

Spontaneously hypertensive rats: further evaluation of age-related memory performance and cholinergic marker expression

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Objective: The spontaneously hypertensive rat (SHR), often used to study cardiovascular disease processes, may also be utilized to model certain central nervous system changes associated with memory disorders. Previous work in our laboratory indicated that central nicotinic acetylcholine receptors are markedly diminished and that memory-related task performance is impaired in this rodent phenotype. Due to the well-documented importance of the central cholinergic system to memory processes and its vulnerability to the effects of aging, it was of interest to measure other cholinergic markers and to further evaluate memory function in older SHRs. **Method:** Radial arm maze performance was used to assess working memory, quantitative receptor autoradiography with [³H]-pirenzipine, [³H]-AFDX-384 and [³H]-epibatidine (combined with cytisine) was used to determine the densities of muscarinic-M1 and -M2 and nicotinic cholinergic $\alpha 3$ receptors, respectively. Immunoblotting experiments were also used to determine the expression of the presynaptic cholinergic markers, choline acetyltransferase and the vesicular acetylcholine transporter. **Results:** Radial arm maze performance was impaired in hypertensive (compared with normotensive Wistar and Wistar-Kyoto) rats, regardless of age. M1 binding was increased in frontal and prefrontal cortical areas in SHR ($p < 0.05$), whereas M2 densities were higher in the hypertensive phenotype in the caudate putamen. A lower expression of $\alpha 3$ -containing nicotinic receptors was observed in the superior colliculus in SHRs. Age-related differences in the expression of the vesicular acetylcholine transporter were noted in the hippocampus. **Conclusion:** The SHR may be useful to model some aspects (particularly hypertension-related) of memory disorders, especially those in which cholinergic function is altered.

Objectif : Le rat spontanément hypertendu (RSH), souvent utilisé pour étudier les phénomènes morbides cardiovasculaires, peut aussi servir à modéliser certains changements du système nerveux central associés à des troubles de la mémoire. Des travaux antérieurs réalisés dans notre laboratoire ont indiqué que

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l'activité des récepteurs centraux de l'acétylcholine nicotinique diminue sensiblement et que l'exécution des tâches reliées à la mémoire est réduite chez ce phénotype de rongeurs. En raison de l'importance bien documentée du système cholinergique central pour les processus de la mémoire et de sa vulnérabilité aux effets du vieillissement, il s'avérait intéressant de mesurer d'autres marqueurs cholinergiques et d'évaluer davantage la fonction mémoire chez des RSH plus âgés. **Méthodes** : On a utilisé les résultats du test du labyrinthe en étoile pour évaluer la mémoire au travail, l'autoradiographie des récepteurs quantitatifs avec la [³H]-pirenzepine, [³H]-AFDX-384 et [³H]-épibatidine (combinée à la cytisine) pour déterminer les densités des récepteurs muscariniques M1 et M2 et cholinergiques nicotiques $\alpha 3$ respectivement. On a aussi utilisé des expériences de transfert pour déterminer l'expression des marqueurs cholinergiques présynaptiques, de la choline-acétylase et du transporteur vésiculaire de l'acétylcholine. **Résultats** : Les résultats du test du labyrinthe en étoile ont diminué chez les rats hypertendus (comparativement à des rats Wistar et Wistar-Kyoto normotendus), sans égard à l'âge. La fixation de M1 dans les régions du cortex frontal et préfrontal était accrue chez les RSH ($p < 0,05$), tandis que les densités de M2 étaient plus élevées dans le putamen caudé chez le phénotype hypertendu. On a observé une expression réduite des récepteurs nicotiques contenant de l' $\alpha 3$ dans le colliculus supérieur chez les RSH. Dans l'hippocampe, on a constaté des différences liées à l'âge dans l'expression du transporteur de l'acétylcholine vésiculaire. **Conclusion** : Le RSH peut être utile pour modéliser certains aspects des troubles de la mémoire (reliés particulièrement à l'hypertension), spécialement dans les cas où la fonction cholinergique est modifiée.

Introduction

A number of epidemiologic studies support the premise that hypertension is a risk factor for late life cognitive impairment.^{1,2} Although the risk for memory dysfunction and dementia associated with strokes resulting from hypertension and vascular disease (i.e., vascular dementia³) is clearly apparent, evidence is emerging that these factors may also play a role in the pathogenesis of other forms of dementia such as Alzheimer's disease (AD),^{4,5} progressive supranuclear palsy^{6,7} and dementia with Lewy bodies.⁸ In vascular dementia and hypertension-related dementia, the contribution of factors other than overt infarcts is also gaining interest. These include alterations in cerebrovascular autoregulation, cytokine activation and increased inflammatory processes in the brain, atherosclerosis, elevated plasma homocysteine and white matter changes.^{4,9} It is conceivable that any of these processes could negatively alter neural transmission and thus play either a direct or indirect role in cognitive dysfunction. It is important, therefore, to further investigate the role of these factors, as well as other hypertension-related CNS changes, in appropriate animal models.

For several decades, the spontaneously hypertensive rat (SHR) has been used extensively as a model of human hypertension and cardiovascular disease. Certain unique phenotypic characteristics (e.g., hyperactivity, deficits in sustained attention) have also resulted in the use of the SHR as a model of attention-deficit hyper-

activity disorder.^{10,11} In addition, observations of inferior performance on a variety of memory-related tasks, including conditioned avoidance¹²⁻¹⁴ and appetitively¹⁵⁻¹⁷ and non-appetitively motivated¹⁸⁻²⁰ spatial learning tasks, have led to interest in using the SHR to study the effects of hypertension on cognitive function. Few studies have, however, investigated the effects of aging on memory function in the SHR.

As early as 6 months of age, SHRs demonstrate hypertensive brain damage (e.g., cytoskeletal breakdown, astrogliosis and atrophy in the hippocampus) reminiscent of vascular dementia.^{21,22} SHRs show other signs of significant morbidity and spontaneous deaths after 15 months of age, as well as a reduced mean lifespan²³⁻²⁵ compared with other rat strains. These findings combined with observations of significant deficits in central nicotinic cholinergic receptors (nAChRs) that worsen with age²⁰ suggest that older SHRs (in particular) could be used to model certain aspects of several age-related human diseases in which cholinergic function is altered.

The cholinergic system appears to be particularly vulnerable to the effects of aging, as determined by immunostaining techniques, neurotransmitter turnover studies and receptor measurements²⁶⁻³⁰ in aged animals and humans, as well those who suffer from age-related neurodegenerative diseases such as AD.^{31,32} There is also evidence of a decline in the integrity of the cholinergic system in patients with vascular dementia (e.g., decreased choline acetyltransferase

[ChAT] and a depletion of cerebrospinal fluid acetylcholine levels.^{33–35}). Recent clinical trials indicate that acetylcholinesterase inhibitors (e.g., galantamine) may be effective in improving memory function in patients with vascular dementia.^{1,36,37} These observations highlight the value of identifying animal models that show age-related cardiovascular changes and deficits in cholinergic function and memory. Such models will be useful for investigate the interactions among these factors as well as for preclinical drug testing.

Given the well-documented importance of the central cholinergic system to memory processes and the unique phenotypic features of SHR, we were interested in extending our previous investigations on memory performance and central cholinergic function in older SHR. We evaluated SHR in a memory-related task known to depend on cholinergic function (the radial arm maze³⁸) and measured the expression of muscarinic cholinergic receptors (mAChRs) and $\alpha 3$ -containing nAChRs via quantitative receptor autoradiography. Immunoblotting experiments were conducted to evaluate potential phenotypic and age-related differences in the expression of 2 well described (presynaptic) cholinergic markers:^{39,40} ChAT and the vesicular acetylcholine transporter (VACHT).

Methods

Male SHR and Wistar-Kyoto rats (WKYs), 3 months old and 9-month-old retired breeders, were purchased from Taconic Farms (Germantown, NY). Male Wistar rats (3 months old) were purchased from Harlan (Indianapolis, Ind.). Animals were housed individually in a temperature-controlled room (25°C) with a 12-hour light–dark cycle. Upon arrival, each animal was provided with water and food (Teklad rodent feed or Purina Rat Chow) ad libitum. Retired breeder WKY and SHR were allowed to age an additional 5 months. All rats were handled for 2 weeks before behavioural testing or whole brain tissue extraction.

All procedures employed during this study were reviewed and approved by the Medical College of Georgia Committee on Animal Use for Research (CAURE) and the Veterans Affairs Medical Center Subcommittee on Animal Use and were consistent with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

Unless otherwise noted, all non-radioactive chemicals were obtained from Sigma–Aldrich (St. Louis) and

all radiolabelled compounds were obtained from Perkin Elmer (formerly NEN Lifesciences, Boston).

Radial arm maze

Three days before radial arm maze (RAM) testing, rats were restricted to a daily feeding of 18 g (approximately 80% of their ad libitum consumption); they were immediately returned to free food access upon completion of RAM testing. RAM testing was conducted in an 8-arm maze constructed of polyvinyl chloride plastic and plexiglass. Each arm (12 cm × 70 cm) extended radially from a central arena (30-cm diameter) and had a food cup 2 cm from the distal end. The maze was positioned 90 cm above the floor in a testing room with a number of extra-maze cues. Rats were tested 7 days per week, 2 trials per day. Before testing, each arm was baited with pieces of sugar-coated cereal.

Results were manually recorded by the experimenter, who was blinded to the phenotype and age of the rats, was located within 30 cm of the maze and able to clearly observe the food cups and entry points of each maze arm and remained stationary between the same 2 arms for all trials. At the beginning of the study, each rat was placed in the central arena and given one 10-min session each day until 2 food pieces were consumed (i.e., during 1 session). These initial sessions were used to allow a period of acclimation (shaping) to the novel environment. Actual testing began on the day after the rat reached criterion.

Test sessions began by placing the rat in the central arena and recording arm choices as the rat entered each arm to consume food rewards. A correct entry was recorded when the rat located and consumed a reward; reinforcements were not replaced during the session. All subsequent entries into an arm (defined as all 4 paws past the threshold of the proximal end) were scored as incorrect entries (errors). A session continued until all 8 pellets were consumed or until 5 minutes elapsed. The dependent measures were the percent correct of the total arms entered and “efficiency,” defined as the number of correct (i.e., reinforced) entries of the first 8 arms entered.

Blood pressure (BP) measurements

Systolic BPs (10 readings/rat) were obtained via a tail-cuff method after behavioural testing. Rats were placed in a restraining chamber and warmed to an ambient temperature of approximately 37°C, typically

taking about 30 minutes. Automatic data collection was performed using a MacLab (World Precision Instruments, Sarasota, Fla.) system synchronized to trigger an electrospphygmomanometer (Narco Biosystems, Austin, Tex.) and to inflate and deflate the tail-cuff to a calibrated pressure at 2-minute intervals. Tail pressure pulsations were detected with a pneumatic pulse transducer.

Brain tissue preparation

Rats were killed by decapitation at 4 and 15 months of age for whole brain tissue extraction. Brains were removed immediately and frozen in isopentane at -35°C . All brains were stored at -70°C for at least 24 hours before sectioning for quantitative autoradiography or dissection of cortex and hippocampus for immunoblotting.

Quantitative receptor autoradiography

Preparation of standards

To define the response of the radiosensitive films to increasing amounts of radioactivity, tissue paste standards containing increasing amounts of radioactivity were prepared and included in all film exposures. Whole rat brains were homogenized in ice-cold phosphate buffer (50 mmol/L sodium phosphate). Aliquots of [^3H]-choline were individually added to prepare a range of 0.5–30.0 nCi/mg ($1\text{ Bq} = 2.7 \times 10^{-11}\text{ Ci}$) of brain homogenate. The specific activity of each standard was determined using a liquid scintillation counter. Standards were flash frozen and 16- μm slices were serially sectioned onto chrome-alum gelatin coated slides in an IEC-Minotome cryostat held at -18°C . Tissue paste standard slides were stored at -70°C until used.

Tissue preparation and sectioning

Frozen whole brains from 15-month-old WKY ($n = 6$) and SHR ($n = 6$) were sectioned at a thickness of 16 μm using a Leica-Jung 1800 cryostat/microtome set at -18°C . Each brain was coronally sectioned from prefrontal cortex through the medulla onto chrome-alum gelatin coated microslides. All slides were stored overnight in a dessicator at 4°C and then stored at -70°C until all brains had been sectioned. All slides containing brain sections were stored for at least 24 hours at -70°C before undergoing radioligand binding assays.

Radioligand binding assay

[^3H]-Epibatidine and cytisine: Measurement of $\alpha 3$ -containing nAChRs was accomplished via the method of Marks et al⁴¹ in which [^3H]-epibatidine ([^3H]-EPB) and cytisine are combined. Slides were preincubated with 50 mmol/L Tris-HCl buffer containing NaCl, KCl, CaCl_2 and MgCl_2 at pH 7.5 for 10 minutes at 25°C . After preincubation, slides were incubated in 450 pmol/L [^3H]-EPB and 150 nmol/L cytisine for 60 minutes at 25°C . Incubation with the radioligand was followed by two 5-minute washes in Tris-HCl buffer at 0°C . Nonspecific binding was determined by adding 300 $\mu\text{mol/L}$ nicotine bitartrate to the incubation buffer prior to the ligand.

[^3H]-Pirenzepine and AFDX-384: Comparisons between the rodent phenotypes were made for M1 and M2 mAChR subtypes using [^3H]-pirenzepine ([^3H]-PRZ) and [^3H]-AFDX 384 ([^3H]-AFX), respectively. Slides were preincubated in 50 mmol/L Tris-HCl buffer (pH 7.4) for 15 minutes. After preincubation, slides were incubated with 5 nmol/L [^3H]-PRZ or 10 nmol/L [^3H]-AFX for 90 minutes at room temperature. Incubation with the radioligand was followed by a series of washes at 4°C : three 4-minute 50 mmol/L Tris-HCl washes, one 5-minute 5 mmol/L Tris-HCl and one 10-second in deionized water. Nonspecific binding was determined by adding 10 $\mu\text{mol/L}$ atropine to the incubation buffer before the muscarinic radioligands.

Film exposure and development

After rinsing, slides were air-dried at room temperature and stored overnight in a vacuum dessicator. Autoradiograms were made by exposing the slides to [^3H]-sensitive Amersham Hyperfilm in Fisher Biotech aluminum autoradiographic cassettes for 20 days ([^3H]-PRZ), 4 weeks ([^3H]-AFX) and 10 weeks ([^3H]-EPB and cytisine). All films were manually developed in Kodak D-19 Developer (5 min), Indicator Stop Bath (30 s) and Rapid Fixer (5 min) according to package instructions.

Quantification of receptor binding (densitometry)

Autoradiographic analyses were made using NIH Image Software and an imaging station (Macintosh Power PC 8100/100I computer, Data Translation

QuickCapture imaging board, Sony SC-77 CCD camera and a Northern Lights Precision Desktop Illuminator). Receptor binding was quantified as optical density in all brain areas and nuclei that had a signal greater than background. Each area was measured bilaterally in at least 4 sections for each rat, with an average number of 20 measurements per area per rat. A calibration curve (optical density versus the known molar quantities of radioligand) was generated from the tissue paste standards. From the curve, molar quantities of bound ligand were obtained. To better visualize and discriminate between structures and boundaries, brain sections were stained with cresyl violet. All brain images were referenced to Paxino and Watson's *Rat Brain in Stereotaxic Coordinates*, 4th ed.⁴²

Immunoblotting

The hippocampus and cortex were dissected from 4- and 15-month-old WKY and SHR ($n = 3$ per group) and then homogenized in ice-cold lysis buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 10% glycerol, 1 mmol/L ethylene glycol-bis(2-aminoethylether)-N,N,N¹,N¹-tetraacetic acid [EGTA], 1 mmol/L sodium orthovanadate, 5 μ mol/L ZnCl₂, 100 mmol/L NaF, 1% Triton X-100, 10 μ g/mL aprotinin, 1 μ g/mL leupeptin and 1 mmol/L phenylmethanesulfonyl fluoride [PMSF]). Homogenates were centrifuged at 16 000 \times g for 15 minutes at 4°C. The protein content of the supernatants was determined using a colorimetric method with Bradford reagent (Bio-Rad Laboratories, Hercules, Calif.). Total hippocampal (50 μ g) or total cortical (100 μ g) protein was boiled for 90 seconds in loading/sample buffer (0.5 mol/L Tris-HCl, 20% glycerol, 10% sodium dodecyl sulfate [SDS], 1% bromophenol blue and 5% 2-mercaptoethanol). Samples were size-fractionated on 10% SDS polyacrylamide gels, then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass.) at 30 V overnight at 4°C. Membranes were blocked overnight in 5% nonfat milk (Bio-Rad) in Tris-buffered saline (TBS; 20 mmol/L Tris, 500 mmol/L NaCl, pH 7.6) and then probed overnight at 4°C incubation with 1:500 goat anti-ChAT (Chemicon International, Temecula, Calif.) or 1:1000 goat anti-VAcHT (Santa Cruz Biotechnology, Santa Cruz, Calif.) in milk-TBS. Membranes were then washed with TBS, and the primary antibody was detected using a horseradish peroxidase (HRP)-conjugated secondary antibody in milk-TBS (1:10 000,

60 min, 25°C). HRP activity was revealed with the enhanced chemiluminescence procedure, according to manufacturer's instructions (Supersignal West Pico Substrate, Pierce Endogen, Rockford, Ill.). Chemiluminescent signal was detected using Amersham Hyperfilm-ECL, and films were developed in an automatic Kodak X-OMAT processor. Band densitometries were measured using the same imaging station used for quantitative autoradiography experiments.

Statistical analyses

Comparisons of rat phenotypes and age for RAM (overall) efficiency scores, systolic blood pressures and immunoblotting experiments were made using 2-way analysis of variance (ANOVA). In the case of daily RAM performance comparisons, group differences, the effects of the day of testing and group by day interactions were compared using 2-way repeated measures ANOVA. Densitometry measurements from autoradiographic experiments were compared using 1-way ANOVA, since only older WKY and SHRs were compared. Statistical significance was assessed at an alpha level of 0.05, except in the case of the autoradiographic results where an alpha level of 0.01 was utilized, to account for multiple measurements. The Student-Newman-Keuls method for post hoc analysis was used for all multiple comparisons.

Results

Radial arm maze

The ability of younger and older WKY and SHRs to navigate an 8-arm RAM for food reinforcement across 7 days of training is depicted in Fig. 1A. A group of young Wistar rats were included in the experiments so that comparisons between WKY and SHRs and a normal (outbred) rat strain (known to efficiently perform the RAM test) could be made. Each group (with the exception of older SHRs) learned to enter the baited arms to locate food with progressively fewer errors of re-entry (i.e., into unbaited arms) on successive days of training. For the percent correct of the total arms entered (by day) comparisons, statistical analyses revealed the following: group effect $F_{4,48} = 5.20$, $p < 0.001$; day effect $F_{6,24} = 29.49$, $p < 0.001$; group by day interaction $F_{284,366} = 3.25$, $p < 0.001$. Post hoc comparisons indicated that both WKY and SHRs (regardless of age)

exhibited a significantly ($p < 0.05$) lower level of performance on several days than the younger Wistar rats. All other group differences were not statistically significant ($p > 0.05$). For the overall efficiency comparisons (collapsed across the days of testing), however, (Fig. 1B) both younger and older SHR demonstrated inferior performance ($p < 0.01$) compared with younger Wistar and WKYs of both age groups. There were no age-related differences among SHRs.

Blood pressure (BP)

Systolic BPs were measured after behavioural testing (Fig 2). There were both phenotypic ($F_{95.55}, p < 0.0001$) and age-related ($F_{7.12}, p < 0.02$) differences in systolic BP. Post-hoc comparisons indicated: systolic BPs were significantly ($p < 0.05$) higher in SHRs than WKYs regardless of age, although the margin of difference was wider among old animals, and systolic BPs were higher in older than younger SHRs ($p < 0.05$).

Receptor autoradiography

Representative autoradiograms and receptor densitometry comparisons between older (15 month old) WKY and SHR for are presented in Fig. 3 and in Table 1 ($[^3\text{H}]\text{-PRZ}$), Table 2 ($[^3\text{H}]\text{-AFX}$) and Table 3 ($[^3\text{H}]\text{-EPB} + \text{cytisine}$).

$[^3\text{H}]\text{-Pirenzepine}$

The autoradiographic distribution of $[^3\text{H}]\text{-PRZ}$ binding to central M1 mAChRs in older WKY and SHRs was similar. Binding was widely distributed in the neocortex and hippocampal formation and minimally represented in the thalamus, hypothalamus and midbrain. The highest $[^3\text{H}]\text{-PRZ}$ binding densities were observed in the CA1 region of the hippocampus, dentate gyrus, nucleus accumbens and the basolateral amygdala. Moderate binding was observed in the cortex, caudate putamen, olfactory tubercle, anterior olfactory nuclei and dorsal peduncular nuclei. The lowest $[^3\text{H}]\text{-PRZ}$ binding densities were found in the subiculum, lateral septal nuclei, centromedial amygdaloid nuclei and thalamus. This distribution profile is similar to what has been reported in other studies where SHRs and other rat strains were examined.⁴³ Overall, SHRs demonstrated higher binding densities in 38 of the 46 areas measured; however, significant differences ($p < 0.05$) were limited to cortical areas.

$[^3\text{H}]\text{-AFX 384}$

The autoradiographic distribution of $[^3\text{H}]\text{-AFX}$ binding to central M2 mAChRs in 15-month-old WKY and SHRs was also quite similar. Like $[^3\text{H}]\text{-PRZ}$ binding,

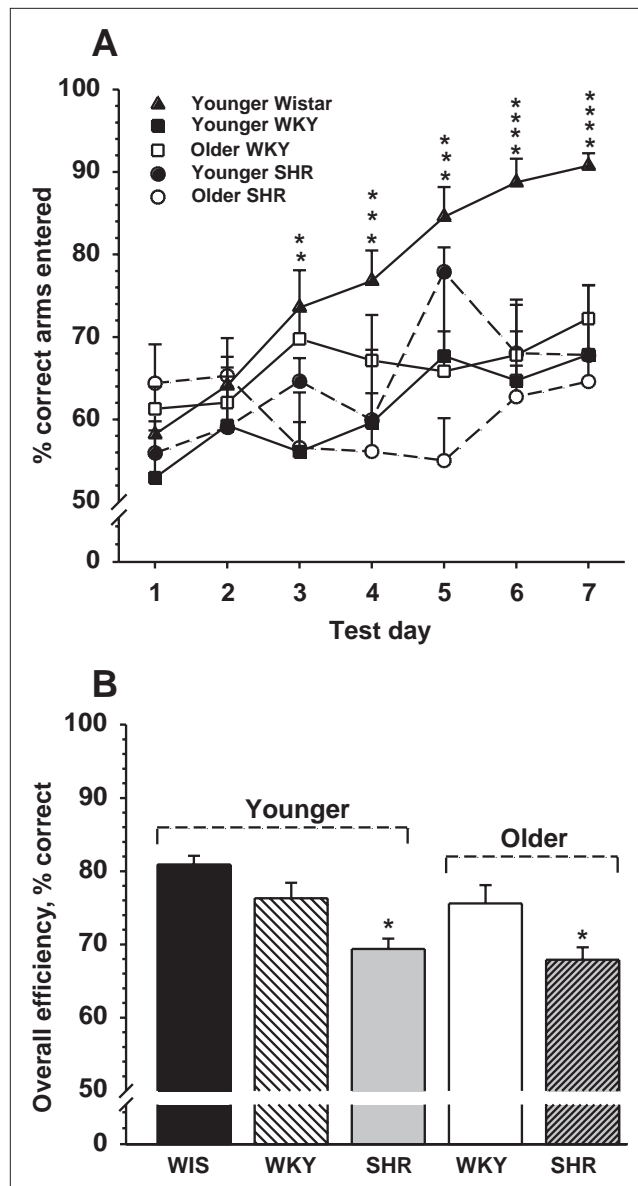


Fig. 1A: Performance of a win-shift task in an 8-arm radial arm maze across 7 consecutive days of testing (2 trials per day per animal) by the various rat groups ($n = 9-12$). Each point represents the mean percent correct (and SEM) of the total arms entered during the 5-min trial. * = Wistar performance significantly different from 1 or more of the other groups.

B: Overall efficiency (collapsed across all days) defined as the number of correct (i.e., reinforced) entries of the first 8 arms entered.

[³H]-AFX binding was widely distributed in the cortex and hippocampal formation for both phenotypes. Unlike [³H]-PRZ binding, [³H]-AFX binding was also

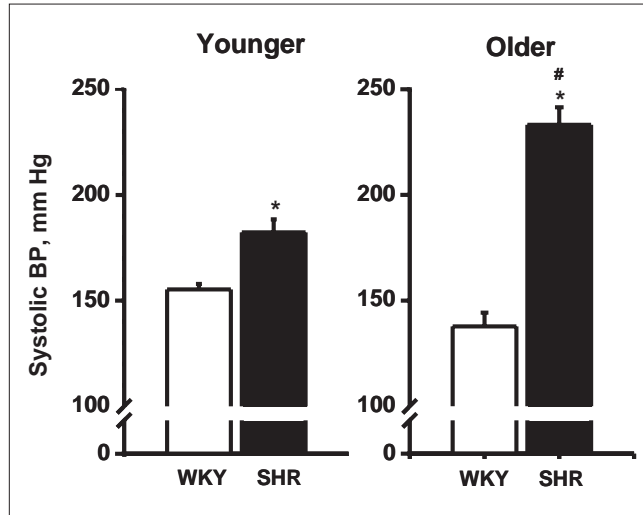


Fig. 2: Mean (and SEM) systolic blood pressure of young and old 15-month-old WKY and SHR rats obtained via a tail cuff method after behavioural training ($n = 10\text{--}12$ rats per group).

* = significant difference between the 2 rat phenotypes ($p < 0.05$); # = significant age-related difference ($p < 0.05$).

distributed across the thalamus, hypothalamus and hindbrain. The highest [³H]-AFX binding densities were observed in the caudate putamen, nucleus accumbens and olfactory tubercle. Moderate binding was found in the cortex, basolateral amygdala and hippocampal formation. Lower [³H]-AFX binding densities were found in the hypothalamus, thalamus and hindbrain. This pattern of [³H]-AFX binding is similar to that reported in previous studies.⁴³ In the present study, a significant difference in binding density was detected in only the caudate putamen (SHR > WKY, $p = 0.003$).

[³H]-Epibatidine and cytisine

The autoradiographic distributions of [³H]-EPB and cytisine binding to central $\alpha 3$ -containing nAChRs in 15-month-old WKY and SHRs were again similar. The highest [³H]-EPB and cytisine binding densities were observed in the medial habenular nuclei and interpeduncular nuclei, and moderate binding densities were found in the fasciculus retroflexus. Lower [³H]-EPB and cytisine binding densities were found in the geniculate nuclei and mammillary nuclei. This pattern of binding is similar to that reported in previous studies.²

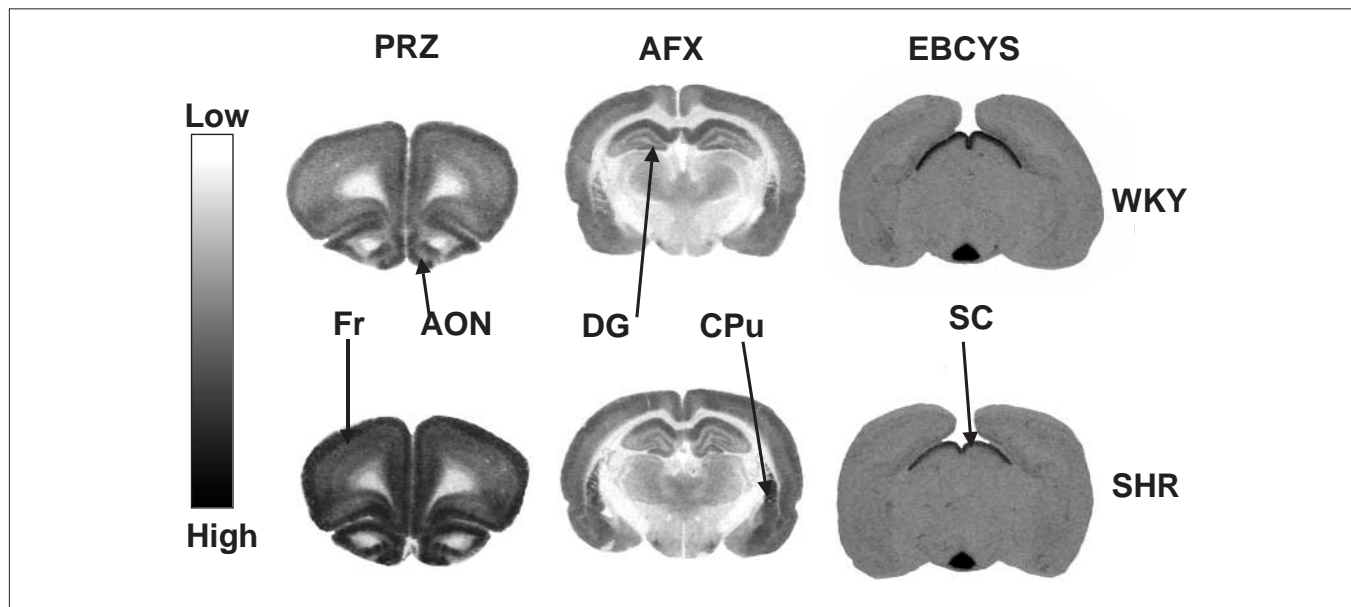


Fig. 3: Representative autoradiograms illustrating receptors labeled by [³H]-pirenzepine (PRZ, M1 muscarinic cholinergic receptors), [³H]-AFDX-384 (AFX, M2 muscarinic cholinergic receptors) and [³H]-epibatidine + 150 nM cytisine (EBCYS, $\alpha 3$ -containing nicotinic cholinergic receptors) in coronal sections of brains from 15-month-old SHR and age-matched controls (WKY). Fr = frontal cortex, AON = anterior olfactory nucleus, DG = dentate gyrus, CPu = caudate putamen, SC = superior colliculus. Sections in which significantly different binding densities between SHR and WKY were found are presented.

* = significant difference between the 2 rat phenotypes ($p < 0.05$).

Table 1: Distribution of [³H]-pirenzepine binding sites in selected brain regions of 15-month-old Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

Brain area	Rat strain; mean (and SEM) binding, nCi bound [³ H]/mg wet tissue, n = 5–6	
	WKY	SHR
Amygdaloid nuc., basolateral	10.51 (0.18)	10.42 (0.11)
Amygdaloid nuc., central	4.75 (0.22)	4.43 (0.17)
Accumbens nuc., core	11.04 (0.14)	10.85 (0.30)
Accumbens nuc., shell	10.36 (0.22)	10.36 (0.25)
Caudate putamen	9.04 (0.16)	9.62 (0.23)
Cerebral cortex		
Auditory	9.49 (0.32)	10.22 (0.27)
Cingulate area 1	8.85 (0.22)	8.92 (0.26)
Cingulate area 2	9.08 (0.23)	8.71 (0.30)
Entorhinal, lateral	9.75 (0.34)	10.39 (0.30)
Frontal association	9.56 (0.13)	10.24 (0.16)
Medial*	8.72 (0.16)	9.48 (0.12)
Outer	10.11 (0.13)	10.63 (0.11)
Infralimbic	9.91 (0.14)	10.32 (0.08)
Insular*	9.63 (0.15)	10.28 (0.10)
Prefrontal*	9.24 (0.15)	10.10 (0.14)
Medial*	8.88 (0.16)	9.75 (0.17)
Outer	9.94 (0.13)	10.52 (0.17)
Prelimbic	9.62 (0.11)	10.05 (1.34)
Motor area 1	8.43 (0.18)	9.16 (0.26)
Motor area 2	8.93 (0.15)	9.50 (0.25)
Orbital*	9.17 (0.11)	10.04 (0.20)
Parietal entire	9.26 (0.33)	9.89 (0.27)
Layer I	9.93 (0.35)	10.50 (0.28)
Layer II–VI	9.00 (0.35)	9.75 (0.17)
Piriform	9.30 (0.14)	9.43 (0.33)
Retrosplenial	4.60 (0.22)	5.12 (0.29)
Somatosensory	7.49 (0.13)	8.11 (0.29)
Temporal	8.43 (0.20)	8.80 (0.16)
Layer I	9.42 (0.20)	9.76 (0.29)
Layer II–VI	7.89 (0.19)	8.16 (0.13)
Visual	9.32 (0.35)	10.00 (0.27)
Anterior olfactory nuc.	9.95 (0.11)	10.42 (0.10)
Dentate gyrus inner blade		
Anterior	11.62 (0.20)	11.20 (0.14)
Posterior	10.65 (0.21)	10.81 (0.17)
Dentate gyrus outer blade		
Anterior	11.23 (0.16)	11.32 (0.22)
Posterior	10.94 (0.18)	11.18 (0.11)
Dentate polymorph layer		
Anterior	9.63 (0.16)	10.05 (0.13)
Posterior*	9.54 (0.19)	10.20 (0.11)
Hippocampal formation		
CA1 anterior	11.97 (0.13)	11.83 (0.21)
CA1 posterior	10.90 (0.14)	11.15 (0.13)
CA2 and 3 anterior	8.35 (0.15)	8.32 (0.22)
CA2 and 3 posterior	9.14 (0.25)	9.48 (0.25)
Indusium griseum	8.96 (0.27)	8.99 (0.48)
Olfactory tubercle	9.74 (0.20)	10.13 (0.22)
Subiculum	5.64 (0.23)	5.96 (0.19)
Septal nuc., lateral	0.70 (0.09)	1.40 (0.13)
Dorsal peduncular nuc.	9.91 (0.18)	10.48 (0.10)
Thalamus	1.00 (0.08)	0.98 (0.14)

Whole brains were sectioned from the prefrontal cortex through the pons.
*Significant difference between phenotypes (SHR > WKY, $p < 0.01$).

Table 2: Distribution of [³H]-AFDX-384 binding sites in selected brain regions of 15-month-old WKY and SHR rats

Brain area	Rat strain; mean (and SEM) binding, nCi bound [³ H]/mg wet tissue, n = 5–6	
	WKY	SHR
Amygdaloid nuc., basolateral	8.29 (0.83)	9.73 (0.05)
Amygdaloid nuc., central	5.41 (1.12)	3.76 (0.05)
Accumbens nucleus	10.44 (0.08)	10.67 (0.05)
Caudate putamen*	9.92 (0.07)	10.37 (0.06)
Diagonal band	3.69 (0.36)	3.93 (0.25)
Substantia inominata	2.20 (0.22)	2.68 (0.54)
Cerebral cortex		
Cingulate	5.63 (0.67)	7.00 (0.86)
Cingulate area 1	5.92 (0.66)	5.73 (0.65)
Frontal association	8.30 (0.71)	8.20 (0.66)
Infralimbic	7.88 (0.78)	8.81 (0.17)
Insular	7.96 (0.60)	8.40 (0.39)
Motor area 1	6.78 (0.59)	7.24 (0.49)
Motor area 2	7.08 (0.60)	8.09 (0.47)
Orbital	7.74 (0.88)	8.55 (0.48)
Parietal	7.08 (0.47)	7.15 (0.66)
Piriform	4.83 (0.28)	5.49 (0.51)
Prefrontal	8.37 (0.59)	8.38 (0.65)
Retrosplenial	4.92 (0.17)	6.38 (0.86)
Somatosensory	6.95 (0.60)	7.44 (0.58)
Anterior olfactory nuc.	6.24 (0.69)	6.47 (0.23)
DG outer and inner blade	5.94 (0.39)	4.43 (0.34)
DG polymorph layer	4.07 (0.13)	3.71 (0.12)
Hipp. form. CA1 region	8.31 (0.27)	7.83 (0.57)
Hipp. form. CA2 and 3 region	3.69 (0.05)	3.47 (0.18)
Olfactory tubercle	10.40 (0.11)	10.60 (0.07)
Bed nuc. of stria terminalis	2.60 (0.14)	2.85 (0.29)
Septal nuc., lateral	4.47 (0.78)	3.56 (0.22)
Septal nuc., medial	3.31 (0.46)	2.45 (0.23)
Dorsal peduncular nuc.	7.59 (0.77)	8.27 (0.15)
Hypothalamic nuc.		
Dorsomedial	2.62 (0.18)	2.64 (0.59)
Lateral	2.36 (0.14)	2.66 (0.19)
Ventromedial	2.24 (0.17)	2.57 (0.34)
Medial preoptic area	2.55 (0.15)	2.64 (0.30)
Zona incerta	2.41 (0.19)	2.43 (0.11)
Thalamic nuc.		
Anteroventral	5.04 (0.35)	5.44 (0.78)
Laterodorsal	3.25 (0.16)	3.13 (0.28)
Mediodorsal	3.42 (0.16)	3.54 (0.23)
Paraventricular	2.87 (0.16)	2.95 (0.13)
Rhomboidal	3.19 (0.22)	3.73 (0.44)
Ventromedial	2.49 (0.12)	2.43 (0.20)
Ventroposterior	2.02 (0.09)	1.98 (0.11)
Central periaqueductal gray	2.29 (0.20)	2.04 (0.20)
Dorsal tegmental nuc.	2.49 (0.25)	2.84 (0.26)
Parabrachial nuc.	3.37 (0.39)	3.91 (0.31)
Pontine nuc.	5.74 (1.13)	8.31 (0.62)
Trigeminal nuc.	5.79 (0.85)	7.03 (1.07)
Gigantocellular nuc.	1.91 (0.25)	2.25 (0.21)
Paragigantocellular nuc., dorsal	1.85 (0.13)	1.85 (0.20)
Reticular nuc. RVL	4.40 (0.51)	6.08 (1.46)
Vestibular nuc.	2.27 (0.16)	2.80 (0.25)

Note: DG = dentate gyrus, RVL = rostroventrolateral; hipp. form. = hippocampal formation.
Whole brains were sectioned from the prefrontal cortex through the pons.
*Significant difference between phenotypes (SHR > WKY, $p < 0.01$).

Of the 10 areas measured, a statistically significant difference was found only in the superior colliculus, with the WKY rats having greater mean density ($p < 0.001$). Binding was slightly higher ($p > 0.05$) than SHR in 7 areas of the 10 areas measured, however.

Immunoblotting

Choline acetyltransferase

The antibody to ChAT recognized a 70-kD band in both the cortex and hippocampus of younger and older WKY and SHRs (Fig. 4B and 4C). There were no significant age- or phenotype-related differences detected in ChAT immunoreactivity in the cortex or hippocampus. There was a notable trend ($p < 0.1$) toward higher ChAT immunoreactivity in the cortex in younger WKYs compared with younger SHRs, however.

Vesicular acetylcholine transporter

The antibody to VACHT recognized a 70-kD band in both the cortex and hippocampus of younger and older WKY and SHRs (Fig. 5B and 5C). Within the hippocampus, VACHT immunoreactivity significantly ($p < 0.05$) decreased with age for both phenotypes (Fig. 5A). In addition, VACHT immunoreactivity was somewhat higher in older SHRs than older WKYs in the hippocampus. Significant differences in VACHT immunoreactivity between age and phenotype were not observed in the cortex.

Table 3: Distribution of [³H]-epibatidine plus 150 nmol/L cytosine binding sites in selected brain regions of 15-month-old WKY and SHR rats

Brain area	Rat strain; mean (and SEM) binding, nCi bound [³ H]/mg wet tissue, $n = 5-6$			
	WKY		SHR	
Cerebral cortex	0.10	(0.02)	0.09	(0.02)
Habenular nucleus, medial	11.29	(0.02)	11.10	(0.18)
Geniculate nuclei				
Dorsal lateral	0.39	(0.04)	0.36	(0.04)
Ventral lateral	0.38	(0.04)	0.33	(0.04)
Medial	0.13	(0.01)	0.13	(0.01)
Superior colliculus				
Superficial gray layer*	1.33	(0.06)	0.94	(0.06)
Interpeduncular nucleus	11.11	(0.05)	11.14	(0.07)
Fasciculus retroflexus	1.06	(0.05)	0.92	(0.08)

Whole brains were sectioned from the prefrontal cortex through the pons.

*Significant difference between phenotypes (SHR > WKY, $p < 0.01$).

Discussion

In previous experiments, we demonstrated that older SHRs exhibit both cognitive deficits in a water maze task (compared with normotensive controls) and a decrease in $\alpha 4$ -containing nAChRs with increasing age.²⁰ An objective of this study was to extend these findings and evaluate both age-related and phenotypic differences in memory performance in an appetitively motivated spatial learning task (i.e., the RAM). The results (of overall efficiency in the RAM) indicated that memory was impaired in SHRs compared with normotensive animals, regardless of age.

One potential confounding factor in this analysis was the observation that younger and older WKYs were unable to reach an asymptotic level of performance (as did Wistar rats) over the 7 days of training. Furthermore, both younger and older WKYs were difficult to shape to the novel test environment in the RAM. In contrast to younger and older SHRs (and younger Wistar rats), which generally habituated to the RAM in only 1 or 2 days, WKYs of both ages took several days on average. A reduced level of investigatory behaviour has been observed in this strain of rat previously.⁴⁴ Furthermore, daily variation in RAM performance was also quite high in the WKYs. These observations highlight possible limitations of using the WKY rats as a so-called "normal control" for SHRs (particularly in some behavioural tasks).

Other factors that should be considered when interpreting memory-related behavioural differences between SHR and WKY strains include level of anxiety, attentional processes and level of motivation. SHRs have been reported to have lower anxiety levels than WKYs, arguing against a major role of anxiety in impaired RAM performance.^{45,46} However, SHRs do demonstrate poorly sustained attention and impaired reward/reinforcement mechanisms,⁴⁷⁻⁴⁹ which could, in fact, contribute to decreased efficiency in the RAM. Many researchers contend that acetylcholine plays a pivotal role in attentional processes,⁵⁰ therefore, observations of deficits in attention and reduced expression of cholinergic markers in SHRs may be of particular importance to the interpretation of memory-related task performance.

The use of the WKY as a normotensive control for SHRs has been challenged on the basis of the variety of genetic differences observed both between the strains and within colonies of each strain (many of which may

not be hypertension related). From a different perspective, it has also been hypothesized that the normotensive Wistar strain may be more appropriate as controls for hypertension-related changes, because the SHR strain was derived from WKY and thus likely carries some of the same genes responsible for hypertension.⁵¹ In the present study, normotensive Wistar rats were clearly superior in performance in the RAM compared with both SHRs and WKYs, although the effects of aging were not assessed.

A second objective of the study was to further analyze the cholinergic system in SHRs to compare the relative densities of (G-protein coupled) mAChRs and

$\alpha 3$ -containing nAChRs.⁵² M1 and M2 receptors are the most prominent of the 5 known mAChR subtypes (M1–M5). Post-synaptic M1 mAChRs have a high affinity for the M1 agonist pirenzepine and stimulate phosphatidylinositol turnover. M2 muscarinic receptors are thought to primarily exist as presynaptic autoreceptors that bind AFDX-384 with high affinity and inhibit the release of adenylate cyclase.^{34,53} Both of these receptors are known to play an important role in a number of mnemonic processes.^{34,52,54,55} Although the functional role of $\alpha 3$ -containing nAChRs in the CNS is poorly understood at present outside of developmental

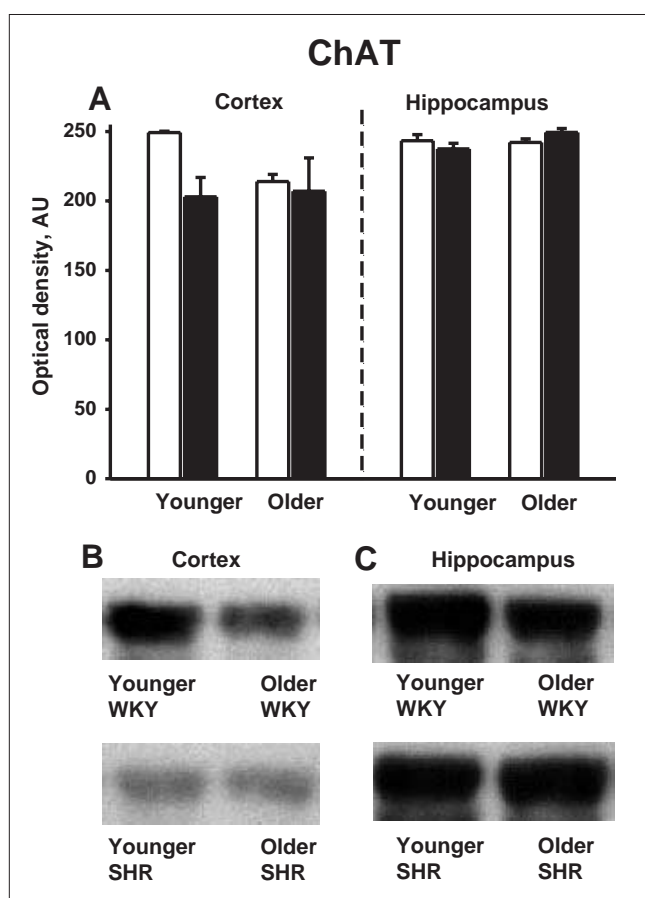


Fig. 4: Choline acetyltransferase (ChAT) immunoreactivity in the cortex and hippocampus of younger (4-month-old) and older (15-month-old) SHR (black bars) and WKY (white bars) rats ($n = 3$ per group).

A: Quantitative densitometry results from ChAT immunoblotting experiments.

B: Representative immunoblots of ChAT in the cortex.

C: Representative immunoblots of ChAT in the hippocampus.

AU = arbitrary densitometric units.

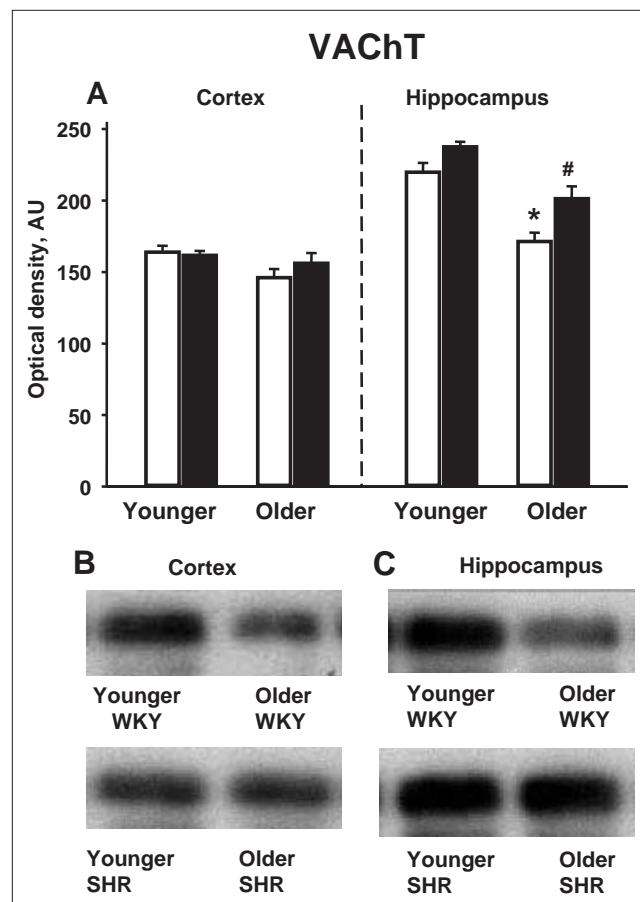


Fig. 5: Vesicular acetylcholine transporter (VACHT) immunoreactivity in the cortex and hippocampus of younger (4-month-old) and older (15-month-old) SHR (black bars) and WKY (white bars) rats ($n = 3$ per group).

* = younger SHR > older SHR ($p < 0.05$); # = younger WKY > older WKY ($p < 0.05$).

A: Quantitative densitometry results from VACHT immunoblotting experiments.

B: Representative immunoblots of VACHT in the cortex.

C: Representative immunoblots of VACHT in the hippocampus.

and autonomic processes,^{56–58} the need to accurately differentiate the expression of these receptors (in hypertensive animals) will become increasingly important as more functional data are obtained.

The density of M1 binding sites was slightly higher in SHR than in WKYs across most of the brain regions analyzed ($p < 0.05$ in prefrontal and frontal cortical areas). However, given the modest nature of these differences (i.e., 7%–8%), the functional significance of this finding is unclear. The slightly increased level of M1 receptor expression in SHR might represent a post-synaptic upregulation after depletion of cholinergic projections in the cortex with age or, alternatively, a compensatory mechanism that follows the nAChR deficiency in older SHR. Interestingly, an increase in M1 AChR expression is observed in humans with dementia with Lewy bodies,⁵⁹ a disease noted for its decrease in nAChRs and other cholinergic markers. As opposed to the observed differences in M1 binding sites, the phenotypic difference in the density of M2 binding sites was minimal; only the caudate putamen was statistically different (i.e., SHR > WKY).

Only 1 area was significantly different in $\alpha 3$ -containing nAChRs (i.e., binding in the superior colliculus was markedly higher in WKYs). Interestingly, the density of $\alpha 4$ - and $\alpha 7$ -containing receptors was also reported to be higher in older WKYs²⁰ in this brain region. Although the functional significance of these differences is unknown, nAChRs in the superior colliculus have been documented to play a significant role in visual responses.⁶⁰ However, studies in our laboratory demonstrated no significant differences in performance of a water maze visible platform task between SHR and WKYs at either 4 or 15 months of age.^{18–20}

Immunoblotting experiments showed no statistically significant phenotypic or age-related differences in cortical and hippocampal ChAT, although there was a strong trend toward a lower expression of ChAT in the cortex of young SHR (see Fig. 4A). Both strains demonstrated a loss of VAcHT in the hippocampus with age, however. In cholinergic presynaptic terminals, ChAT is responsible for synthesizing acetylcholine, and VAcHT is responsible for the transport of acetylcholine into synaptic vesicles for regulated exocytotic release.⁶¹ Decreases in VAcHT in an area important for memory, such as the hippocampus, could contribute to the inferior performance of SHR compared with WKYs often observed in memory-related tasks.

In summary, the autoradiographic results of this

study (measuring $\alpha 3$ -containing nAChRs and mAChRs) and previous studies (in which $\alpha 4$ - and $\alpha 7$ -containing nAChRs were measured²⁰) indicate no marked differences between SHR and WKYs (at a younger or older age) in the expression of $\alpha 3$ - and $\alpha 7$ -containing nAChRs or mAChRs in important memory areas. However, age-related alterations in $\alpha 4$ -containing nAChRs and the cholinergic marker VAcHT in important memory areas, combined with the behavioural results of this study, indicate that the SHR may be useful to model some aspects (particularly hypertension-related) of memory disorders, especially those in which cholinergic function is altered.

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