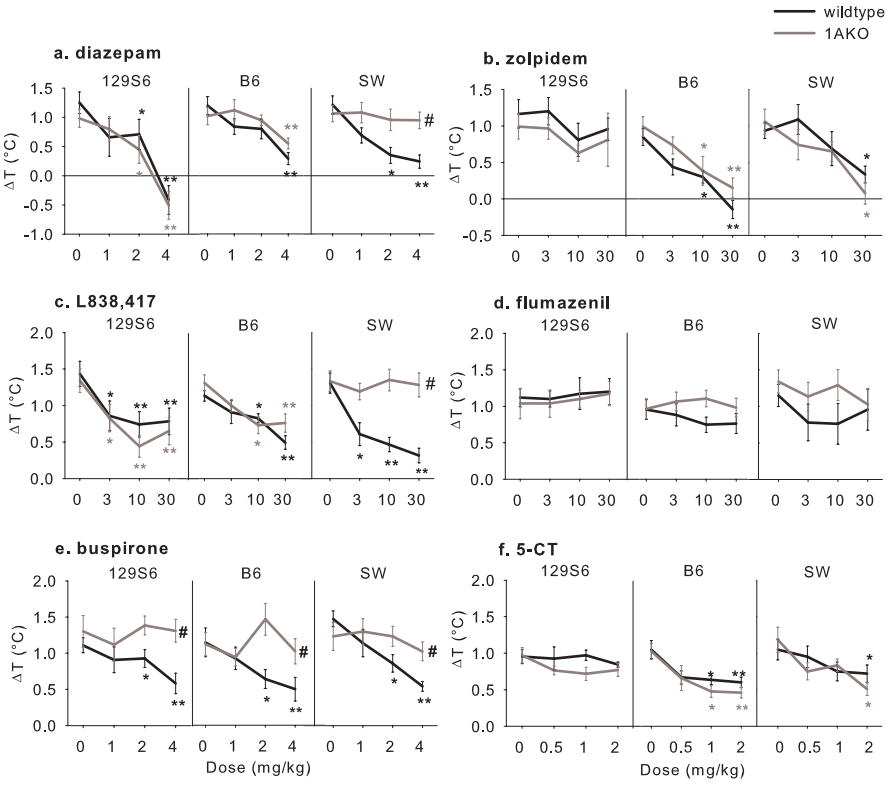

Figure 2: Drug effects on stress-induced hyperthermia, ΔT

Anxiolytic-like effects (ΔT) of diazepam (a), zolpidem (b), L838,417 (c), flumazenil (d), buspirone (e) and 5-CT (F) on the stress-induced hyperthermia procedure in 5-HT_{1A} receptor knockout and wildtype mice on three different background strains (129S6, B6, SW). * $p<0,05$ negative difference in ΔT from vehicle condition, ** $p<0.01$ negative difference in ΔT from vehicle condition. # $p<0.05$ dose x genotype interaction difference.

in any strain or genotype ($F[3,60]=2.0$, NS).

As expected, a dose×genotype interaction was observed in ΔT following buspirone (fig. 2e, $F[3,60]=5.9$, $p=0.001$). ΔT was unchanged in all 1AKOs mice to buspirone administration ($F[3,29]=1.9$, NS), while ΔT in wildtype animals of all strains showed reduction at 2 and 4 mg/kg of buspirone ($F[3,29]=18.4$, $p<0.001$). No difference in the overall response of ΔT following buspirone of the three background strains (wildtype) was found ($F[2,31]=0.42$, NS).

5-CT administration (fig. 2f) resulted in no differences in ΔT between 1AKO and wildtype mice in any strain, but different responses were seen in the three strains. 129S6 mice showed no response in ΔT to any dose of 5-CT ($F[3,18]=0.99$, $p=0.42$ NS), SW mice showed a decrease in ΔT at 2 mg/kg ($F[3,18]=4.47$, $p=0.04$) and B6 mice showed reduced ΔT at both 1 and 2 mg/kg of 5-CT ($F[3,20]=8.10$, $p=0.001$).

Discussion

We studied the effects of pharmacological and genetic manipulations (5-HT_{1A} receptor knockout (1AKO) and wildtype mice) on temperature regulation in mice on three genetic background strains at two levels. First level was regulation of basal body temperature (T_1), the second was temperature regulation following stress (ΔT) using the SIH paradigm. Both processes can be modulated by the $GABA_A$-ergic and serotonergic systems, illustrated by drugs acting on 5-HT_{1A}, 5-HT_7 and $GABA_A$ receptors. An overall lower T_1 is observed in 1AKO mice on the 129S6 strain only and this decrease is also observed during undisturbed radio-telemetry measurements (unpublished data). However, no effect on T_1 can be obtained by stimulation of 5-HT_{1A} receptors in wildtype mice of the 129S6 strain. Moreover, selective 5-HT_{1A} receptor agonists like flesinoxan reduced T_1 in wildtype mice of this strain (Pattij et al., 2002a). This indicates that 5-HT_{1A} receptors are not tonically involved in T_1 regulation in this strain, but that absence of this receptor might influence modulating genes in 129S6 mice only.

Based on results with the 5-HT_{1A} receptor agonist 8-OH-DPAT, it was thought that stimulation of 5-HT_{1A} receptors resulted in hypothermia (Hjorth, 1985; Moser, 1991), but 8-OH-DPAT also has 5-HT_7 receptor agonistic activity (Hoyer et al., 1994; Wesolowska, 2002). More recently it was shown that both 5-HT_7 and 5-HT_{1A} receptors play a role in 5-HT mediated hypothermia after 8-OH-DPAT (Hedlund et al., 2004). 5-CT has high affinity for 5-HT_7 receptors (Wesolowska, 2002; Yamada et al., 1998), but also displays affinity for several other 5-HT receptors, including 5-HT_{1A} receptors (Hoyer et al., 1994). Using selective 5-HT_{1A} receptor antagonists and 5-HT_7 receptor knockout mice, it was found that 5-CT induces its hypothermic effects through 5-HT_7 receptors. A strain dependent interaction effect between 5-HT_{1A} and 5-HT_7 receptors on T_1 was observed following 5-CT. Depending on the genetic background, 5-HT_{1A} receptors are required for the full hypothermic effect of a 5-HT_7 receptor agonist. In the 129S6 strain, 5-HT_{1A} receptors appear not to be involved in hypothermic effects of 5-CT, which remarkably also is the only strain that shows changes in T_1 after elimination of 5-HT_{1A} receptors.

The present study provides no evidence that tonic activation of $GABA_A$ receptors is

involved in basal T_1 or ΔT processes. However, both diazepam and zolpidem result in a reduced basal body temperature and stimulate α_1 subunits of the $GABA_A$ receptor, while L838,417 does not affect the α_1 subunit of the $GABA_A$ receptor (McKernan et al., 2000) and does not affect T_1 either. We show that T_1 can be modulated by stimulation of $GABA_A$ receptor α_1 subunits, but not α_2, α_3, or α_5 subunits. We furthermore show that 1AKO mice on the SW strain display normal sensitivity of $GABA_A$ receptor α1 subunits with regard to T_1. $GABA_A$ receptor α_1 subunits are present in high densities throughout the brain, while α_2 subunits are predominantly found in limbic structures, cerebral cortex and striatum (Fritschy and Mohler, 1995; Wisden et al., 1992). The primary brain area involved in the regulation of homeostatic body temperature is the pre-optic area of the hypothalamus (Boulant, 2000; Nagashima et al., 2000; Simon et al., 1986) and involvement of $GABA_A$ receptors in this area is well established (Osaka, 2004). Changes in $GABA_A$ receptor α subunits were observed in 1AKO mice on the SW background in several brain areas, but were unchanged in the hypothalamus (Sibille et al., 2000).

In wildtype mice of all background strains, but none of the 1AKO mice, buspirone reduces ΔT to the same extent. This indicates that buspirone modulates its anxiolytic-like effects via 5-HT_{1A} receptors. ΔT can also be modulated via 5-HT_7 receptors, but is genetic background dependent in contrast to modulation via 5-HT_{1A} receptors. Although it has been suggested that 5-HT_7 receptors might be involved in anxiety processes, so far little evidence has been found (Guscott et al., 2005; Thomas and Hagan, 2004). As mentioned earlier, 5-CT displays not only affinity for 5-HT_7 receptors, but also for 5-HT_{1A} receptors, implying the possibility of anxiolytic-like effects (Hoyer et al., 1994). However, 5-CT is able to reduce ΔT in 1AKO mice, excluding the role of 5-HT_{1A} receptors in this process. Therefore it is more likely that either strong hypothermic effects on T_1 are responsible for the reduction in ΔT and not stimulation of 5-HT_{1A} receptors by 5-CT. As previously suggested, disturbance in homeostatic mechanisms might interfere with the SIH procedure (Olivier et al., 2003).

Modulation of ΔT can also be obtained through $GABA_A$ receptors. This is α_2, α_3 or α_5 subunit dependent in the 129S6 strain, since in this strain no modulation of ΔT via the α_1 subunit (zolpidem) is obtained. It appears that in B6 mice, both α_1 and α_2, α_3 or α_5 are involved in the modulation of ΔT, since stimulation of both these subunit groups induces decreased ΔT. However, it was previously suggested that stimulation of α_1 subunits of the $GABA_A$ receptor complex results in sedative effects, but has little effect on anxiety (Elliot and White, 2001; Kralic et al., 2002; Rudolph et al., 1999). Similar to the effect of 5-CT on ΔT, strong hypothermic effects on T_1 might explain reduced ΔT values. This strong hypothermic effect of zolpidem could also explain the results of $GABA_A$ receptor agonists in 1AKO and wildtype mice on the SW strain. In this strain, 1AKO mice are insensitive to stimulation of all $GABA_A$ receptor subunits and of α_2, α_3 or α_5 subunits, but show reduced ΔT after stimulation of only α_1 subunits. Effects on ΔT of L838,417 and diazepam can most likely be attributed to stimulation of the α_2 and α_3 subunits of the $GABA_A$ benzodiazepine receptor (Dias et al., 2005; Low et al., 2000; Rowlett et al., 2001). Since reduced levels of only $GABA_A$ receptor α_2 subunits were observed in 1AKO mice on the SW strain (Sibille et al., 2000), this subunit might be primarily responsible for the reduction of ΔT in the SIH procedure. The lack of effect

of the $GABA_A$ receptor antagonist flumazenil was previously reported for the 129S6 strain (Pattij et al., 2002b). Apparently intrinsic activity of $GABA_A$ receptor antagonists is unchanged, even when changes in sensitivity towards $GABA_A$ receptor agonists were observed, like in 1AKO mice on the SW strain.Our data on buspirone and L838,417 show that ΔT and T_1 can be independently manipulated by drugs acting on different receptor (sub)types. Furthermore, we found that drug sensitivity depends to a great extent on the genetic background strain used.

Together, the data imply that genetic background has an enormous modulatory effect on how drugs affect temperature regulation, either basal temperature or following stress. This effect of genetic background can modulate both serotonergic and $GABA_A$-ergic regulation of these processes. Moreover, depending on the genetic background, these two systems interact. We show that 5-HT_{1A} receptors are essential for the effects of 5-CT on T_1 in SW and B6 mice and for the effects of diazepam and L838,417 on ΔT in the SW strain.