

Lewis and Fischer 344 strain differences in α_2 -adrenoceptors and tyrosine hydroxylase expression

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Abstract

Lewis and Fischer 344 (F344) rats differ in their pharmacological responses to a variety of drugs such as opioids, which has been partially attributed to differences in the endogenous opioid tone. Since opioid and α_2 -adrenergic mechanisms closely interact in nociception and substance abuse, a comparative study of the endogenous α_2 -adrenergic system in both inbred strains is of interest. Alpha-2 adrenoceptor subtypes and tyrosine hydroxylase, the rate-limiting enzyme of the catecholamine biosynthesis, were studied by Taqman RT-PCR analysis of gene expression in four brain areas of F344 and Lewis rats: hypothalamus, hippocampus, striatum and cortex. No differences were found in the mRNA levels of α_{2A} - and α_{2C} -adrenoceptors in any of the areas examined, however F344 rats exhibited lower levels of α_{2B} -adrenoceptor transcripts in the hippocampus and higher levels in the hypothalamus. Tyrosine hydroxylase gene expression was found to be higher in hippocampus and striatum of F344 rats compared to Lewis, and a consistent 2-fold increase of the protein levels was detected by Western blots only in the case of the hippocampus. These results together with previous studies strongly suggest that the hippocampal noradrenergic activity of Lewis and F344 rats could be involved in their different responses to pain, stress and drug addiction.

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Introduction

Fischer 344 (F344) and Lewis rats are two inbred strains that have been comparatively studied in the field of drug addiction, since the latter seems to be more sensitive to the addictive effects of drugs of abuse such as opioids (Guitart et al., 1992; Suzuki et al., 1992; Martin et al., 1999). Furthermore, differences between F344 and Lewis rats have been also reported concerning opioid analgesia (Herradon et al., 2003a). It is known that the degree of activation of the Hypothalamic Pituitary Adrenal (HPA) axis is much higher in F344 rats (Chaouloff et al., 1995; Chisari et al., 1995; see review by

Kosten and Ambrosio, 2002), which may contribute to the above differences since a link between stress and its concomitant HPA axis responses with behavioural sensitivity to psychoactive drugs is known (Kosten and Ambrosio, 2002). Recent evidences suggest that at least part of these differences can be directly attributed to heterogeneous function of the endogenous opioid system, since Lewis rats show a reduced endogenous opioid activity (Sternberg et al., 1989a,b; Nylander et al., 1995; Martin et al., 1999), including a less effective activation pathway in the case of the μ -opioid receptor (Selley et al., 2003; Herradon et al., 2003b).

Extensive basic and clinical data are now available to affirm that opioid receptors and α_2 -adrenoceptors are closely interrelated, which apply both to addiction and pain control (see review by Alguacil and Morales, 2004). It is widely known that α_2 -adrenergic agonists limit opioid withdrawal symptoms (Tseng et al., 1975; Gold et al., 1978; Gowing et al., 2004; Bailey, 2004), an effect related to reduction of central

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noradrenergic hyperactivity (Williams et al., 2001). Conversely, a single administration of α_2 -adrenergic antagonists increases opioid withdrawal symptoms (Stine et al., 2002), while chronic coadministration decreases opioid dependence both in rodent models (Iglesias et al., 1992, 1998; Laorden et al., 2000) and humans (Hameedi et al., 1997). These interactions are extendable to antinociception where additive or synergistic interactions between opioids and α_2 -adrenoceptor agonists have been described in rodents (Harada et al., 1995; Ossipov et al., 1997) and have been used to manage pain in humans specially when high levels of opioid tolerance are present (Walker et al., 2002).

Although the endogenous opioid system has been widely studied in F344 and Lewis rats, a deep comparative study of the noradrenergic system in these two inbred strains is lacking, even when there is some evidence to think that there could be some differences. For example, the activity of the rate-limiting enzyme of catecholamine biosynthesis, tyrosine hydroxylase, is much lower in the locus coeruleus of F344 rats, whose neurons also exhibit less spontaneous firing rate (Beitner-johnson et al., 1991; Guitart et al., 1993). We have recently reported that the acute antinociceptive effect of the α_2 -adrenoceptor agonist clonidine is significantly higher in F344 rats (Herradón et al., 2003a). However, direct evidence of strain-related differences concerning the α_2 -adrenergic system is not available at the molecular level. In this work we have combined gene and protein expression studies to determine possible strain differences concerning α_2 -adrenergic system in the brain of Lewis and F344 rats, mainly focusing on some brain areas clearly involved in drug reward and nociception according to the literature (Di Chiara and Imperato, 1988; McEwen, 2001; Millan, 2002; Kosten and Ambrosio, 2002; Maihofner et al., 2004).

Methods

Animals and drugs

Male Lewis and F344 rats (7 weeks old; Harlan) were used. The animals had free access to water and standard diet and were maintained in a controlled environment (20–22 °C, 12 h/12 h dark/light cycle) until the day of the experiment. The assays were carried out in accordance with the NIH guidelines for care and use of laboratory animals.

Analysis of gene expression

Tissue acquisition and RNA isolation

After sacrifice four brain areas (cortex, striatum, hypothalamus and hippocampus) were rapidly dissected in all animals (5/strain), frozen in dry ice and stored to – 80 °C until the RNA isolation procedure. Frozen tissues were directly homogenized in 1 ml TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50–100 mg tissue and total RNA was extracted following manufacturer's suggested protocol. The concentration of each sample was obtained from A₂₆₀ measurements. RNA integrity was confirmed using agarose

gel electrophoresis. RNA samples were purified with DNases enzymes (Ambion, Austin, TX) following manufacturer's protocol.

cDNA synthesis

One microgram of total RNA was mixed with 10× buffer RT, 25 mM MgCl₂, 10 mM dNTPs, 50 μM oligodT, 50 μM random hexamers, 20 U/μl RNases inhibitor and 50 U/μl Multiscribe. All the reagents were purchased to Applied Biosystems (Foster City, CA). The reaction conditions were 10 min/25 °C, 60 min/42 °C, 5 min/95 °C.

Taqman RT-PCR

Probe and primer sequences of α_2 -adrenoceptors and tyrosine hydroxylase genes were designed using Primer Express software (Applied Biosystems, Foster City, CA) as close as possible to the 3'-coding region of target gene sequences obtained from Genbank and are presented in Table 1.

Primers and probes were purchased from PE Applied Biosystems and each probe was synthesized with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and a quencher dye TAMRA (6-carboxy-tetramethylrhodamine) to the 3'-end.

We performed Taqman RT-PCR assays to measure the relative expression of our target genes following the protocol previously described (Medhurst et al., 2000). As housekeeping gene expression we used 18S (human rRNA, Applied Biosystems, Foster City, CA). Briefly, the results quantification is obtained through standard curves for each primer/probe set that were plotted showing the threshold cycle (C_t) vs. log initial copy number of genomic DNA in order to extrapolate our target genes C_t and generate an estimate copies/ng RNA (Wang et al., 1998). To correct for both RNA quality and quantity, data were also normalized by dividing target genes copies/ng by the copies/ng of an assay-dependent 18S gene, and expressed as a percentage (relative expression). We performed three experimental determinations for each gene.

Table 1
Taqman probes and primers sequences for every target gene

<i>Gene</i>	Taqman probe
α_{2A} -Adrenoceptor	TTTCAACCACGACTTCCGCCGC
α_{2B} -Adrenoceptor	TTGTGCTGGCCGTGGTCATTGG
α_{2C} -Adrenoceptor	ACGGTCTTCAACCAGGATTTCCGGC
Tyrosine hydroxylase	TGTCCGAGAGCTTCAATGACGCCAA
<i>Gene</i>	Forward primer
α_{2A} -Adrenoceptor	AGCTCGCTGAACCCGTGTTATCT
α_{2B} -Adrenoceptor	GCCGGGAGAAGAGGTTTAC
α_{2C} -Adrenoceptor	CTCGCTCAACCCGGTTCAT
Tyrosine hydroxylase	ATCAAACCTACCAGCCTGTGTACT
<i>Gene</i>	Reverse primer
α_{2A} -Adrenoceptor	CACGGCAGAGGATCTTCTTGA
α_{2B} -Adrenoceptor	GAAACCAGCAGACCACGAAAA
α_{2C} -Adrenoceptor	CTCCGTCGGAAGAGGATGTG
Tyrosine hydroxylase	GAGGCATAGTTCCTGAGCTTGTG

Analysis of tyrosine hydroxylase protein expression

Western blots

After sacrifice the same four brain areas (cortex, striatum, hypothalamus and hippocampus) were rapidly dissected in all animals (2/strain), frozen in dry ice and stored to -80°C until the protein extraction procedure. Tissue samples were homogenized in RIPA buffer and protein extracted in presence of protease inhibitors. Total protein was quantified by the BCA protein assay (Pierce, Rockford, IL). Equilibrated protein samples were mixed with loading buffer (60 mM Tris pH 6.8, 10% glycerol, 5% SDS, 0.65% β -mercaptoethanol, and 0.01% bromophenol blue), boiled for 5 min, and loaded on to 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) as appropriate. The gels were transferred to nitrocellulose membranes that were blocked with 50 mM Tris, 150 mM NaCl, 0.1% Tween-20 (TBS-T) and 5% non-fat milk for 1 h and either probed with rabbit anti-tyrosine hydroxylase antibody (Chemicon, Temecula, CA) at a 1:1000 dilution, and rabbit anti-actin antibody at a 1:2000 dilution (Chemicon, Temecula, CA), detected with goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody diluted 1:5000 (Santa Cruz, Santa Cruz, CA), and the immunoreactive proteins visualized using the ECL enhanced method (Amersham, San Francisco, CA). We quantified protein levels by densitometry in three independent experiments using quantity one software (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical analysis of comparisons between F344 and Lewis rats was performed by Student *t*-test. Significance was considered at the 0.05 level.

Results

Gene expression experiments did not reveal prominent strain differences regarding α_{2A} - or α_{2C} -adrenoceptor transcripts in any of the brain areas examined (data summarized in Table 2A and C). However, in the case of the α_{2B} subtype, Lewis rats exhibited higher gene expression in hippocampus and lower in hypothalamus (summarized in Table 2B).

Fischer 344 and Lewis rats also exhibited differences in the expression of tyrosine hydroxylase, the rate-limiting enzyme of the catecholamine biosynthesis, in some of the brain areas studied. The difference of gene expression was slight and not significant in the brain cortex and was absent in the hypothalamus (Table 2D), but a sharp increase in the levels of mRNA of tyrosine hydroxylase was observed in the striatum of F344 rats compared to Lewis; this same tendency was noted in the hippocampus, however differences did not achieve statistical significance in this brain area (Fig. 1A and C, summarized in Table 2D). The densitometry of the Western blots confirmed the lack of important strain differences of tyrosine hydroxylase protein levels in cortex and hypothalamus (data not shown) and also revealed that the marked differential

Table 2

The table summarizes the results of the comparison of the three α_2 -adrenoceptor subtypes (A–C) and tyrosine hydroxylase (D) gene expression levels in four brain areas (cortex, hypothalamus, hippocampus and striatum) of F344 and Lewis rats

A		
α_{2A} -Adrenoceptor gene expression		
Brain area	Lewis	F344
Cortex	0.01 \pm 0.003	0.008 \pm 0.002
Hypothalamus	0.03 \pm 0.010	0.03 \pm 0.009
Hippocampus	0.005 \pm 0.002	0.006 \pm 0.002
Striatum	0.012 \pm 0.003	0.009 \pm 0.003
B		
α_{2B} -Adrenoceptor gene expression		
Brain area	Lewis	F344
Cortex	0.003 \pm 0.0009	0.0011 \pm 0.0003
Hypothalamus	0.023 \pm 0.0023*	0.045 \pm 0.0044
Hippocampus	0.019 \pm 0.0029*	0.008 \pm 0.0014
Striatum	0.11 \pm 0.0085	0.11 \pm 0.0063
C		
α_{2C} -Adrenoceptor gene expression		
Brain area	Lewis	F344
Cortex	0.040 \pm 0.016	0.022 \pm 0.010
Hypothalamus	0.025 \pm 0.012	0.029 \pm 0.017
Hippocampus	0.069 \pm 0.026	0.075 \pm 0.023
Striatum	0.030 \pm 0.012	0.040 \pm 0.025
D		
Tyrosine hydroxylase gene expression		
Brain area	Lewis	F344
Cortex	0.025 \pm 0.011	0.033 \pm 0.014
Hypothalamus	0.254 \pm 0.094	0.257 \pm 0.101
Hippocampus	0.002 \pm 0.0007	0.005 \pm 0.002
Striatum	0.183 \pm 0.055*	0.476 \pm 0.085

Gene expression levels were measured by Taqman RT-PCR and values (mean \pm S.E.M) represent gene expression relative to 18S (housekeeping gene). **p* < 0.05 vs. F344.

gene expression observed in the striatum only translated into a 10% increase in the tyrosine hydroxylase protein levels, which did not achieve statistical significance (Fig. 1D). In contrast, tyrosine hydroxylase protein expression was highly increased in the hippocampus of F344 rats (Fig. 1B), thus confirming the tendency of gene expression data.

Discussion

We describe in this study some differences in central noradrenergic pathways between Lewis and F344 rats that could be playing a significant role in their heterogeneous behaviour regarding nociception and vulnerability to drug addiction. A detailed analysis of α_2 -adrenoceptor mRNA levels in four brain areas, cortex, hypothalamus, hippocampus and striatum, revealed strain differences of gene expression in the case of α_{2B} -adrenoceptor subtype. At present, these changes are difficult to confirm functionally and to interpret, since the physiology and pharmacology of α_2 -adrenoceptor subtypes are

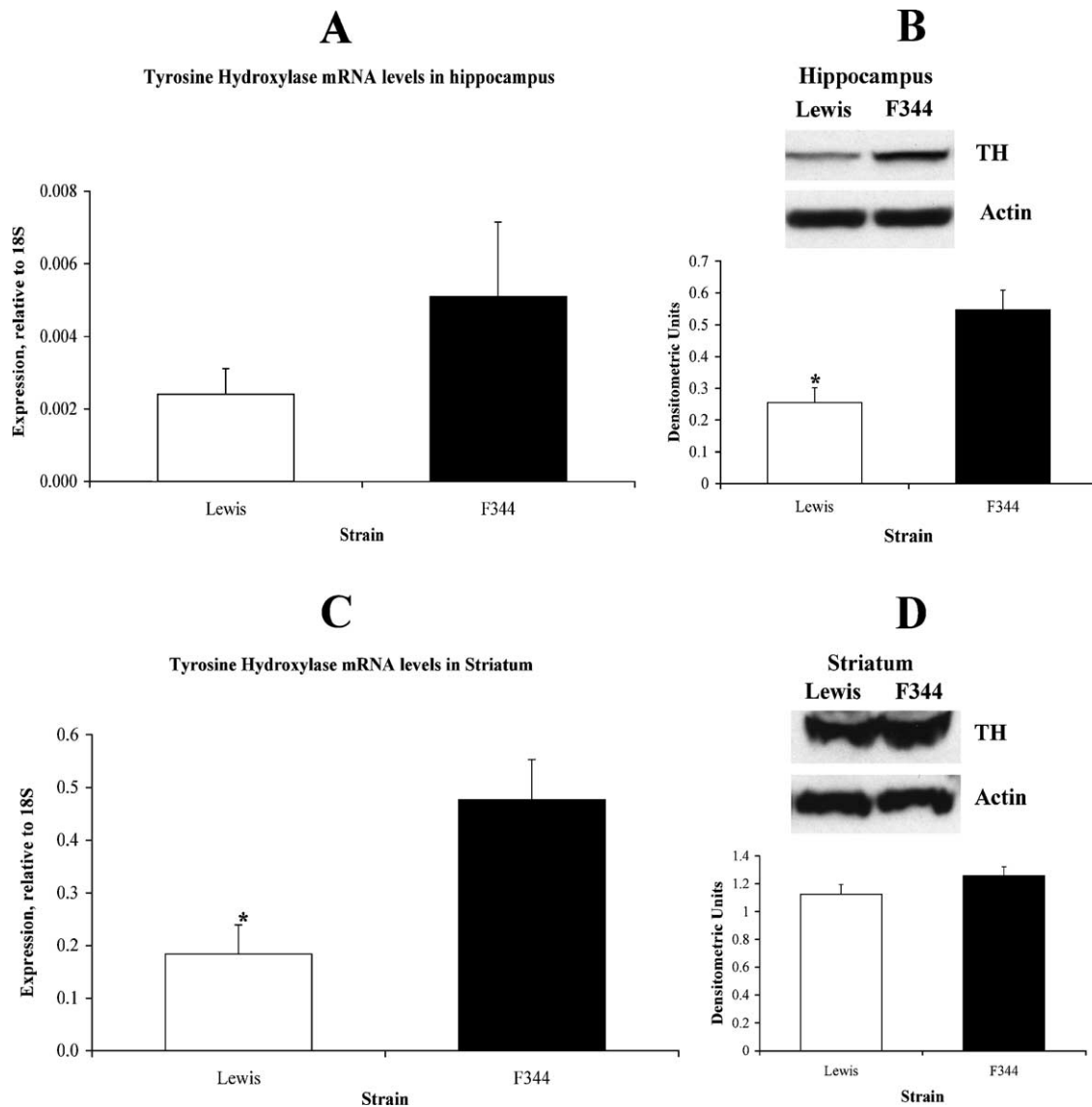


Fig. 1. Tyrosine hydroxylase mRNA and protein levels in hippocampus (A, B) and striatum (C, D) of F344 and Lewis rats. Messenger RNA levels were measured by Taqman RT-PCR and bars (mean \pm S.E.M) represent gene expression relative to 18S (housekeeping gene). White bars show gene expression in Lewis rats and black bars in F344 rats. Protein was studied by Western blots using specific rabbit anti-tyrosine hydroxylase and anti-actin antibodies. Protein levels were quantified by densitometry and values are expressed in densitometry arbitrary units. * $p < 0.05$ vs. F344.

not widely known and there is a lack of selective pharmacological tools to progress in this direction (see for instance Stone et al., 2003). Thus, in the field of nociception, the differential contribution of each one of the α_2 -adrenoceptor subtypes to the antinociceptive effect of their ligands remains to be clearly established. There is substantial evidence to think that α_{2A} - and α_{2C} -adrenoceptors play a major role in induction of antinociception (Stone et al., 1997; Li and Eisenach, 2001; Kingery et al., 2002; Leiphart et al., 2003; Ozdogan et al., 2004), however the contribution of α_{2B} -adrenoceptors is poorly understood (Millan, 2002). The specific differences in the α_{2B} -adrenoceptor expression levels in hypothalamus and hippocampus of F344 and Lewis rats lead us to hypothesize the possible involvement of α_{2B} -adrenoceptor in pain transmission processes since both areas are involved in the descending control of pain transmission (Millan, 2002), and F344 and Lewis rats

exhibit different sensitivity to clonidine analgesia (Herradón et al., 2003a).

In addition to α_{2B} -adrenoceptors, we also found important strain differences in tyrosine hydroxylase gene expression in striatum (and a similar tendency in the hippocampus), a finding that confirms and extends previous data on the differential rate of catecholamine biosynthesis in Lewis and F344 rats (Beitner-johnson et al., 1991; Guitart et al., 1992; Haile et al., 2001). In contrast, we failed to detect significant changes in tyrosine hydroxylase gene and protein expression in the cortex and hypothalamus, even when F344 rats exhibited higher levels of hypothalamic dopamine and norepinephrine in a previous study (Lowry et al., 2003). A possible explanation for this apparent discrepancy is that we examined gene expression in the whole hypothalamus while Lowry et al. circumscribed their study to the dorsomedial portion; therefore, a more detailed

comparative study of noradrenergic function within the hypothalamus should be interesting since this brain region plays an important role both in addiction and pain sensitivity, as explained above. Concerning the striatum, we identified a highly significant increase in the tyrosine hydroxylase gene transcripts in F344 rats compared to Lewis, suggesting a higher rate of catecholamine synthesis which is in agreement with previous observations circumscribed to the ventral striatum (nucleus accumbens) (Beitner-johnson et al., 1991; Guitart et al., 1992; Haile et al., 2001). However, this difference translated into a modest, not significant 10% increase of the protein levels that do not permit to achieve clear conclusions and recommend a separate study of protein expression in the ventral and dorsal striatum, i.e. by immunohistochemical methods.

Much more consistent are the findings obtained in the hippocampus, where the overexpression tendency of the TH gene in F344 rats was confirmed by a 2-fold increase of the protein levels. It must be taken into account that the accuracy of the Taqman RT-PCR technique runs in parallel with the mRNA levels of the samples (Medhurst et al., 2000), therefore it seems probable that the lower TH gene expression of the hippocampus could have led to high data dispersion and therefore insufficient resolution to achieve significant strain differences. However, the combined results of gene and protein expression strongly suggest an important increase of TH function in F344 rats. These findings, together with α_2B -adrenoceptor data, reveal a marked difference in hippocampal catecholaminergic function between Lewis and F344 rats that could help to explain some of the strain differences described in previous studies, such as those related to pain transmission. In this way, F344 and Lewis rats display prominent differences in their responses to both acute and chronic neuropathic pain (Yoon et al., 1999; Lovell et al., 2000; Shir et al., 2001; Herradón et al., 2003a, 2004), and the severity of this last condition has been shown to correlate with suppression of hippocampal noradrenergic neurotransmission (Ignatowski et al., 1999; Covey et al., 2000; McEwen et al., 2001). Since F344 rats simultaneously exhibit a higher resistance than Lewis to neuropathic pain and higher tyrosine hydroxylase levels, an inverse correlation between basal levels of catecholamines in the hippocampus and severity of neuropathic pain could be suggested and is consistent with the referenced studies.

The hippocampal function is also crucial in learning and memory and therefore in drug addiction, where associative and operational learning plays a critical role (Nestler, 2004). Hippocampal connections with the nucleus accumbens gate cortical input in this brain area and enable contextual cues to modulate drug seeking behaviours (O'Donnell and Grace, 1995; Jentsch and Taylor, 1999); it is therefore not surprising that differences in hippocampal function between Lewis and F344 rats could play a role in the observed strain variations related to drug seeking behaviours. There is also a direct link between hippocampal noradrenergic projections and HPA axis regulation that could be relevant to explain F344 and Lewis dissimilar behavioural and neuroendocrine responses to external stimuli (Kosten and Ambrosio, 2002). Thus, the connec-

tions between the hippocampus and the bed nucleus of the stria terminalis (BNST) are essential for responses to contextual stimuli (Sullivan et al., 2004), and it has been shown that noradrenergic terminals in the BNST coming from the hippocampus influence on the HPA axis activation (Forray and Gysling, 2004). The possibility exists that Lewis and F344 rats could exhibit broad differences in learning and memory abilities affecting a variety of tasks not necessarily circumscribed to drug seeking behaviours. A detailed comparative study on this subject is lacking up to our knowledge, but important strain differences have not emerged from the behavioural studies performed so far.

Conclusion

The work presented here demonstrates that the α_2 -adrenergic system and tyrosine hydroxylase activity differ in F344 and Lewis rats depending on the brain area considered. We hypothesize that differences in the basal hippocampal noradrenergic activity of Lewis and F344 rats could be involved in their different behavioural and neuroendocrine responses to a variety of external stimuli, such as nociceptive stimulation, stress or exposure to drugs of abuse, but further functional experiments are needed to confirm the physiological and pharmacological relevance of these findings. Moreover, strain differences concerning noradrenergic function in the striatum and hypothalamus cannot be totally excluded and will be interesting to check in the ventral and dorsomedial portions of both brain areas by using complementary techniques.

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