

Male contraception via simultaneous knockout of α_{1A} -adrenoceptors and P2X1-purinoceptors in mice

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Therapeutic targets for male contraception are associated with numerous problems due to their focus on disrupting spermatogenesis or hormonal mechanisms to produce dysfunctional sperm. Here we describe the dual genetic deletion of α_{1A} -adrenergic G protein-coupled receptors (adrenoceptors) and P2X1-purinoceptor ligand gated ion channels in male mice, thereby blocking sympathetically mediated sperm transport through the vas deferens during the emission phase of ejaculation. This modification produced 100% infertility without effects on sexual behavior or function. Sperm taken from the cauda epididymides of double knockout mice were microscopically normal and motile. Furthermore, double knockout sperm were capable of producing normal offspring following intracytoplasmic sperm injection into wild-type ova and implantation of the fertilized eggs into foster mothers. Blood pressure and baroreflex function was reduced in double knockout mice, but no more than single knockout of α_{1A} -adrenoceptors alone. These results suggest that this autonomic method of male contraception appears free of major physiological and behavioral side effects. In addition, they provide conclusive proof of concept that pharmacological antagonism of the P2X1-purinoceptor and α_{1A} -adrenoceptor provides a safe and effective therapeutic target for a nonhormonal, readily reversible male contraceptive.

sympathetic neurotransmission | noradrenaline | ATP

Development of a male contraceptive is a major medical challenge with numerous barriers in its path. Historically, most therapeutic targets have been hormonal and therefore likely to have intolerable sexual, behavioral, physiological, and psychological side effects. More recently, efforts have concentrated on spermatogenic mechanisms that can render sperm dysfunctional and therefore incapable of fertilization (1, 2). These reports identified a number of problems associated with such strategies, including (i) the enormous number of spermatozoa produced by men (~1,000 per s) (1); (ii) the difficulty in suppressing all of these many sperm compared with only one ovum per female (1); (iii) concern that affecting production of cells in the germ line may alter genetics in offspring (1); (iv) finding a molecule that is able to cross the blood–testis barrier (1); and (v) being able to readily reverse this process (2). These challenges are in addition to the usual issues faced in the development of pharmaceuticals such as large financial expenditure, regulatory hurdles, preference for oral availability, and unexpected adverse events.

An alternate mechanism is to block sperm transport. During ejaculation, sperm is transported from its storage site in the cauda epididymis to the urethra via the vas deferens (3, 4). This process of propulsion of sperm into the ejaculate is essential for males to produce an adequate sperm count for fertilization. Sympathetically innervated smooth muscle cells surround the vas deferens and contract in response to ATP and noradrenaline due to activation of P2X1-purinoceptor ligand-gated ion channels and α_{1A} -adrenergic G protein-coupled receptors (adrenoceptors), respectively (5–7). Consequently, conjoint pharmacological

antagonism of these receptors has been shown to inhibit nerve-mediated contraction of the vas deferens in isolated tissue experiments. This suggests a potential mechanism for male contraception, but critical studies in the context of whole-organism physiology are yet to be performed.

Administration of the selective α_{1A} -adrenoceptor antagonist tamsulosin (8) to male rats decreases the number of sperm in the ejaculate as well as reducing the number of embryos per pregnancy; however, full infertility has not been achieved. Furthermore, whereas genetic deletion of α_{1A} -adrenoceptors (9) or P2X1-purinoceptors (10) impairs fertility in mice, the observed percentage of infertile male mice was only 50% for α_{1A} -adrenoceptors (9) and 86% for P2X1-purinoceptors (10). Such impairment of fertility suggests that these receptor targets are indeed important for reproduction. However, male infertility would have to be 100% in preclinical studies for a novel therapeutic target for male contraception to be considered viable in humans.

It has been proposed that simultaneous blockade of both of these receptors may produce adequate inhibition of sperm transport for male contraception (11); however, this strategy is also likely to fail if compensatory mechanisms arise to counteract α -adrenoceptor blockade, as has been observed following genetic deletion of P2X1-purinoceptors in male mice (10). Furthermore, it is unknown whether dual pharmacological blockade of receptors can be exploited as a safe and effective male contraceptive, because the distribution of P2X1-purinoceptors and α_{1A} -adrenoceptors is widespread and they act in concert to control the

Significance

The search for a viable male contraceptive target has been a medical challenge for many years. Most strategies have focused on hormonal or germ-line strategies to produce dysfunctional sperm that are incapable of fertilization. The problem with such approaches is that they have intolerable side effects such as affecting male sexual activity or causing long-term irreversible effects on fertility. In addition, some strategies may transmit detrimental changes to future offspring. This manuscript describes a male contraceptive target within the autonomic nervous system, which would not affect the long-term viability of sperm nor the sexual or general health of males. In addition, due to the nature of the target, the contraceptive has the potential to be orally administered.

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diameter of resistance arteries and thus blood pressure homeostasis (12, 13). This study demonstrates that dual knockout of α_{1A} -adrenoceptors and P2X1-purinoreceptors renders male mice infertile without compromising physiology and behavior.

Results

Generation of Double Knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ Mice. First-generation mice were produced by crossing female α_{1A} -adrenoceptor knockout ($^{-/-}$) mice with male P2X1-purinoreceptor heterozygous ($^{+/-}$) mice. Double knockout mice were then generated at a Mendelian frequency of 1:16 in the second generation by breeding female $\alpha_{1A}^{+/-}$ /P2X1 $^{+/-}$ with male $\alpha_{1A}^{+/-}$ /P2X1 $^{+/-}$ mice (Fig. 1A). Thereafter, because double knockout female $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice were fertile, they were crossed with double heterozygous male $\alpha_{1A}^{+/-}$ /P2X1 $^{+/-}$ mice (Fig. 1A), producing an average litter size of 6.6. The resulting double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice occurred at an expected Mendelian genotype frequency of 1:4 (sex ratio 1.11 male to 1 female; $n = 240$), indicating that there is no selective fertilization or mortality in utero and that female fertility is unaffected by this simultaneous gene deletion.

Confirmation of the deficiency of both the α_{1A} -adrenoceptor and P2X1-purinoreceptor in double knockout mutant $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice was obtained at the genomic and protein levels. PCR genotyping as previously described for α_{1A} -adrenoceptor knockout $\alpha_{1A}^{-/-}$ (13) and P2X1-purinoreceptor knockout P2X1 $^{-/-}$ (10) mice indicated that mRNA for either gene is not amplified in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 1B). A commercially available P2X1-purinoreceptor antibody (Alomone Labs) produced high levels of immunoreactivity in the smooth muscle layer of wild-type vas deferens (Fig. 2A), as previously reported (10). However, as observed in P2X1-purinoreceptor single knockout P2X1 $^{-/-}$ mice, no P2X1-purinoreceptor immunoreactivity was seen in vasa deferentia taken from double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 2B). Similarly, β -galactosidase staining of the Lac Z reporter gene that had been inserted at the $\alpha_{1A/C}$ gene translational start site of the α_{1A} -adrenoceptor gene in these knockout $\alpha_{1A}^{-/-}$ mice (13) showed positive staining in vasa deferentia from double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 2D), which was absent in vasa deferentia taken from wild-type mice (Fig. 2C). This was despite the continued presence of noradrenaline-containing nerve fibers within the smooth muscle of the vas deferens of double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 2E

A Breeding strategy

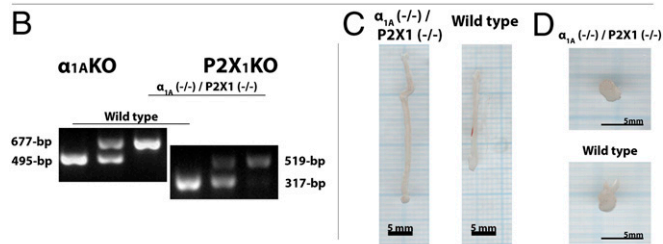
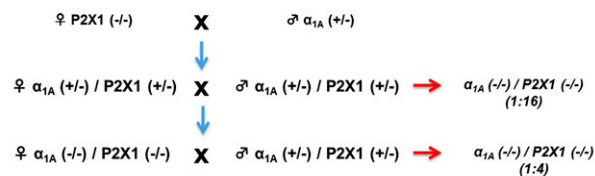


Fig. 1. Breeding and gross morphological effects of $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ double knockout in male mice. (A) Breeding strategy. (B) Genotyping by PCR. (C) Photos of vas deferens from wild-type and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice. (D) Photos of whole prostates from wild-type and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice.

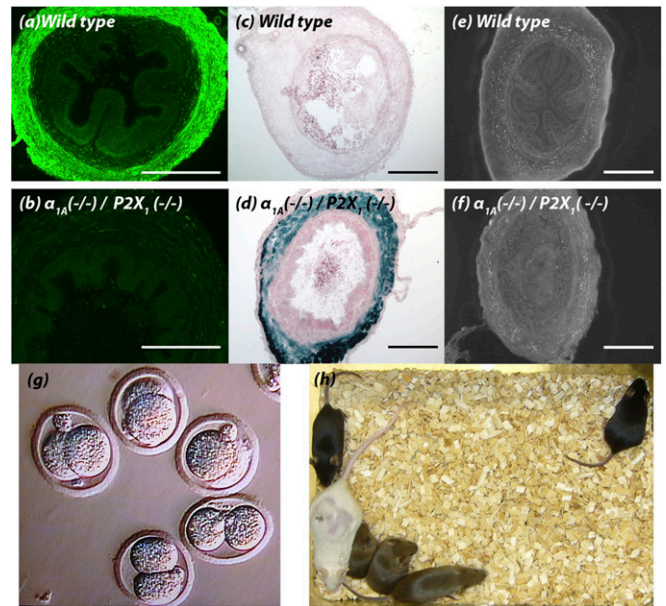


Fig. 2. Vas deferens histochemistry and sperm viability. P2X1-purinoreceptor immunohistochemistry of cross-sections of vas deferens from (A) wild-type male mice ($n = 3$) and (B) double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice ($n = 3$). X-gal staining for β -galactosidase in cross-sections of vas deferens from (C) wild-type male mice ($n = 3$) and (D) double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice ($n = 3$). SPG histochemical fluorescence of cross-sections of vas deferens from (E) wild-type male mice ($n = 3$) and (F) double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice ($n = 3$). (Scale bars, 100 μ m.) (G) Fertilization of wild-type ova in vitro by intracytoplasmic injection of sperm extracted from cauda epididymides of male double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice; the photomicrograph was taken at 3 d postinjection. (H) Weanlings (black/agouti) 7 wk after implantation of fertilized ova into a foster mother (white).

and F), as demonstrated by sucrose potassium glyoxylic acid (SPG) histochemistry (14).

Sexual Behavior. In contrast to the lack of effect of this simultaneous gene deletion on female fertility, when double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ males were mated with wild-type $\alpha_{1A}^{+/+}$ /P2X1 $^{+/+}$ females, no pregnancies resulted [$n = 29$ matings of 17 double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice]. This was despite coitus having taken place to the point of ejaculation during 28/29 matings (Movie S1). In addition, when paired with wild-type $\alpha_{1A}^{+/+}$ /P2X1 $^{+/+}$ female mice, all double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice exhibited normal sexual behavior for this background strain of mouse in terms of pre-coital chasing, sniffing, mounting of mates, pelvic thrusting with appropriate vigor, and frequency, followed by a period of postejaculation latency and loss of interest in the female mate (Movie S1).

Fertility Evaluation. Vaginal coagulum plugs that are normally formed in rodents after copulation were not observed in any wild-type females where copulation to the point of ejaculation had been confirmed via video surveillance following matings with male double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice ($n = 29$). The male infertility in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice did not result from problems with spermatogenesis or sperm quality, because sperm were recovered from the cauda epididymides of wild-type and double knockout mice in similar numbers and with similar motility (Movies S2 and S3). Furthermore, sperm extracted from the cauda epididymides of double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice were able to fertilize wild-type ova in vitro following intracytoplasmic sperm injection [Fig. 2G; success rate at 3 d, $78.0 \pm 6.5\%$; $n = 140$ embryo injections of sperm from three double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$

($^{-/-}$) mice]. In addition, implantation of fertilized ova into wild-type α_{1A} ($^{+/+}$)/P2X1 ($^{+/+}$) foster mothers produced normal litters that survived until weaning (28 d after birth) (Fig. 2H; $n = 3$).

Vas Deferens Pharmacology. As has been observed previously (9, 10), both α_{1A} ($^{-/-}$) and P2X1 ($^{-/-}$) single knockout mice have compromised vas deferens contractility following electrical nerve stimulation (Fig. 3 A and B), and these previously observed effects were additive in double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice (Fig. 3 A and B). Interestingly, a residual contractile response to electrical field stimulation remained in double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice that was abolished by the noradrenergic neuron-blocking drug guanethidine (10 μ M) and inhibited by the selective α_{1D} -adrenoceptor antagonist BMY 7378 (100 nM) (Fig. 3C).

In isolated vasa deferentia taken from wild-type α_{1A} ($^{+/+}$)/P2X1 ($^{+/+}$) mice, the purinoceptor agonist ATP and the adrenoceptor agonist noradrenaline evoked concentration-dependent contractions, as previously described (9, 10) (Fig. 3 E and G). Noradrenaline also elicited concentration-dependent contractile responses in vasa deferentia taken from P2X1 ($^{-/-}$) knockout and to a much lesser extent in double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice but not α_{1A} ($^{-/-}$) single knockout mice (Fig. 3E). As was observed previously in single P2X1 ($^{-/-}$) knockout mice (10), noradrenaline was more potent in vasa deferentia taken from P2X1 ($^{-/-}$) knockout ($-\log$ effective concentration required to produce 50% of maximal response: pEC_{50} , 6.11 ± 0.14) than wild-type mice (pEC_{50} , 4.78 ± 0.06); however, the maximum responses produced were not different (Fig. 3E). In contrast, despite no change in the maximum contractile response mediated by KCl (80 mM; Fig. 3D), the maximum contractile response mediated by noradrenaline in double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice was only 24% of that observed in wild-type mice (Fig. 3E) and was inhibited by the selective α_{1D} -adrenoceptor antagonist BMY 7378 (100 nM; Fig. 3F; $P < 0.001$), as was the residual response to electrical field stimulation in double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice (Fig. 3C). Furthermore, expression of mRNA that encodes for the α_{1D} -adrenoceptor is up-regulated in vasa deferentia from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice (Fig. S1).

Similarly, ATP elicited concentration-dependent contractile responses in vasa deferentia taken from α_{1A} ($^{-/-}$) knockout mice (pEC_{50} , 3.90 ± 0.14) more potently than in wild-type mice (pEC_{50} , 2.50 ± 0.48) (Fig. 3G), whereas ATP did not produce contractile responses in vasa deferentia taken from P2X1 ($^{-/-}$) knockout mice (Fig. 3G). However, a small contractile response was observed to high concentrations of ATP in vasa deferentia taken from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice (Fig. 3G).

Vas Deferens Electrophysiology. Using intracellular electrophysiological techniques (15), vas deferens smooth muscle cells were characterized by resting membrane potential that was -65.2 ± 1.7 mV for tissue taken from wild-type ($n = 25$ impalements from 10 vasa deferentia) and -64.9 ± 1.7 for tissue taken from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) ($n = 19$ impalements from 8 vasa deferentia) mice, respectively. Spontaneous excitatory junction potentials (EJPs) (amplitude, 5.3 ± 0.5 mV; frequency, 14.3 ± 4.9 events per min) were recorded from greater than 80% of impalements of the wild-type vas deferens ($n = 20$ impalements from vasa deferentia from five mice) (Fig. 3H). In addition, exogenously applied ATP (1 mM) evoked electrical depolarizations in vasa deferentia taken from wild-type mice (Fig. 3I) with an amplitude $39 \pm 7\%$ ($n = 7$) of the depolarization evoked by 20 mM K^+ (Fig. 3J) and a rise time of 9 mV/s (Fig. 3I). In contrast, vasa deferentia from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice evoked smaller electrical depolarizations to ATP (1 mM) (Fig. 3I) that were only $14 \pm 3\%$ ($P < 0.05$ compared with wild type; $n = 4$) of that evoked by 20 mM K^+ (Fig. 3J), with a slower rise time of only 1 mV/s (Fig. 3I). In contrast to what has previously been described for P2X1 ($^{-/-}$) single knockout mice (10),

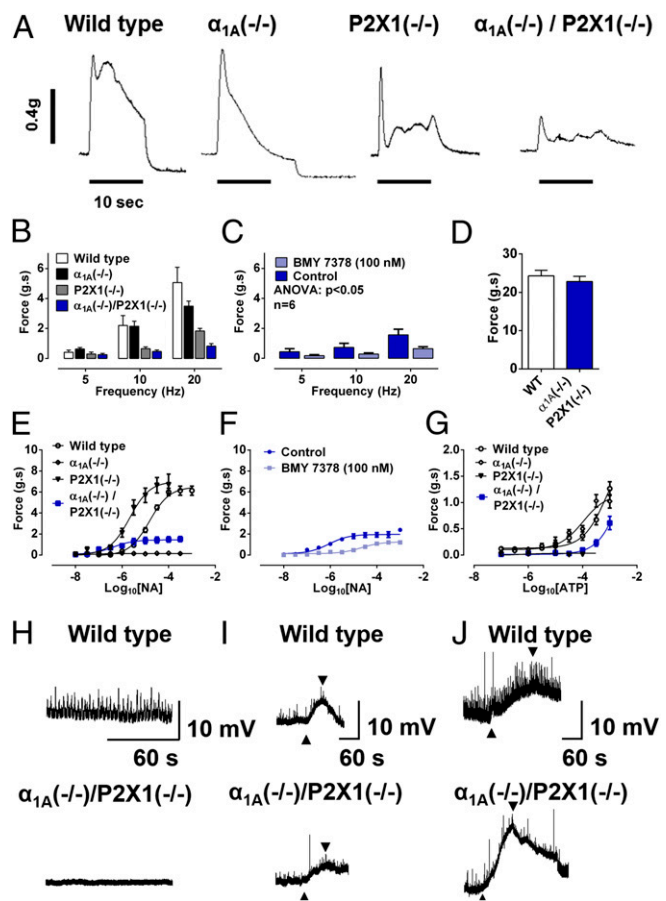


Fig. 3. Vas deferens contractility and electrical activity. (A) Typical recording of contractile responses to electrical field stimulation of isolated vasa deferentia taken from wild-type, α_{1A} ($^{-/-}$) single knockout, P2X1 ($^{-/-}$) single knockout, and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice. (B) Mean frequency–response curve to electrical field stimulation of isolated vasa deferentia taken from wild-type ($n = 7$), α_{1A} ($^{-/-}$) single knockout ($n = 10$), P2X1 ($^{-/-}$) single knockout ($n = 9$), and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) ($n = 21$) mice. (C) Mean frequency–response curve to electrical field stimulation of isolated vasa deferentia taken from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice in the absence and presence of BMY 7378 ($n = 7$). (D) Mean contractile response to 80 mM KCl of isolated vasa deferentia taken from wild-type ($n = 10$; open columns) and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) ($n = 21$; filled columns) mice. (E) Mean concentration–response curve to exogenous administration of noradrenaline (NA) in isolated vasa deferentia taken from wild-type ($n = 6$), α_{1A} ($^{-/-}$) single knockout ($n = 10$), P2X1 ($^{-/-}$) single knockout ($n = 10$), and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) ($n = 5$) mice. (F) Mean concentration–response curve to exogenous administration of noradrenaline in isolated vasa deferentia taken from double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice in the absence and presence of BMY 7378 ($n = 6$). (G) Mean concentration–response curve to exogenous administration of ATP in isolated vasa deferentia taken from wild-type ($n = 10$), α_{1A} ($^{-/-}$) single knockout ($n = 7$), P2X1 ($^{-/-}$) single knockout ($n = 4$), and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) ($n = 5$) mice. (B–G) Y-axes: Force (g.s) = integral of force (g) \times time (s). Error bars represent SEM. (H) Representative intracellular spontaneous electrical activity recordings of vas deferens smooth muscle from wild-type and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice. (I) Representative intracellular electrical responses to exogenous administration of ATP (upward arrow, 1 mM) of vas deferens smooth muscle from wild-type and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice. (J) Representative intracellular electrical responses to exogenous administration of K^+ (upward arrow, 20 mM) of vas deferens smooth muscle from wild-type and double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice. (I, J) Downward arrows signify washout.

spontaneous EJPs of similar frequency and amplitude to wild type were still detected in 3 of 19 impalements of vasa deferentia from four double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) mice.

Cardiovascular Function. Resting heart rate of double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice was not different from wild type when measured consciously using a tail cuff method (Fig. 4B). In contrast, there was a small decrease ($\sim 10\%$) in resting systolic arterial pressure when measured consciously using the tail cuff method (Fig. 4A). However, this decrease in arterial pressure was not different from the decrease in resting arterial pressure seen in single $\alpha_{1A}^{-/-}$ knockout mice (13) (Fig. 4A). In double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice, the pressor response to the selective α_{1A} -adrenoceptor agonist A61603 (0.2–2.0 $\mu\text{g}/\text{kg}$) was absent (Fig. 4C), as had been previously reported in single $\alpha_{1A}^{-/-}$ knockout mice (13). Consequently, A61603 (0.2–2.0 $\mu\text{g}/\text{kg}$) had only a minimal effect on heart rate (Fig. 4D). The subtype nonselective α_1 -adrenoceptor agonist phenylephrine (5–100 $\mu\text{g}/\text{kg}$) produced pressor responses in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice that were $\sim 60\%$ lower than those produced in wild-type mice (Fig. 4E), again similar to what has been reported in single $\alpha_{1A}^{-/-}$ knockout mice (14).

The baroreflex heart rate response to phenylephrine (5–100 $\mu\text{g}/\text{kg}$) was also altered in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 4F), as seen in single $\alpha_{1A}^{-/-}$ knockout mice (13). Cardiovascular responses to the muscarinic receptor antagonist atropine and the β -adrenoceptor agonist isoprenaline were not different in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice compared with wild type (Fig. 4 G and H), indicating that the baroreflex resetting that occurs in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice does not mask differences in pressor responsiveness or signaling in other systems.

Gross Anatomy. In nonvascular tissues, there were no observable differences between double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ and wild type (Table S1), with the exceptions of the vas deferens and seminal vesicles. The vas deferens was longer and heavier in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Fig. 1 C and E) and the seminal vesicles were heavier in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice (Table S1). The increased weight of seminal vesicles appeared to be due to engorgement with fluid, presumably caused by a loss of contractile function, as in pharmacological electrical field stimulation studies the contractile response of isolated seminal vesicles to 10-Hz stimulation was reduced by 47% [wild-type response, 0.25 ± 0.04 g, compared with double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ response, 0.13 ± 0.06 g; $P < 0.001$; $n = 12$ and 6, respectively]. A smaller 26% decrease in the contractile response to electrical field stimulation of the coagulating gland was also observed (at 10 Hz: wild-type response, 59.5 ± 7.3 mg, compared with double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ response, 44.2 ± 12.5 mg; $P < 0.001$; $n = 9$ and 6, respectively). The prostate gland (Fig. 1D and Table S1) and urinary bladder (Table S1) were of similar weight in wild-type and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice and appeared to function in a relatively normal fashion, with decreases of only 32% and 31%, respectively, in the contractile response to electrical field stimulation at 10 Hz (prostate responses: wild type, 0.13 ± 0.01 g, compared with double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$, 0.09 ± 0.01 g; $P < 0.001$; $n = 8$ and 14, respectively; bladder responses: wild type, 0.95 ± 0.14 g, compared with double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$, 0.66 ± 0.13 g; $P < 0.001$; $n = 12$ for each).

Discussion

Both $\alpha_{1A}^{-/-}$ and P2X1 $^{-/-}$ single knockout mice have compromised vas deferens contractility following electrical nerve stimulation (9, 10), and these previously observed effects are additive in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice. This explains why in all wild-type females mated with double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice no evidence of ejaculated sperm in the form of a vaginal plug was found. Thus, the infertility of double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ male mice observed in this study was not due to dysfunctional sperm but rather from a lack of sperm being ejaculated. Coagulum plugs form in rodents following copulation to occlude the vaginal

opening. They are formed by the catalytic action of type IV transglutaminase from the coagulating gland (16, 17) cross-linking with semenoclotin (seminal vesicle secretion 2 protein) from the seminal vesicles (18, 19). The lack of a coagulum plug following mating of double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice with wild-type females indicates a lack of transport of not only sperm but also secretions from the seminal vesicle, coagulating gland, and, to a lesser extent, the prostate gland. This absence of ejaculated sperm circumvents a major problem associated with other strategies for producing male contraception, which is that

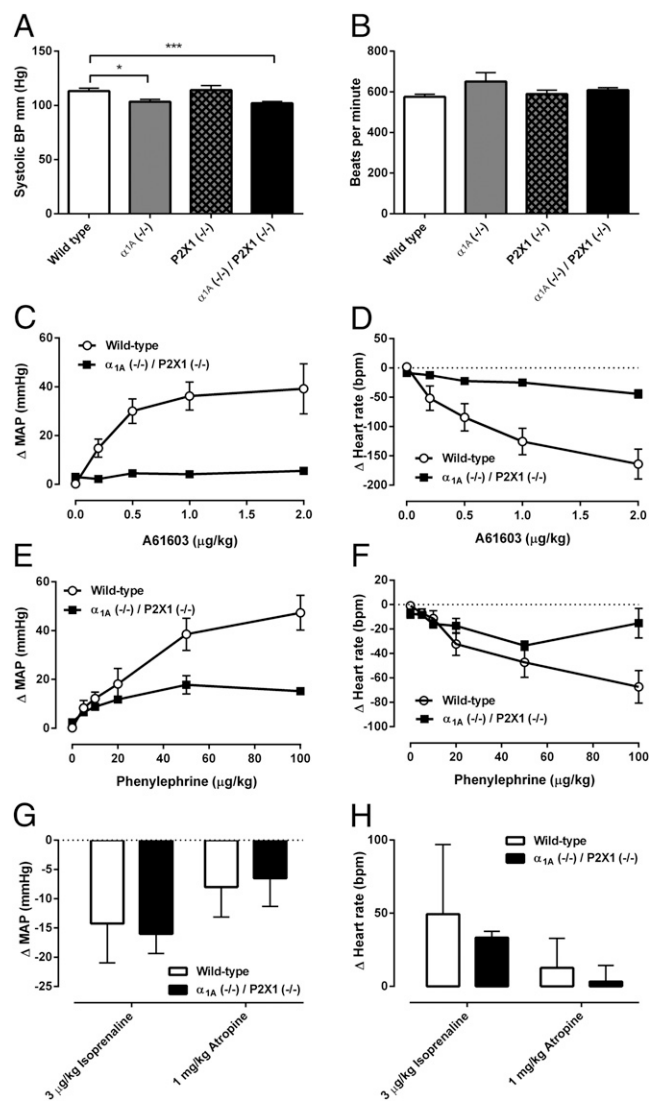


Fig. 4. Cardiovascular responses in double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ mice. (A) Resting systolic arterial blood pressure (BP) and (B) resting pulse rate as measured by the tail cuff method in conscious wild-type ($n = 8$), $\alpha_{1A}^{-/-}$ single knockout ($n = 11$), P2X1 $^{-/-}$ single knockout ($n = 11$), and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ ($n = 14$) male mice. Asterisks represent a significant difference, * $P < 0.05$, *** $P < 0.001$. Mean effect of A61603 (0.2–2.0 $\mu\text{g}/\text{kg}$) in wild-type ($n = 6$; open circles) and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ ($n = 5$; filled squares) male mice on (C) carotid artery pressure and (D) heart rate. bpm, beats per min; MAP, mean arterial pressure. Mean effect of phenylephrine (5–100 $\mu\text{g}/\text{kg}$) in wild-type ($n = 6$; open circles) and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ ($n = 5$; filled squares) male mice on (E) carotid artery pressure and (F) heart rate. Mean changes to isoprenaline (3 $\mu\text{g}/\text{kg}$) and atropine (1 mg/kg) in wild-type ($n = 6$) and double knockout $\alpha_{1A}^{-/-}$ /P2X1 $^{-/-}$ ($n = 5$) male mice to (G) mean arterial pressure and (H) heart rate. Error bars represent SEM.

Materials and Methods

Study Approval. Prior approval for animal breeding and experimentation was granted by the Monash University Standing Committee on Animal Ethics, ethics nos. BCSV 2009.03 for the maintenance and breeding of the knockout mouse colonies and VCPA 2009.14 and VCPA 2009.15 for the use of genetically modified and wild-type mice, respectively. All studies conformed to the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Breeding. Breeding pairs of α_{1A} -adrenoceptor knockout mice purchased from JAX Mice (Jackson Laboratory) and breeding pairs of P2X1-purinoceptor knockout mice generated in-house were used to establish a colony of double α_{1A} -adrenoceptor/P2X1-purinoceptor double knockout mice by selective cross-breeding of mice containing the mutated *Adra1a* or *P2rx1* gene (Fig. 1A). All mice had C57BL/6 genetic backgrounds. Mice for experimentation were produced by crossing heterozygous α_{1A} ($^{+/-}$)/P2X1 ($^{+/-}$) males with double knockout α_{1A} ($^{-/-}$)/P2X1 ($^{-/-}$) females generated by the initial crossings of mice from the parental colonies maintained on a C57BL/6 background (Fig. 1A). Wild-type controls and, where required, single knockout controls were obtained from the parental single receptor knockout mouse colonies. Offspring were genotyped by PCR (10, 13). Male mice were used when sexually mature (10–12 wk old).

Histochemistry. Detection of P2X1-purinoceptors by immunohistochemistry used a commercially available antibody (Alomone Labs; APR-001) as previously described (25). X-gal staining for β -galactosidase was as described (13). SPG fluorescence histochemistry was as described (14).

Behavioral Studies. Double knockout male mice were mated with wild-type female mice in cages fitted with infrared video-recording equipment for 2 h/d for up to 9 consecutive days or until copulation and ejaculation had occurred. Following ejaculation, male mice were given 2 d of rest before being mated with another wild-type female. Female mice were checked for vaginal plugs immediately after mating sessions. Following mating sessions in which copulation and ejaculation had taken place, female mice were kept for 14 d before being killed and their uteri checked for pregnancies.

Sperm Analysis. Sperm were extracted from cauda epididymides and placed on single concave microscope slides with two drops of physiological saline before coverslipping and viewing under a conventional light microscope (Olympus BX60) fitted with a SPOT RT slider digital camera. Intracytoplasmic sperm injections and implantation of fertilized ova into foster mothers were conducted at the Monash Animal Research Platform, Monash University.

Physiological Studies. Isolated organ bath experiments were conducted as described (25, 26). Intracellular electrical recordings of vas deferens smooth muscle were as described (15). Resting blood pressure and pulse rate were measured in conscious mice using the tail cuff method with a noninvasive blood pressure analysis system (SC1000; Hatteras Instruments). Analysis was performed over a period of 4–5 consecutive days, with each day comprising 20 repeat blood pressure and heart rate measurements. The first and/or second day was used for acclimatization. Intraarterial pressure under isoflurane anesthesia was measured via a carotid artery catheter with the jugular vein catheterized for i.v. drug infusion, as described (27).

Statistical Methods. In all cases, means were calculated from data pooled from n experiments, where n is equal to the number of mice used. Results are expressed as mean \pm SEM. Mean data were analyzed by ANOVA, followed by Bonferroni posttest for multiple comparisons where required or Student t test. Tests were carried out using GraphPad Prism version 6.00, and $P < 0.05$ was considered significant.

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