Canadian Journal of Cardiology 35 (2019) 661–670

Basic Research

Functional Dosage of Muscarinic Cholinergic Receptor 3 Signalling, Not the Gene Dose, Determines Its Hypertension Pathogenesis

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ABSTRACT

Background: Multiple quantitative trait loci for blood pressure (BP) have been localized throughout human and rodent genomes. Few of them have been functionally identified especially in humans, and little is known about their pathogenic directionality when identified. We focused on *Chrm3* encoding the muscarinic cholinergic receptor 3 (M3R) as the causal gene for *C17QTL1* in the Dahl salt-sensitive rat model.

Methods and Results: Congenic knock-ins, gene-specific knockout, and *ex vivo* and *in vivo* function studies were applied in the Dahl salt-sensitive rat model of polygenic hypertension. A *Chrm3* missense T1667C mutation in the last intracellular domain functionally correlated with a rise in BP increased the M3R signalling and resensitization, and adrenal epinephrogenesis. Gene targeting that abolished the M3R function without affecting any of noncoding *Chrm3* variants

RÉSUMÉ

Contexte : De nombreux loci de caractères quantitatifs associés à la pression artérielle (PA) ont été localisés dans le génome des humains et de rongeurs. Nous avons découvert la fonction de quelques-uns d'entre eux seulement, surtout chez les humains, mais nous savons peu de choses sur la directionnalité de leurs propriétés pathogènes. Nous nous sommes penchés sur *Chrm3*, le gène codant pour le récepteur muscarinique 3 (M3) et le gène en cause dans le locus de caractères quantitatifs *C17QTL1* chez un modèle de rat Dahl sensible au sel.

Méthodologie et résultats : Des *knock-in* congéniques, l'invalidation d'un gène précis (*knock-outs*) et des études fonctionnelles menées *ex vivo* et *in vivo* ont été réalisés sur un modèle murin d'hypertension artérielle polygénique, le rat Dahl sensible au sel. La mutation faux-sens T1667C de *Chrm3* dans le dernier domaine intracellulaire

Localizing quantitative trait loci (QTLs) has uncovered chromosome regions and/or genomic markers that are statistically associated with blood pressure (BP) in humans.[1] So far, hundreds of QTL signals have appeared from genome-wide association studies (GWASs)[1] and more are expected. Obvious questions to follow are, first, because GWAS signals are mostly found in noncoding regions of genes that have unknown functions, how do we go from a statistic association of a QTL to identifying a gene capable of biologically controlling BP? Second, what is the directionality of each QTL in hypertension pathogenesis? That is, whether it is pro- or antihypertensive. Finally, one reason for expanding the search for more QTLs by GWASs is that one assumes that if each QTL affects a protein, which in turn would directly act on adult BP with a minor effect, they could cumulatively reach a BP threshold.[2] We addressed the relevance of this quantitative premise *in vivo*.

Experimental studies of polygenic hypertension showed that QTLs biologically affect BP in modularity; namely, combined effects from multiple QTLs are noncumulative by function.[3] When a master regulator is absent,[4] the BP genetic architecture is composed of various QTLs as building blocks functionally organized into separate epistatic modules (EMs). Mechanistically, the function modularity suggests a common pathway for multiple QTLs to act within the same EM,[5] regardless of whether underlying genetic bases are structural or at the level of gene expression. In EM2/pathway2 of 8 members, *Chrm3* encoding the muscarinic cholinergic receptor 3 (M3R) is *C17QTL1* in Dahl salt-sensitive (DSS) rats.

Received for publication August 17, 2018. Accepted December 7, 2018.

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See page 670 for disclosure information.

https://doi.org/10.1016/j.cjca.2018.12.029

caused a decrease in BP, indicating that the M3R-mediated signalling promotes hypertension. In contrast, removing 8 amino acids from the M3R first extracellular loop had no effect on BP.

Conclusions: The M3R-specialized signalling constitutes a new pathway of hypertension pathogenesis within the context of a polygenic and quantitative trait. Increased epinephrine in the circulation and secreted from the adrenal glands are suggestive of a molecular mechanism partially mediating M3R to promote hypertension. The structure-function relationships for various M3R domains in their effects on BP pave the way for identifying missense mutations that impact functions on BP as potential diagnostic targets.

ayant une corrélation fonctionnelle avec une hausse de la PA a entraîné une amplification des signaux médiés par le récepteur M3 et de sa resensibilisation, ainsi qu'une épinéphrogenèse surrénalienne. Le ciblage du gène visant à abolir la fonction du récepteur M3 sans altérer les variants non codants de *Chrm3* a entraîné une baisse de la PA, ce qui montre que les signaux médiés par le récepteur M3 favorisent l'hypertension artérielle. En revanche, le retrait de 8 acides aminés de la première boucle extracellulaire du récepteur M3 n'a eu aucun effet sur la PA.

Conclusions : Les signaux spécialisés du récepteur M3 constituent une nouvelle voie dans la pathogenèse de l'hypertension artérielle dans l'optique d'un caractère quantitatif et polygénique. La hausse de l'adrénaline dans la grande circulation et de celle sécrétée par les glandes surrénales laisse entrevoir un mécanisme moléculaire poussant partiellement le récepteur M3 à favoriser l'hypertension artérielle. Les liens structure-fonction de divers domaines du récepteur M3 en ce qui a trait à leurs effets sur la PA pavent la voie à l'identification de mutations faux-sens ayant une incidence sur les fonctions agissant sur la PA en tant que cibles thérapeutiques possibles.

The mechanistic directionality of the M3R-mediated pathway is to raise BP, even at the expense of M3R-dependent vasodilation.[6]

On the basis of the prohypertensive property of M3R,[6,7] we posed further questions. First, can 1 gene, not a cluster of genes, encode 1 BP QTL? Second, modularity/noncumulativity of QTLs in BP control[3] molecularly implicates a pathway for an EM.[5] We addressed whether or not the M3R-mediated signalling constitutes a pathway for EM.[3] Third, we compared the structural integrity of M3R with its signalling itself in hypertension pathogenesis. Finally, we attempted to identify potential mechanistic connections to the M3R signalling.

Methods

Congenic animals

Protocols for handling, maintaining, and treating animals have been approved by our institutional animal committee (Comité Institutionnel de Protection des Animaux du Chum).

Isolated smooth muscle preparation

Strips of rat urinary bladder detrusor smooth muscle were prepared and incubated as previously described[8] and were stimulated with KCl (120 mM) for 5 minutes to determine intrinsic contractility. After a washout period of 30 minutes, the strips were then exposed to carbachol, as described in the Figure 1 legend, in the absence or presence of the M3R-selective antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP). This protocol was applied to a subset of tissues using KCl (120 mM) or serotonin (1 mM) instead of carbachol.

Heart rate variability assessment

The short-term heart rate variability (HRV) was examined in conscious, unrestrained rats instrumented 10 days by telemetry. Spectral analysis[9] was performed, and statistical differences in HRV were analysed by a Kuskall-Wallis test followed by Dunnett's multiple comparison post-test.

Catecholamine measurements

Catheters that minimize the stress and disturbance to the free-moving animals were implanted into the carotid artery of male rats aged 10 weeks, which were fed a high-salt (2% NaCl) diet since 5 weeks of age. A week later after recuperation from the surgery, 3.5 mL of blood was withdrawn remotely from each rat without disturbing them and centrifuged immediately. The serum of each sample was frozen and analysed for catecholamines, aldosterone, and cortisol by the hospital biochemical services at the Centre hospitalier de l'Université de Montréal, or vasopressin at the Sacre-Coeur hospital at Montreal. The plasma renin activity was determined by Elisa using a kit (DBC #CAN-RA-7070; Diagnostics Biochem Canada, London, ON), according to the manufacturer's instructions.

Measuring adrenal epinephrine release *ex vivo*

Intact fresh adrenals from 10-week-old rats (as in BP studies) were incubated with carbachol by culture according to a modified protocol[10] at 37°C with agitation. Two separate aliquots of 500 μL solution incubated for various times (Table 1) were kept at −80°C to be measured for catecholamines (see the section above). The difference was compared by Student's *t*-test.

Gene-targeting by zinc finger nuclease in generating *Chrm3*-distinct deletions from DSS rats was as detailed previously.[6] BP experimental protocols and analyses were principally the same as reported previously.[5,6] Repeated measures' analysis of variance followed by Dunnett's test compared parameters in BP between 2 groups and the power and sample size calculations were as given previously.[4]

Results

Molecular characterization of rat *Chrm3*

Aligning the rat genomic DNA sequence with that of the human *CHRM3* cDNA[11] suggested probable rat exon positions (Fig. 2). The first 4 exons define 5′ untranslated *Chrm3* regions that are identical between DSS and Lewis rats. A single fifth

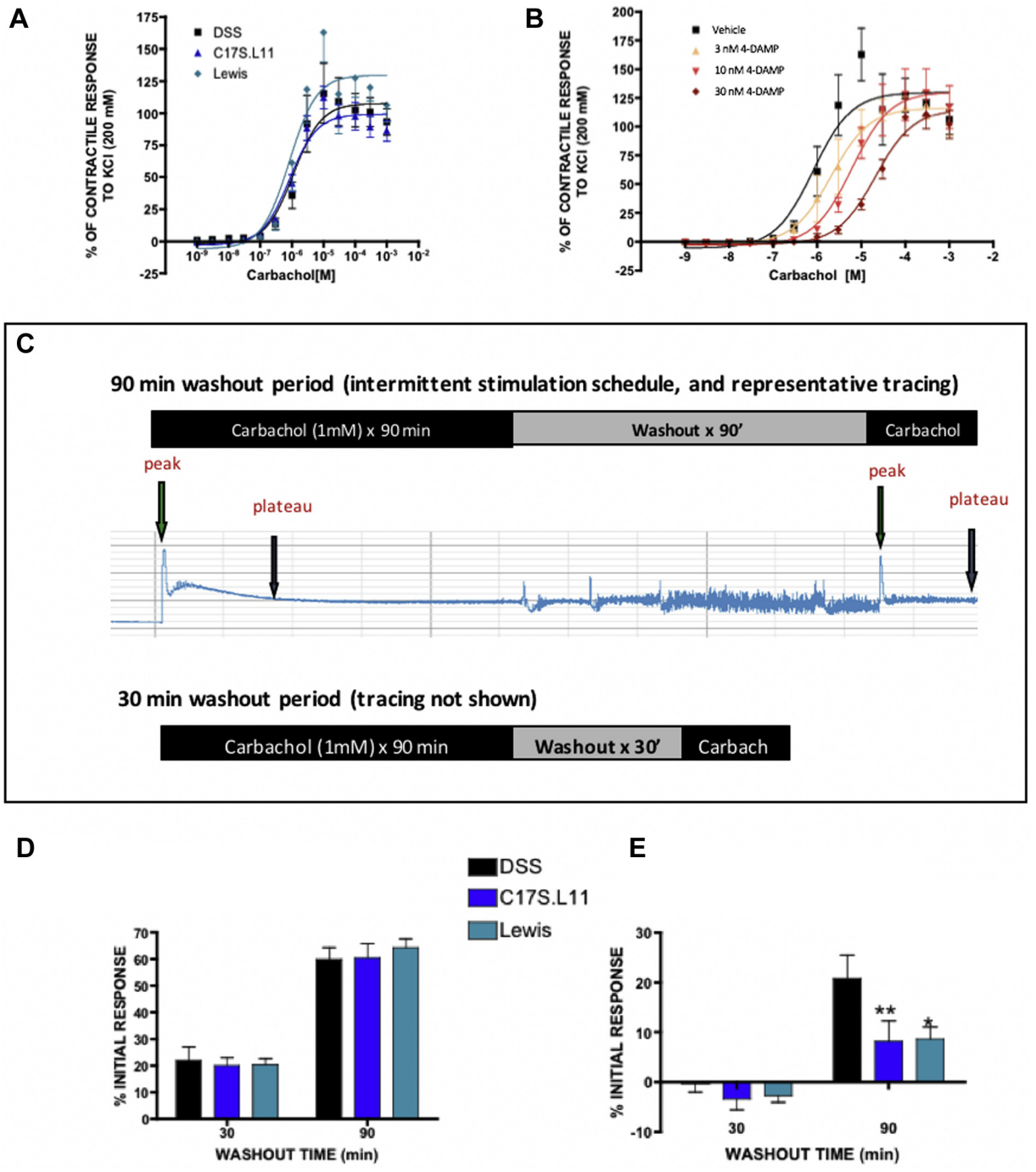

Figure 1. Muscarinic cholinergic receptor 3 (M3R)-T556M increases receptor resensitization without affecting receptor apparent affinity or efficacy: bladder strips were contracted with cumulative concentrations of carbachol to determine that (**A**) apparent agonist affinity (EC_{50}) and receptor efficacy (maximal response) were similar between preparations from the Dahl salt-sensitive (DSS), C17S.L11 congenic, and Lewis strains. (**B**) Responses to carbachol were inhibited dose dependently by the muscarinic receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP). (**C**) Representative tracing of the typical biphasic contractile response to a first maximal carbachol treatment, showing a peak (within 1 minute) and a plateau (within 20 minutes), and a second treatment with carbachol after a washout period of 90 minutes or 30 minutes (not shown). On restimulation, bladder strips showed a time-dependent recovery of responsiveness to carbachol for both (**D**) the peak phase and (**E**) the plateau phase of the response. The magnitude of recovery was significantly faster in strips from the DSS strain carrying the mutant M3R-T556M as compared with the strains bearing the wild-type M3R (Lewis and C17S.L11 congenic). All data are expressed as mean ± standard error of the mean. Statistical differences were analysed by a 2-way analysis of variance followed by the Bonneferroni multiple comparison post-test. n = 10-15 for each strain. $*P < 0.01$; $**P < 0.05$.

Table 1. Plasma epinephrine levels and adrenal epinephrogenesis are diminished in the congenic strain C17S.L11 compared with DSS rats

(A) Biochemical parameters in the circulation

	DSS		C17S.L11		
	n	Value	n	Value	*P* value
Epinephrine (pmol/L)	9	6324 ± 848	8	2830 ± 700	**0.0015**
Norepinephrine (pmol/L)	9	1046 ± 134	8	894 ± 126	0.45
Vasopressin (pg/μL)	7	3.0 ± 0.8	9	1.9 ± 0.5	0.79
Aldosterone (pmol/L)	5	200 ± 31	5	237 ± 72	0.68
Plasma renin activity (ng(AngI)/mL h)	7	1.22 ± 0.4	9	1.31 ± 0.3	0.45

(B) Adrenal secretions *ex vivo*

	Time	N	DSS	C17S.L11	*P* value
Epinephrine	T0	5	1347 ± 171	954 ± 187	0.196
	T20	5	3377 ± 222	2393 ± 206	**0.012**
	T70	5	7451 ± 453	5852 ± 450	**0.037**
Norepinephrine	T0	5	351 ± 37	279 ± 21	0.163
	T20	5	636 ± 50	633 ± 34	0.957
	T70	5	2160 ± 490	1897 ± 341	0.673

All rats were males. The level of cortisol in the rats was too low to be reliably detected in the serum (data not shown). Because each experiment was performed independently of another, an unpaired Student's *t*-test was used to compare the corresponding data in DSS vs C17S.L11. At the designated point of time, that is, Time 0 (T0), 20 min of incubation with carbachol (T20), and T70, the same adrenal glands were transferred with forceps without damage into the new solution without carbachol for a washout period of 20 min.

Bold numbers are statistically significant.

DSS, Dahl salt-sensitive; n, number of rats.

exon contains M3R's entire coding region that is different between DSS and Lewis (Supplemental Table S1E). Based on the newly defined exon positions, polymerase chain reaction primers spanning several *Chrm3* exons including the sole coding exon were used to define *Chrm3* organ expressions (Fig. 2, Supplemental Table S1). *Chrm3* is prominently expressed in the brain and less in the adrenal glands, but was not detectable in the other organs, probably due to low expressions. The pivotal issue to address next is: does the single $Chrm3^{T1667C}$ mutation causing a T556M transformation (Supplemental Table S1) alter functions of M3R?

Functional characterization of M3R

In complement to our previous *in vitro* results,[6] we sought to determine whether the change in M3R signalling in cultured cells should also alter the M3R function. We assayed smooth muscle contractions *ex vivo* on bladder tissues from rats expressing M3R-T556M (DSS strain) or the wild-type M3R (C17S.L11 congenic strain or Lewis strain). The contractile response to KCl (200 mM) was not different ($P > 0.05$) between strains (in DSS: 3.47 + 0.55 g; in C17S.L11: 3.62 + 0.74 g; in Lewis: 2.66 + 0.43 g).

Stimulations with carbachol induced sustained concentration-dependent contractions that were not significantly different between strains (in DSS: EC_{50}: 1.2 ± 0.6 μM and Emax: 90% ± 19% of KCl-induced contraction; Fig. 1A). Apparent affinity for the antagonist 4-DAMP was also similar between strains and consistent with an interaction with M3R (pA2 in DSS/C17S.L11/Lewis: 8.89/8.67/8.60; Fig. 1B).[12] When applied at a supramaximal concentration, carbachol induced a rapid contractile response that peaked above 225% of the KCl response, followed by a plateau of contraction that remained above 50% of the response (Fig. 1C). After a 90-minute washout period, carbachol-induced peak contractions were reduced similarly (60% of KCl) in tissues from all rat strains. However, the sustained phase of contractions was markedly less (8% of KCl) in tissues from C17S.L11 congenic rats or Lewis rats as compared with tissues from DSS rats (21% of KCl; $P < 0.001$; n = 15), suggesting reduced desensitization of M3R-T556M in DSS rats (Fig. 1, D and E). In contrast, when serotonin (1 mM) or KCl (120 mM) was used instead of carbachol for both pretreatment and treatment, the final contractile response was not reduced in C17S.L11 congenic vs DSS rats (Supplemental Table S2), suggesting a dysregulation specific for the M3R pathway.

In $Chrm3^{-/-}$ rats, contractile responses to carbachol were reduced by approximately 97% as compared with those in $Chrm3^{+/+}$, whereas responses to KCl were not significantly different between strains (not shown), indicating that the contractile effect of carbachol is predominantly M3R dependent in this model.[13] Considering that M1R, M2R, M4R, and M5R are identical between DSS and C17S.L11 (Supplemental Table S1A), the sole function-altering M3R-T556M change appeared to be responsible for resensitization.

Hypertensive etiology of M3R-T556M: from enhanced molecular signalling and reduced desensitization to increased production of a BP-elevating mediator

A peripheral effect of M3R as a vasodilator is thought to be mediated through the release of nitric oxide in the endothelium.[14] The central nervous system role of M3R[15] on BP was not suspected until our current work, despite that *Chrm3* is abundantly expressed in the brain (Fig. 3). How can the elevated signalling by M3R-T556M increase BP via a central nervous system/adrenal mechanism?

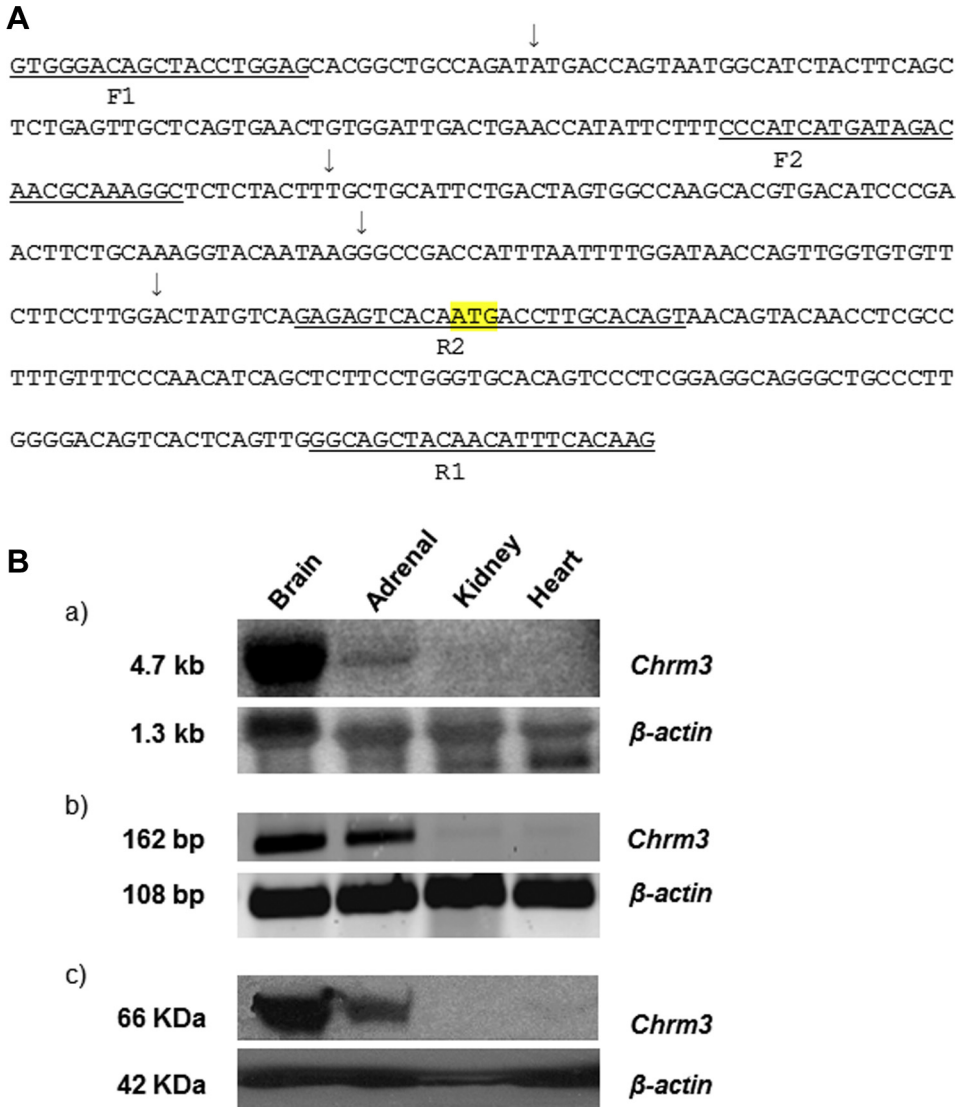

Figure 2. (**A**) Rat *Chrm3* 5′ region. The sequence is identical among Dahl salt-sensitive, C17S.L11, and Lewis rats. Down-pointing arrows indicate predicted starts of exons. The start codon is highlighted. (**B**) Expressions of *Chrm3* exhibiting adrenal and central nervous system specificity. (a) Northern blotting with *Chrm3*. A polymerase chain reactions (PCR)-generated *Chrm3*-specific probe using the F1/R1 combination was used to amplify brain cDNAs and was hybridized to total RNAs from whole organs. (b) *Chrm3* reverse transcriptase-PCR. Because the entire coding *Chrm3* domain resides in exon No. 5, primers F2/R2 are located in separate *Chrm3* exons to rule out amplifications from genomic DNAs. No products were seen from amplifying genomic DNAs. The PCR products from the brain and adrenal glands were sequence-verified. All rats were males. (c) Western blotting with M3 receptor antibodies. Antibodies (cat. No. AB9018-50UL) were purchased from Chemicon (Millipore). Standard protocols and *β-actin* controls for Northern blotting, reverse transcriptase-PCR, and Westerns were used. Organs were from Dahl salt-sensitive rats. C17S.L11 and Lewis exhibited the same patterns. bp, base pairs; kb, kilo base pairs; KDa, kiloDalton.

First, as a measure of autonomic activity, a spectral analysis of the HRV by telemetry[16] showed that the low-frequency component of HRV is decreased in congenic strain C17S.L11 (Fig. 3) carrying the wild-type M3R compared with DSS. Second, the increased signalling and sympathetic output of M3R-T556M in DSS caused a rise in the circulating epinephrine (Table 1A), a hormone, neurotransmitter, and vasoconstrictor.[17] The effect of epinephrine on hypertension is well recognized.[18]

An elevated M3R-T556M-mediated signalling may cause hypertension by enhancing the epinephrine production/release from the adrenal glands (Fig. 2). Because the circulating epinephrine is mostly generated from the adrenal medulla,[17] the adrenal glands may be responsible for the changed epinephrine production/release. Indeed, epinephrine released from the isolated adrenal glands of C17S.L11 congenic rats *ex vivo* is significantly lower than that of DSS rats (Table 1B). This result suggests that adrenal epinephrogenesis is different due to the differing *Chrm3* alleles. The M3R-selective antagonist, 4-DAMP, interfered with catecholamine measurements and could not be used in this assay. In contrast to epinephrine, other hypertensive agents of neuronal or adrenal origins such as norepinephrine, vasopressin, and aldosterone in the circulation (Table 1A) were not influenced by M3R-T556M.

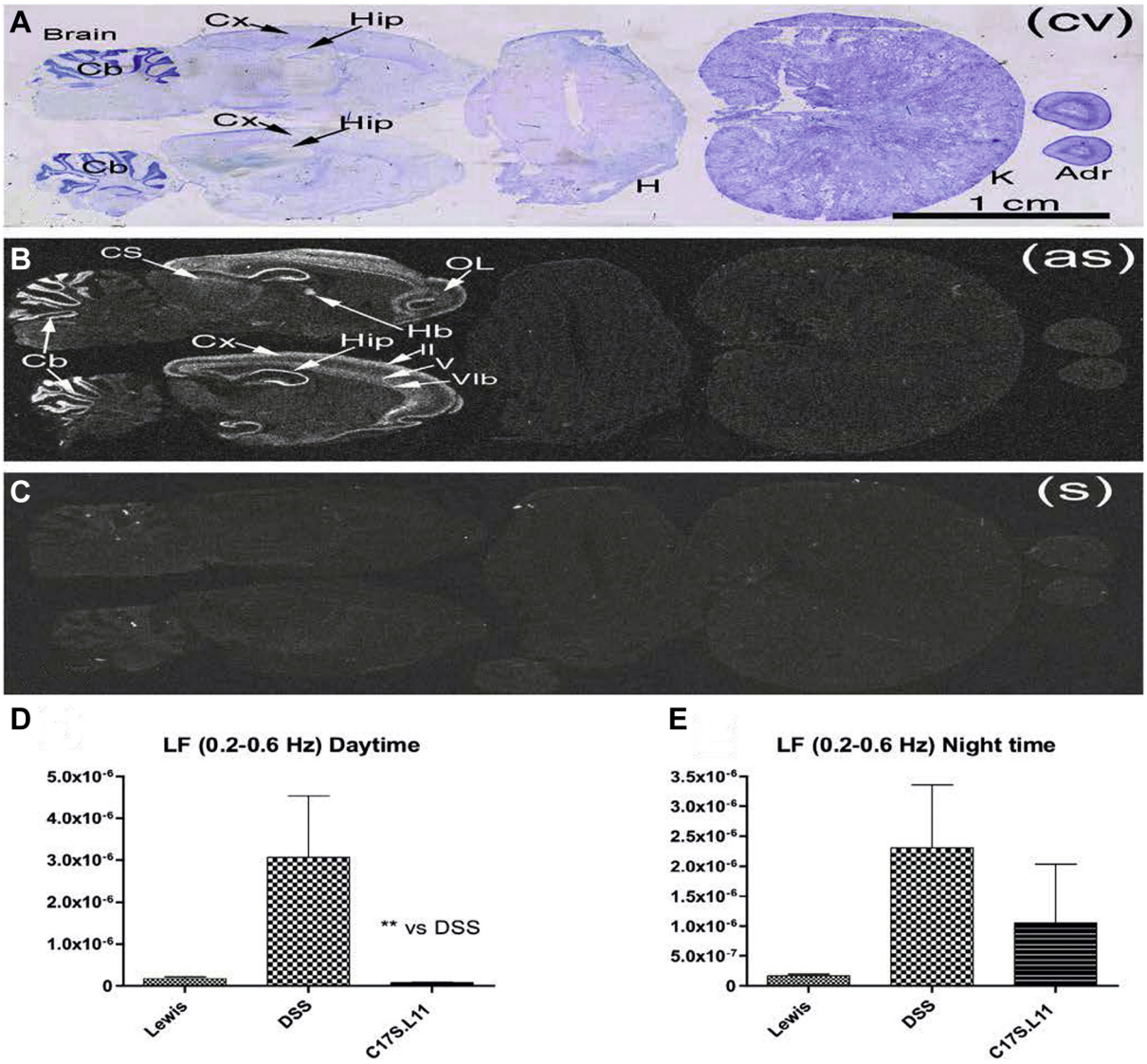

Figure 3. *In situ* hybridization of *Chrm3* and spectral analysis. (**A**) Cryostat sections through the adult rat brain, heart, kidney, and adrenal gland stained with cresyl violet (CV). (**B**) Antisense (as) *Chrm3* hybridization. *Chrm3* is expressed in brain regions, such as the cerebellum (Cb), cerebral cortex (Cx) layers II, V and VIb, colliculus superior (Cs), habenula (Hb), hippocampus (Hip), and olfactory lobe (OL). No detectable *Chrm3* expression is the heart (H), kidneys (K), and adrenal glands (Adr). (**C**) Controls hybridized with the *Chrm3* sense (S) probe. Pulse interval (PI) values (*Y* axis) were extracted from a 60-minute pressure waveform recorded at a rate of 2 kHz between (**D**) 8-10 AM daytime and (**E**) 7-9 PM nighttime. Short-term heart rate variability (*Y* axis) was examined in conscious, unrestrained rats instrumented for intra-arterial blood pressure monitoring by telemetry (Data Sciences Inc). Spectral analysis was performed using Dataquest A.R.T. software (Data Sciences Inc) in the low frequency (LF; 0.2-0.6 Hz) time domain associated with sympathetic activation.[9] All data are expressed as mean ± standard error of the mean. Statistical differences were analysed by a Kuskall-Wallis test followed by Dunnett's multiple comparison post-test. n = 5-8/strain.

The M3R signalling is required for regulating BP as a part of polygenicity

DSS *Chrm3* alleles enhance the signalling and resensitization of M3R and adrenal epinephrogenesis associated with raising BP.[6] This biological insight was possible because DSS rats have lost the buffering capacity for controlling BP fluctuations.[19] When BP-raising *Chrm3* alleles were introgressed into the Lewis resistant background containing a hypertension suppressor, BP could not be changed.[4] Thus, the following function studies on BP were conducted in the DSS genetic background.

A functional M3R is dispensable for embryogenesis. Removing it lowers BP (Fig. 4). The congenic knock-in of the chromosome segment containing the wild-type *Chrm3* allele (Supplemental Table S1A) alone weakens the receptor signalling[6] and resensitization (Fig. 1), and diminishes BP to a less degree than the *Chrm3* nulls (Fig. 4).

The integrity of the first extracellular loop is not critical for regulating BP by M3R

Along with generating a 5-bp-deletion founder (Fig. 5, A and B), we created another founder with an in-frame deletion

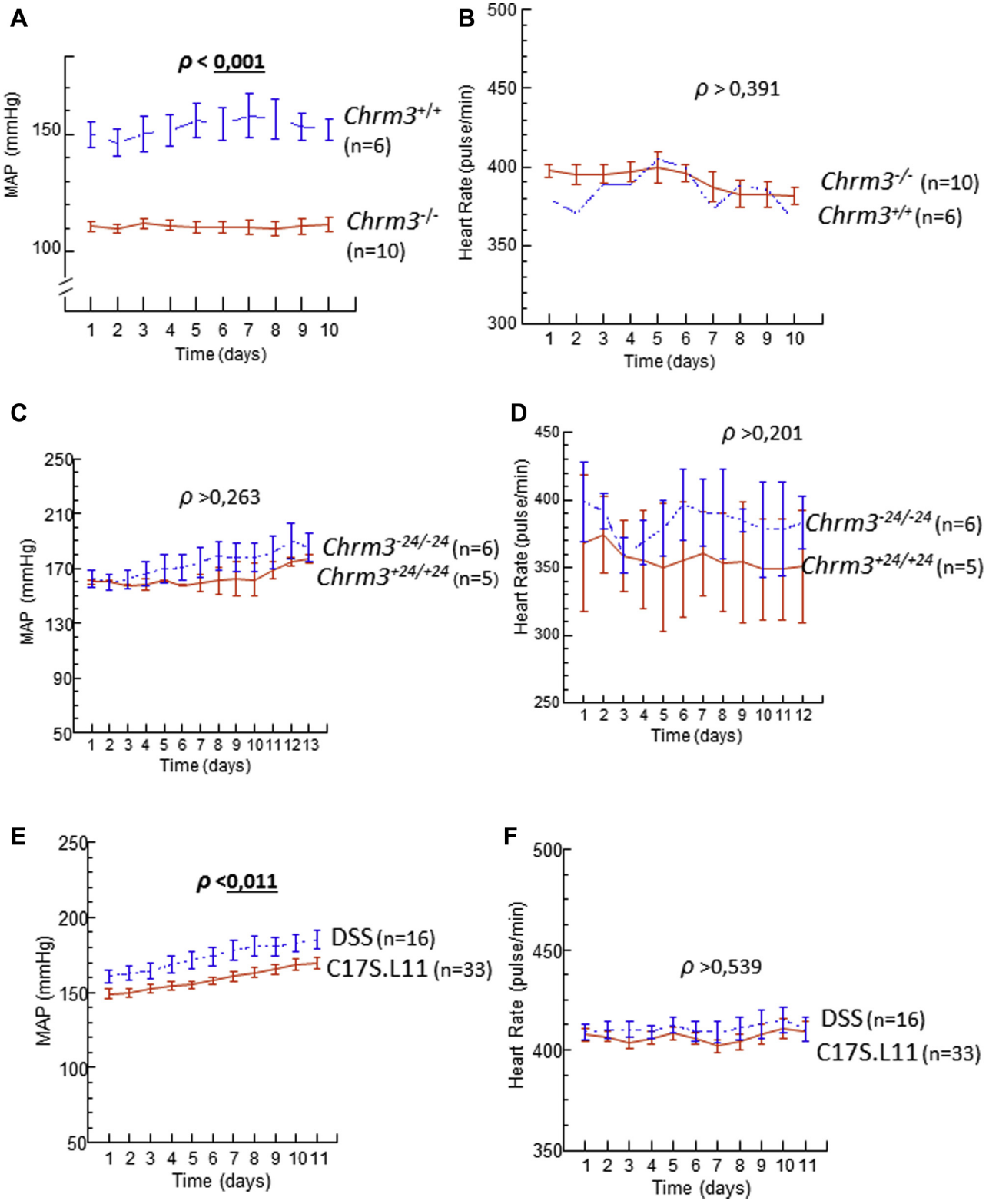

Figure 4. Blood pressures of "Dahl salt-sensitive (DSS)" rats with coding modifications, DSS, and C17S.L11. (**A**, **C**, **E**) Mean arterial pressure (MAP) comparisons as measured by telemetry in males on high-salt (2% NaCl) diet. The results of females on high salt and pooled females and males on low salt as their MAPs on low-salt (0.2% NaCl) diet are given in Supplemental Table S4. *p*, analysis of variance. Their diastolic and systolic pressures are consistent as their MAPs (Supplemental Table S4). (**B**, **D**, **F**) Heart rates are not different. Vasodilation to acetylcholine is impaired in *Chrm3*$^{-/-}$ in cerebral and mesenteric arteries (Supplemental Table S4).

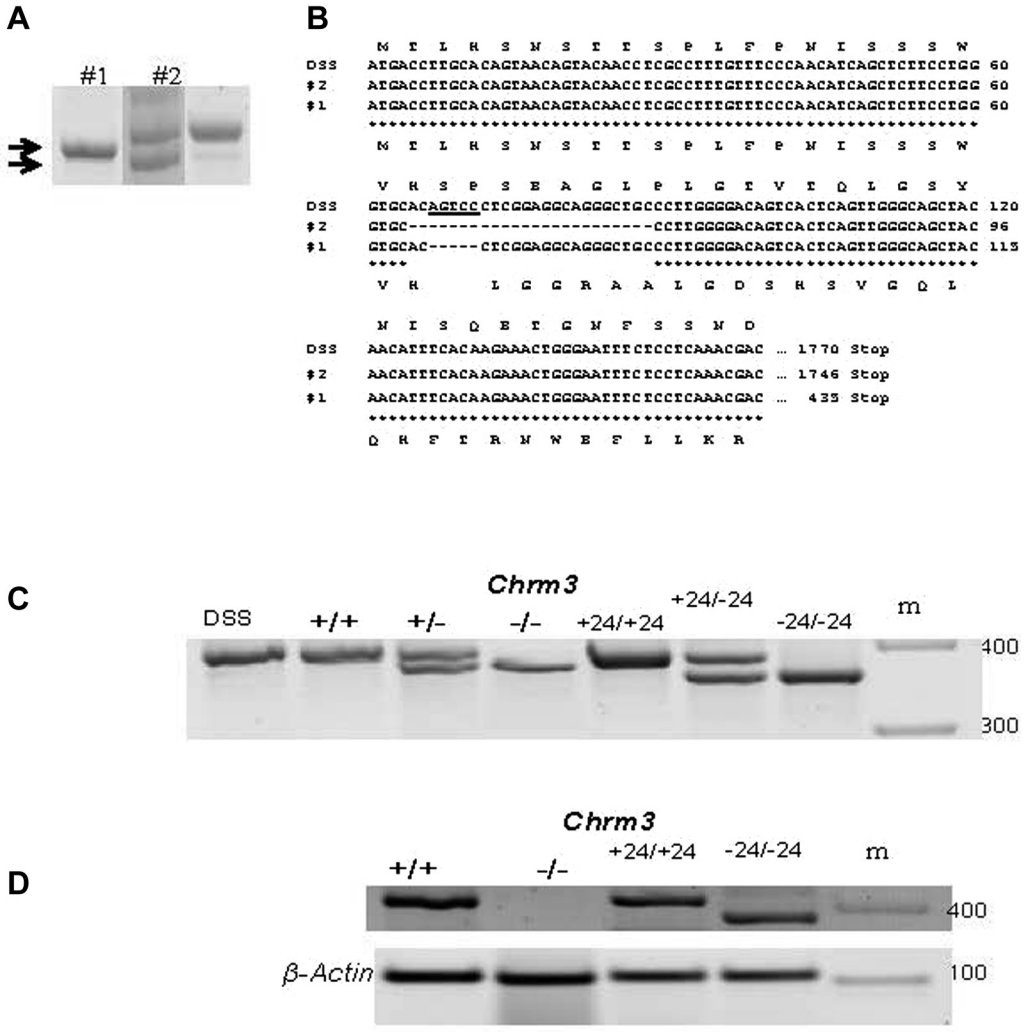

Figure 5. Generation of 2 founders in modifying the *Chrm3* coding domains in Dahl salt-sensitive rats by the zinc finger nuclease technology. Polymerase chain reactions (PCR) primers used for screening for knockout pups are forward 5′-CACGTAATGTAAGCTGCCCA-3′; reverse 5′-ACCTTGAAGGCCACAATGAC-3′. Rats # 1 and #2 contain an out-of-frame and in-frame *Chrm3* knockout, respectively. (**A**) Rat #1 contains a 5-bp deletion that causes the formation of a premature stop codon at nucleotide #435, truncating muscarinic cholinergic receptor 3. Rat #2 carries a 24-bp internal deletion that is in frame. Their sequences are given in (**B**) from the founder rats #1 and # 2. Dotted lines indicate the nucleotides deleted. Thick arrows on the left panel indicate the bands of interesting DNAs, which were excised and sequenced. The sequence underlined is the zinc finger nuclease cutting site. (**C**) Genotyping genomic DNAs showing the size polymorphisms of the 5-bp deletion in $Chrm3^{-/-}$ and 24-bp deletion in $Chrm3^{-24/-24}$, respectively, by PCR. (**D**) From founder #1, $Chrm3^{-/-}$ and $Chrm3^{+/+}$ have been generated and confirmed by reverse transcriptase-PCR using the PCR primers (forward 5′-GTGGGACAGCTACCTGGAGC-3′ and reverse 5′-CTTGTGAAATGTTGTAGCTGCC-3′), and specifically amplify *Chrm3* expressed in adrenals, because they are located in 2 separate *Chrm3* exons (Fig. 2). The PCR products of 400 bp were sequence-verified to be *Chrm3* (data not shown). −/− and +/+ indicate 3 $Chrm3^{-/-}$ knockout rats and 3 $Chrm^{+/+}$ non-knockout littermates, respectively. −24/−24 and +24/+24 are littermates originating from founder #2, lack and carry 24 bp, respectively. m indicates marker.

of 24 bp/8 amino acids in the *Chrm3* codons, designated as $Chrm3^{-24/-24}$. The deleted M3R section is located in the first extracellular loop (ie, o1), which is absent from other M receptors (Supplemental Table S3) and involved in the cell surface localization.[20] $Chrm3^{-24/-24}$ may provide a unique insight into a structure-function relationship for M3R on BP when M3R is modified, not abolished, because only in rare cases a lack a functional M3R is known in humans.[13] In contrast to $Chrm3^{-/-}$ and the C17S.L11 congenic strain, deleting 8 amino acids from M3R in $Chrm3^{-24/-24}$ (Fig. 5) has no effect on BP as compared with $Chrm3^{+24/+24}$ (Fig. 4, Supplemental Table S4). This indicates that the intactness of the first M3R extracellular loop is not required for M3R to control BP.

Discussion

Major findings from this work are as follows: (1) *Chrm3* bears a lone function-changing missense mutation that enhances the M3R-specific resensitization. (2) The *Chrm3* gene dosage is irrelevant to the magnitude of BP elevation. (3) Individual M3R structure domains have distinct functional roles in regulating BP and provide a function-based diagnosis predicated on the importance of *CHRM3* mutations whatever

their prevalence is in human populations. (4) The prohypertensive *CHRM3* mutation is associated with enhanced adrenal epinephrine output.

M3R signalling in hypertension pathogenesis independent of the *Chrm3* gene dose

Although it is yet to be established that the lone missense T1776C mutation of *Chrm3* (Supplemental Table S1) can directly elevate BP, the degree of M3R signalling correlates with the BP level. Using the BP of DSS rats as the reference point, when the signalling was unaffected in $Chrm3^{-24/-24}$, BP was not impacted. When the M3R signalling was diminished by the congenic knock-in of C17S.L11 (Supplemental Table S1), a slight reduction in BP was seen. A complete elimination of the M3R signalling in $Chrm3^{-/-}$ caused a greater reduction in BP (Fig. 4, Supplemental Table S4). Thus, the *Chrm3* gene dose is not a determining factor, because 1 copy of the functional M3R in $Chrm3^{-/+}$ has the same effect on BP as 2 copies in $Chrm3^{+/+}$.[6]

Human BP signals have been marked mostly by noncoding variants,[1] giving them special statistical powers in GWASs. However, what roles they play in the BP biology remains elusive. Based on the *C17QTL1*/*Chrm3* paradigm, clearly it is the M3R function domain, not any of the noncoding variants, that biologically determines the hypertension pathogenesis. In $Chrm3^{-/-}$ and $Chrm3^{+24/24}$, none of the 1096 noncoding *Chrm3* variants was touched, yet BP changes, or a lack of them, completely depend on the integrity of the M3R-mediated signalling. Thus, the central mechanistic determinant of *C17QTL1*/*Chrm3* on BP originates from the functional M3R signalling.

A pathway underlies the QTL functionality

Within the confines of a complex, polygenic, and quantitative trait, the M3R-specialized signalling reinforces the concept that a pathway causing the hypertension pathogenesis is the function basis for a QTL such as *C17QTL1* to influence BP. Thus, *C17QTL1*/*Chrm3* as a QTL is fundamentally equal to 1 gene,[21] not a collection of genes. None of the remaining 3 genes (Supplemental Table S1) in the *C17QTL1*-residing interval bears a function-altering variant either impacting on its structure or quantity.

C17QTL1/*Chrm3*, by itself, "indirectly" affects BP (Fig. 4), despite that BP "physiology" genes such as those involved in the epinephrine synthesis are unaltered. Exactly because etiology genes such as *Chrm3*, not physiology genes (eg, epinephrine-synthesis genes), are causal to hypertension[22] or BP variations[1] and functionally altered such as *Chrm3* (Figs. 1 and 4), the etiology genes are critical in the pathogenesis of polygenic hypertension.

M3R as a potential target for hypertension

To date, M3R has not been recognized as a potential therapeutic target for hypertension.[23] M3R is a target for etiology-based therapy causal to hypertension and is at the forefront of genetics-to-drug therapy discovery. This process involves (1) to detect a QTL GWAS-marked mostly by a noncoding variant with unknown function;[1] (2) to identify a gene candidate; (3) to prove a variant's function; (4) to prove a gene causation to BP and directionality of hypertension pathogenesis; and (5) to develop a drug against/for the product for a clinical proof of concept. So far, except for M3R, no target has passed stage (1). This appears despite potential side effects[23] similar to all drugs, due to a lack of specificity and gene function pleiotropy.

Structure-function characterization of M3R

In the functional framework of M3R signalling as being prohypertensive, our current structure-function study has revealed that the last M3R intracellular domain is important for receptor trafficking[6] and resensitization (Fig. 1). Its first extracellular loop is nonessential for M3R's control on BP (Fig. 4). This starting point of characterization in the M3R structure-function relationships has paved the way for a comprehensive investigation of individual M3R substructures in their distinct roles in BP modulations. Consequently, it will help us identify specific susceptibility sites within the overall M3R structure involved in regulating BP. In doing so, it may facilitate functional identifications of potential structural mutations that render hypertension susceptible in certain humans.

M3R-mediated signalling to BP in the DSS model

$Chrm3^{-/-}$ DSS rats (Supplemental Table S4) and $Chrm3^{-/-}$ mice of 129SvEv/CF-1 derivative[24] show a selective decrease in M3R-dependent vasodilation. In spite of it, elevating M3R activities (Figs. 1 and 3) should cause hypertension, not hypotension that the DSS-based *Chrm3* -null rats have verified. Thus, the hypertension pathophysiology directed by M3R is largely dissociated from the M3R-mediated vasorelaxation. However, the absence of M3R does not mean an endothelial dysfunction, only that the M3R-mediated pathway is inactivated. Many M3R-independent pathways in the endothelium remain active and likely important for BP regulation. Because BP decreases *in vivo* in *Chrm3* nulls (Fig. 4), even if endothelium-derived acetylcholine could contribute to endothelium-dependent dilation, it is likely to be compensated *in vivo* by other mechanisms such as improved renal and cardiac activities.[6] A cautionary note is that a cause-effect relationship was not established between BP and a renal or cardiac function, one way or the other.

We propose a role for accelerated M3R resensitization leading to enhanced signalling and resulting in an increase in epinephrine release and BP elevation (Table 1). Caveats to this interpretation are that they were obtained when the M3R signalling and trafficking are altered, not eliminated. When comparing sympathetic activities of $Chrm3^{-/-}$ with those of $Chrm3^{+/+}$, wide fluctuations are seen (data not shown). The epinephrine level in the circulation and secreted into the cultured medium in $Chrm3^{-/-}$ also showed wide fluctuations among individual rats of the same genotype under similar environmental conditions (data not shown).

These results suggest that the M3R pathway may affect not only epinephrine levels, but also stabilize the internal epinephrine range. When the M3R pathway is absent, other pathways controlling the epinephrine secretion/production into the circulation and in adrenal epinephrogenesis may compensate.

In conclusion, individualizing (equivalent to inbreeding) polygenic properties can elucidate and distinguish one

person's pathogenic mechanisms of hypertension from the heterogeneity of essential hypertension worldwide. After all, treating persons with hypertension is an individual-administered task, not a collective abstraction in lowering BP. Clinically inhibiting the M3R-mediated signalling with existing drugs may provide a novel treatment to reduce essential hypertension without tachycardia, especially in patients with an M3R susceptibility.

Funding Sources

The work was supported by grants from the Canadian Institutes for Health Research (CIHR) and from the Kidney Foundation of Canada to A.Y. Deng. J.-É. Huot-Marchard was a recipient of a Banting and Best doctoral fellowship from CIHR.

Disclosures

The authors have no conflicts of interest to disclose.

References

1. Warren HR, Evangelou E, Cabrera CP, et al. Genome-wide association analysis identifies novel blood pressure loci and offers biological insights into cardiovascular risk. Nat Genet 2017;49:403.
2. Munroe PB, Barnes MR, Caulfield MJ. Advances in blood pressure genomics. Circ Res 2013;112:1365-79.
3. Chauvet C, Crespo K, Menard A, Roy J, Deng AY. Modularization and epistatic hierarchy determine homeostatic actions of multiple blood pressure quantitative trait loci. Hum Mol Genet 2013;22:4451-9.
4. Crespo K, Menard A, Deng AY. Hypertension suppression, not a cumulative thrust of quantitative trait loci, predisposes blood pressure homeostasis to normotension. Circ Cardiovasc Genet 2015;8:610-7.
5. Deng AY. Genetic mechanisms of polygenic hypertension: fundamental insights from experimental models. J Hypertens 2015;33:669-80.
6. Deng AY, deBlois D, Laporte SA, et al. Novel pathogenesis of hypertension and diastolic dysfunction caused by muscarinic cholinergic 3 receptor signalling. Hypertension 2018;72:755-64.
7. Cowley AW. Chrm3 gene and M3 muscarinic receptors contribute to salt-sensitive hypertension, but now a physiological puzzle. Hypertension 2018;72:588-91.
8. Braverman AS, Tibb AS, Ruggieri MR. M2 and M3 muscarinic receptor activation of urinary bladder contractile signal transduction. I. Normal rat bladder. J Pharmacol Exp Therapeut 2006;316:869-74.
9. Janssen BJ, Oosting J, Slaaf DW, Persson PB, Struijker-Boudier HA. Hemodynamic basis of oscillations in systemic arterial pressure in conscious rats. Am J Physiol 1995;269:H62-71.
10. Bibeau K, Battista MC, Houde V, Brochu M. Fetal adrenal gland alterations in a rat model of adverse intrauterine environment. Am J Physiol Regul Integr Comp Physiol 2010;298:R899-911.
11. Forsythe SM, Kogut PC, McConville JF, et al. Structure and transcription of the human m3 muscarinic receptor gene. Am J Respiratory Cell Mol Biol 2002;26:298-305.
12. Krichevsky VP, Pagala MK, Vaydovsky I, Damer V, Wise GJ. Function of M3 muscarinic receptors in the rat urinary bladder following partial outlet obstruction. J Urology 1999;161:1644-50.
13. Weber S, Thiele H, Mir S, et al. Muscarinic acetylcholine receptor M3 mutation causes urinary bladder disease and a prune-belly-like syndrome. Am J Hum Genet 2011;89:668-74.
14. Bruning TA, Hendriks MG, Chang PC, Kuypers EA, van Zwieten PA. In vivo characterization of vasodilating muscarinic-receptor subtypes in humans. Circ Res 1994;74:912-9.
15. Bonner TI, Buckley NJ, Young AC, Brann MR. Identification of a family of muscarinic acetylcholine receptor genes. Science 1987;237:527-32.
16. Thireau J, Zhang BL, Poisson D, Babuty D. Heart rate variability in mice: a theoretical and practical guide. Exp Physiol 2008;93:83-94.
17. Berecek KH, Brody MJ. Evidence for a neurotransmitter role for epinephrine derived from the adrenal medulla. Am J Physiol 1982;242:H593-601.
18. Floras JS. Epinephrine and the genesis of hypertension. Hypertension 1992;19:1-18.
19. Charron S, lambert R, Eliopoulos V, et al. A loss of genome buffering capacity of Dahl salt-sensitive model to modulate blood pressure as a cause of hypertension. Hum Mol Genet 2005;14:3877-84.
20. Kruse AC, Hu J, Pan AC, et al. Structure and dynamics of the M3 muscarinic acetylcholine receptor. Nature 2012;482:552-6.
21. Deng AY. Positional cloning of quantitative trait loci for blood pressure: how close are we?: a critical perspective. Hypertension 2007;49:740-7.
22. Consortium W. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007;447:661-78.
23. Hegde SS. Muscarinic receptors in the bladder: from basic research to therapeutics. Br J Pharmacol 2006;147(suppl 2):S80-7.
24. Gericke A, Sniatecki JJ, Mayer VGA, et al. Role of M1, M3, and M5 muscarinic acetylcholine receptors in cholinergic dilation of small arteries studied with gene-targeted mice. Am J Physiol Heart Circ Physiol 2011;300:H1602-8.

Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at www.onlinecjc.ca and at https://doi.org/10.1016/j.cjca.2018.12.029.