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Preserved fertility despite erectile dysfunction in mice lacking the nitric oxide receptor

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Key points

- Erectile dysfunction may result from reduced or non-functional nitric oxide (NO)/cGMP-mediated signalling. Mice lacking NO synthases are fertile whereas mice deficient in cGMP-dependent protein kinase I suffer from erectile dysfunction.
- To clarify this discrepancy we performed studies on the corpus cavernosum of male mice lacking the NO receptor NO-sensitive guanylyl cyclase (NO-GC) either globally or specifically in smooth muscle cells.
- NO released from NO donors as well as from nitrergic neurons failed to relax precontracted corpus cavernosum from mice lacking NO-GC either globally or specifically in smooth muscle; to our surprise, males from both knockout lines were fertile.
- Our data show that deletion of the NO receptor specifically in smooth muscle cells abolishes NO-induced corpus cavernosum relaxation but does not lead to infertility.

Abstract Nitric oxide (NO) and cGMP have been shown to be important mediators of penile erection. Erectile dysfunction may result from reduced or non-functional signal transduction within this cascade. There is, however, some inconsistency in the available data as mice lacking NO synthases (endothelial and neuronal nitric oxide synthase, or both) appear to be fertile whereas mice deficient in cGMP-dependent protein kinase I (PKGI) suffer from erectile dysfunction. To clarify this discrepancy we performed studies on mice lacking the NO receptor NO-sensitive guanylyl cyclase (NO-GC). In addition, we generated cell-specific NO-GC knockout (KO) lines to investigate the function of NO in individual cell types. NO-GC was specifically deleted in smooth muscle or endothelial cells (SM-guanylyl cyclase knockout (SM-GCKO) and EC-GCKO, respectively) and these KO lines were compared with total knockouts (GCKO) and wild-type animals. We investigated expression of NO-GC, NO-induced relaxation of corpus cavernosum smooth muscle and their ability to generate offspring. NO-GC-positive immunostaining was detected in smooth muscle and endothelial cells of murine corpus cavernosum but not in interstitial cells of Cajal. NO released from NO donors as well as from nitrergic neurons failed to relax precontracted corpus cavernosum from GCKO mice in organ bath experiments. Similar results were obtained in corpus cavernosum from SM-GCKO mice whereas deletion of NO-GC in endothelial cells did not affect relaxation. The lack of NO-induced relaxation in GCKO animals was not compensated for by guanosine 3′,5′-cyclic monophosphate (cGMP) signalling. To our surprise, GCKO males were fertile although their ability to produce offspring was decreased. Our

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data show that deletion of NO-GC specifically in smooth muscle cells abolishes NO-induced corpus cavernosum relaxation but does not lead to infertility.

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Abbreviations CC, corpus cavernosum; DEA-NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide-diethylammonium salt; ECs, endothelial cells; EC-GCKO, EC-specific guanylyl cyclase knockout; EFS, electric field stimulation; eNOS, endothelial nitric oxide synthase; GCKO, guanylyl cyclase knockout; IBMX, 3-isobutyl-1-methylxanthine; ICC, interstitial cells of Cajal; NO, nitric oxide; NO-GC, nitric oxide-sensitive guanylyl cyclase; nNOS, neuronal nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKG, cGMP-dependent protein kinase; α-SMA, α-smooth muscle actin; SMCs, smooth muscle cells; SM-GCKO, smooth muscle-specific guanylyl cyclase knockout; WT, wild-type.

Introduction

To date, it is widely accepted that nitric oxide (NO) is the main neurotransmitter mediating penile erection. It can be produced either by neuronal nitric oxide synthase (nNOS) from nitrergic nerves or by endothelial nitric oxide synthase (eNOS) from sinusoidal endothelial cells (Burnett, 1997; Lue, 2000; Musicki *et al.* 2009; Andersson, 2011). By activation of its main receptor NO-sensitive guanylyl cyclase (NO-GC), NO leads to production of guanosine 3′,5′-cyclic monophosphate (cGMP) in different cell types (Friebe & Koesling, 2003). In turn, cGMP activates cGMP-dependent kinase (Hofmann, 2005) resulting in relaxation of the resistance vessels and trabecular smooth muscle; the resulting arteriolar vasodilatation induces an increase in blood flow, which expands the sinusoidal spaces leading to erection.

Previous studies in knockout (KO) mouse models have already shown the importance of the NO/cGMP cascade in corpora cavernosa (CC) relaxation. All three NOS isozymes have been deleted on the genomic level, either alone or in double or triple combinations (Huang *et al.* 1993, 1995; Tranguch & Huet-Hudson, 2003; Morishita *et al.* 2005). Mice lacking nNOS were shown to be fertile and maintain erectile function. This indicates that NO from eNOS is important for erectile function as inducible NOS (iNOS) was not detected in penile tissue (Burnett *et al.* 1996). Nevertheless, mice lacking eNOS alone or in combination with nNOS or nNOS plus iNOS were still fertile. Making this issue even more complicated, data from another nNOS knockout in which exon 6 was deleted, indicate infertility of male mice attributed to central hormonal dysregulation but not to erectile dysfunction (Gyurko *et al.* 2002). Deletion of cGMP-dependent protein kinase I (PKGI), however, was shown to result in erectile dysfunction (Hedlund *et al.* 2000). The CC of male PKGI-deficient mice failed to relax upon stimulation of the NO/cGMP cascade; in addition, a very low ability to reproduce was observed.

To resolve this paradox we first investigated erectile function in mice lacking NO-GC globally. Neither pharmacological NO nor electrical field stimulation (EFS)-induced NO release from cavernosal neurons led to relaxation of precontracted CC from guanylyl cyclase knockout (GCKO) mice; yet, cAMP-induced relaxation was unchanged. Immunohistochemical analysis reveals NO-GC expression in smooth muscle cells (SMCs) and endothelial cells (ECs) but not in interstitial cells of Cajal. Cell-specific deletion of NO-GC revealed a total loss of NO-induced CC relaxation in smooth muscle GCKO (SM-GCKO) whereas that from endothelial cell GCKO (EC-GCKO) animals was unperturbed. Surprisingly, male GCKO mice were fertile and able to produce offspring albeit with a reduced number of litters. We conclude that NO-GC in smooth muscle is the main contributor to CC relaxation and that, in line with the data on NOS knockout mice, NO/cGMP signalling is not necessary for fertility.

Methods

All experiments were conducted in accordance with the German legislation on protection of animals and were approved by the local animal care committee.

Generation of GCKO, SM-GCKO and EC-GCKO mice

Mice lacking NO-GC globally were generated as described previously (Friebe *et al.* 2007). SM-GCKO and EC-GCKO mice carry a floxed exon (exon 10 of the β_1 subunit of NO-GC; NO-GC-$\beta_1^{flox/flox}$) and are transgenic for either the inducible (SMMHC-CreER^{T2}) or the constitutive Cre recombinase (Tie2-Cre). SM-GCKO mice aged 6–8 weeks were injected with tamoxifen (1 mg I.P.) on five consecutive days to remove the floxed exon. The last day of tamoxifen injection was defined as day 0. SM-GCKO mice were analysed 20, 50 and 150 days after the last tamoxifen injection. In the relevant figures, 'pre' indicates mice without tamoxifen injection. NO-dependent CC relaxation did not differ between SM-GCKO$_{pre}$ (Fig. 3*B*) and wild-type (WT) controls (Fig. 2*B* and *C*), indicating that the SMMHC-CreER^{T2} itself did not

influence NO signalling. After 50 days, deletion of NO-GC was considered complete as judged by the cessation in time-dependent reduction of NO relaxation in SM-GCKO mice. Thus, for experiments with SM-specific knockout CC we used animals >50 days after tamoxifen treatment. In each experiment, wherever feasible WT littermates or respective tamoxifen-injected littermates were used as controls.

Isometric force studies

The animals were killed by inhalation of an overdose of isoflurane. The tunica albuginea was carefully opened and the two CCs were microsurgically excised. Both CCs were immediately transferred to Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 7.5 mM glucose, pH 7.4) and bubbled with 95% O_2 and 5% CO_2.

The CCs were mounted longitudinally on fixed segment support pins in two four-chamber myographs (Myograph 610; Danish Myo Technology, Aarhus, Denmark) containing 5 ml of the Krebs–Henseleit solution and bubbled with 95% O_2 and 5% CO_2. Resting tension was set to 3 mN. For pharmacological studies the strips were contracted with phenylephrine (PE; 3 μM). To investigate the effect of NO and cGMP on the CC of the different mouse strains, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide-diethylammonium salt (DEA-NO; 10 nM–10 μM), 8-Br-cGMP (10 μM), carbachol (1 μM) or forskolin (1 nM–10 μM) were applied into the Krebs–Henseleit solution. To specifically inhibit NO-GC, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM) was administered 20 min before relaxation. To determine maximal relaxation, 3-isobutyl-1-methylxanthine (IBMX; 100 μM) was added at the end of each experiment.

Electrophysiological studies

EFS was measured in CC strips precontracted with PE (3 μM). EFS was applied through two platinum wire electrodes (5 mm distance; supramaximal voltage, 0.5 ms, 0.125–8 Hz, 15 s).

Immunohistochemistry

Mice were killed by inhalation of an overdose of isoflurane and tissues were fixed by transcardiac perfusion with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and tissue was cryoprotected using 20% sucrose and snap frozen. Cryosections (10 μm thick) were cut, air-dried and incubated overnight with an antibody generated in our lab (Friebe *et al.* 2007) against the β_1 subunit of NO-GC raised in rabbit (1:800) together either with a FITC-conjugated mouse anti-α-smooth muscle actin antibody (1:500, clone 1A4; Sigma-Aldrich, Munich, Germany), with a rat anti-ckit antibody (1:1600, clone ACK4; Linaris, Wertheim-Bettingen, Germany) or with a goat anti-caveolin 1 antibody (1:100, polyclonal; Abcam, Cambridge, UK) all diluted in Tris-buffered saline (TBS). Antibodies were detected by incubating the sections for 1 h with the appropriate secondary antibodies diluted in TBS. The following reagents were used – for the rabbit antibody: Alexa 555-conjugated or Alexa 488-conjugated anti-rabbit IgG antibody raised in donkey (both 1:800; Invitrogen, Carlsbad, CA, USA); for the rat antibody: Alexa 488-conjugated donkey anti-rat IgG antibody (1:400; Invitrogen); for the goat antibody: Alexa 555-conjugated anti-goat IgG antibody (1:100; Invitrogen). The sections were coverslipped using Mowiol and were evaluated using an epifluorescence microscope equipped with appropriate filter sets for Alexa 555 and FITC/Alexa 488.

Materials

8-Br-cGMP, DEA-NO and ODQ were purchased from Axxora (Lörrach, Germany). Carbachol, IBMX, PE and tamoxifen were from Sigma (Taufkirchen, Germany).

Individual statistical analyses

For calculation of statistical tests, GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA), was used. The Mann–Whitney *U* test was used for the data in Figs 3*C*, 5*B*, 5*D* and 6 in a predefined sequence and comparison was stopped if P was >0.05 to prevent α inflation. In Figs 2*B*, 3*B*, 4*B*, 4*D* and 4*F*, all groups were compared by the Kruskal–Wallis test. If P was $\leq$0.05 for the global test, two groups were compared by Mann–Whitney *U* test (Figs 4*B*, 4*D* and 4*F*). In Figs 2*B* and 3*B*, P was $\leq$0.05 for the Kruskal–Wallis test and two groups were compared by the Mann–Whitney *U* test in a predefined sequence and comparison was stopped if p was >0.05 to prevent alpha inflation.

Results

Immunohistochemical localization of NO-GC in CC

To visualize the expression of NO-GC in murine CC we performed immunohistochemistry using an antibody against the β_1 subunit as the obligatory subunit for both NO-GC isoforms (Friebe *et al.* 2007). Figure 1*A* shows intense staining in SMCs of the central and helicine artery walls and in smooth muscle cells of the trabecular

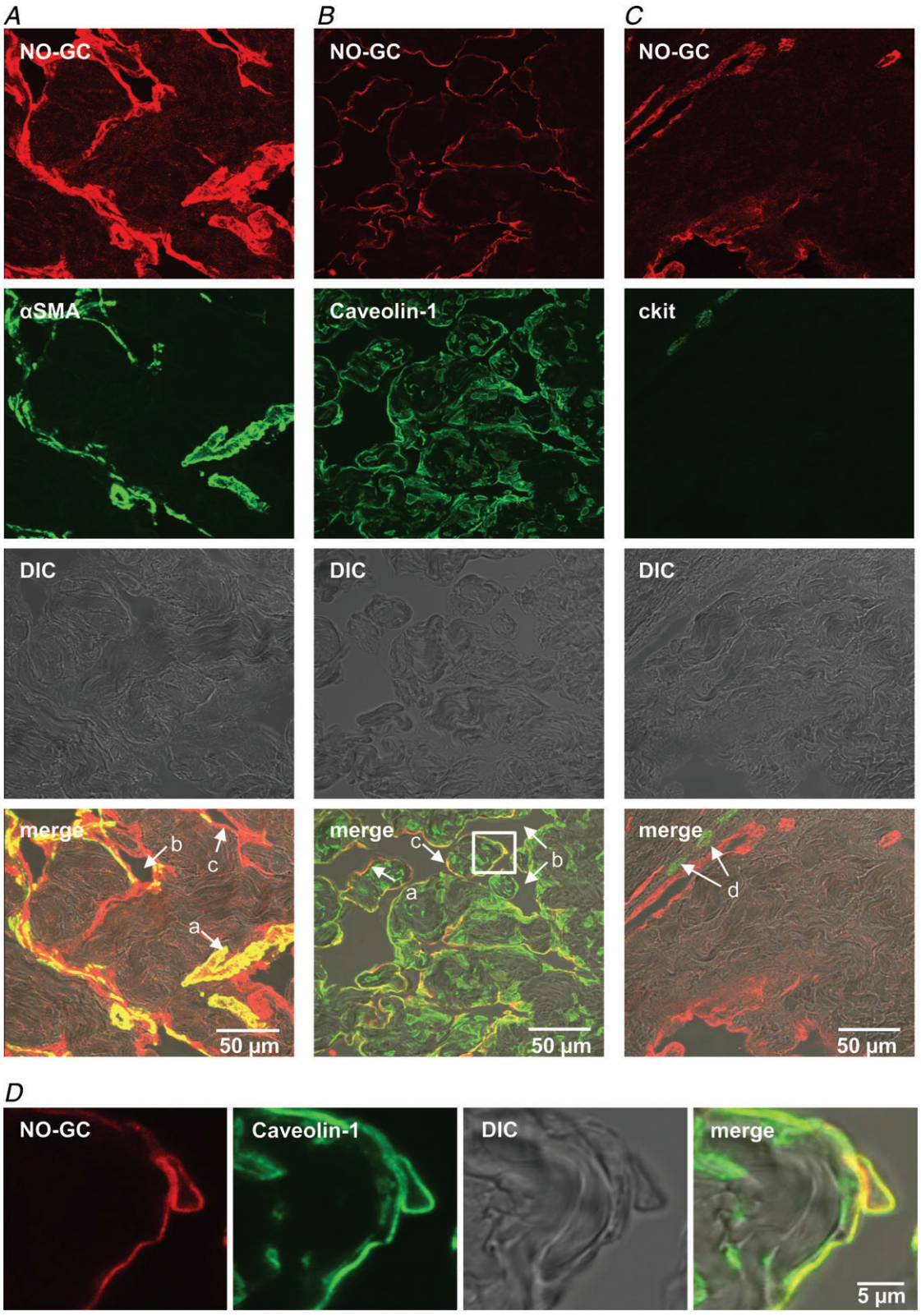

Figure 1. Immunohistochemical analysis of NO-GC expression
Expression of NO-GC (β_1 subunit) in the CC of WT animals. Tissues were co-stained with specific antibodies for α-smooth muscle actin (*A*), caveolin 1 (*B*) or ckit (*C*). Indicated in the merged images are trabecular smooth muscle (*a*), sinusoidal caverns (*b*), sinusoidal endothelium (*c*) and mast cells (*d*). DIC = differential interference contrast.

septa surrounding the cavernous spaces. In addition, endothelial cells lining the cavernosal sinusoids were positive for NO-GC and caveolin-1 was highly expressed in endothelial cells (Fig. 1*B*; enlargement in Fig. 1*D*). In contrast to previous reports, ckit-positive interstitial cells were not detected. The only ckit-immunoreactive cells had the morphology of mast cells, which are known to express ckit (Fig. 1*C*). Taken together, in murine CC, NO-GC is expressed in SMCs and, surprisingly strongly, in ECs.

Lack of NO-induced relaxation in CC from GCKO mice

The functionality of the NO signalling in CC was investigated in organ bath experiments. CCs were isolated and precontracted with PE (3 μM). CC from WT mice showed a concentration-dependent relaxation in response to the NO donor DEA-NO (original trace in Fig. 2*A*, statistical analysis in Fig. 2*B*). The specific NO-GC inhibitor ODQ (10 μM) abolished this NO-induced relaxation, indicating cGMP dependence of NO-induced relaxation. In CC from GCKO animals, DEA-NO up to 10 μM did not lead to relaxation.

NO-induced relaxation in SM-GCKO and EC-GCKO mice

As NO-GC was detected in both SMCs and ECs of the murine CC we next aimed to clarify the contribution of either cell type to NO-mediated relaxation. Mice lacking NO-GC specifically in SMCs (Groneberg *et al.* 2010) and ECs were generated using smooth muscle myosin heavy chain promoter-driven (SM-MHC) and Tie2 promoter-driven Cre expression. In contrast to the constitutively active Tie2 promoter the SMC-specific knockout needed tamoxifen induction as shown in Fig. 3*A*: CCs of homozygous floxed mice carrying the SM-MHC-Cre transgene were investigated before (pre) and 20, 50 and 150 days after tamoxifen injection (indicated as a subscript, e.g. SM-GCKO$_{50}$). In previous reports we have shown that quantitative SMC-specific deletion of NO-GC was reached only after >50 days (Groneberg *et al.* 2010, 2011). NO-induced relaxation of CC from SM-GCKO mice was reduced 20 days and fully abolished 50 days after tamoxifen treatment (Fig. 3*B*) when compared to the control (SM-GCKO$_{pre}$). The relaxing response did not recover as even 150 days after tamoxifen treatment NO still had no effect. As shown in Fig. 3*C*, endothelium-specific deletion of NO-GC did not influence NO-induced CC relaxation. The effective deletion of NO-GC in both KO models is shown in Fig. 3*D*: in CC tissue from SM-GCKO$_{50}$ mice, NO-GC only stains endothelial cells (see also Fig. 1*B*) but not SMCs positive for α-smooth muscle actin (αSMA), whereas in EC-GCKO tissue, endothelial staining is absent and NO-GC colocalizes with αSMA. Thus, NO-induced CC relaxation invariably depends on NO-GC in SMCs. Consequently, further analyses were performed only in the SM-GCKO animals.

Effect of endogenously released NO on CC from GCKO and SM-GCKO mice

Next, we studied whether endogenously produced NO still had an effect on CC tension. To stimulate cholinergic

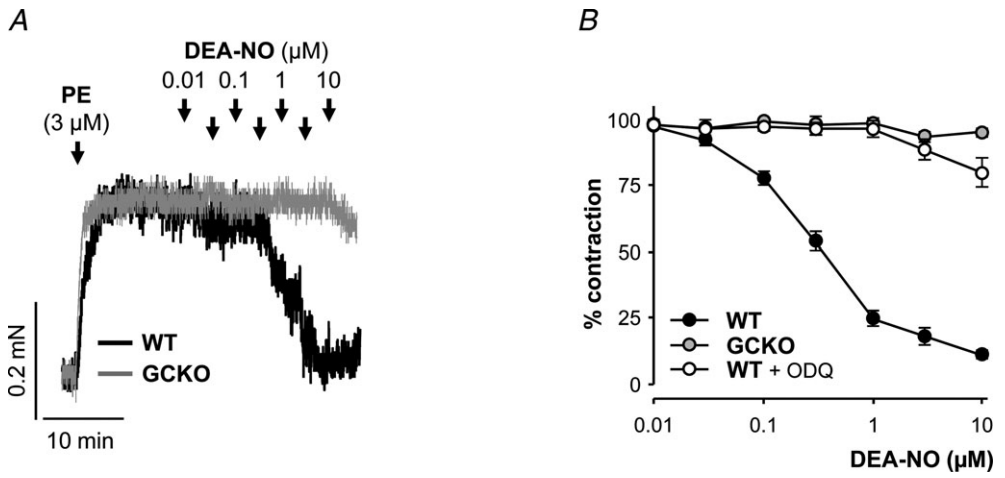

Figure 2. Lack of NO-induced relaxation in CC from GCKO mice
A, original traces of organ bath experiments showing DEA-NO-dependent relaxation in CC from WT and GCKOmice after precontraction with 3 μM PE. The difference between WT and GCKO reached statistical significance ($P < 0.05$, Mann–Whitney *U* test) from 0.1 μmol l^{-1} to 10 μmol l^{-1}. *B*, quantitative analysis of the experiments carried out in *A*. In addition, CC from WT animals was pre-incubated with ODQ (10 μM) to specifically inhibit NO-GC. The difference between WT and WT + ODQ reached statistical significance ($P < 0.05$, Mann–Whitney *U* test) from 0.1 μmol l^{-1} to 10 μmol l^{-1}. Data shown are means ± SEM of n = 5–6 per genotype.

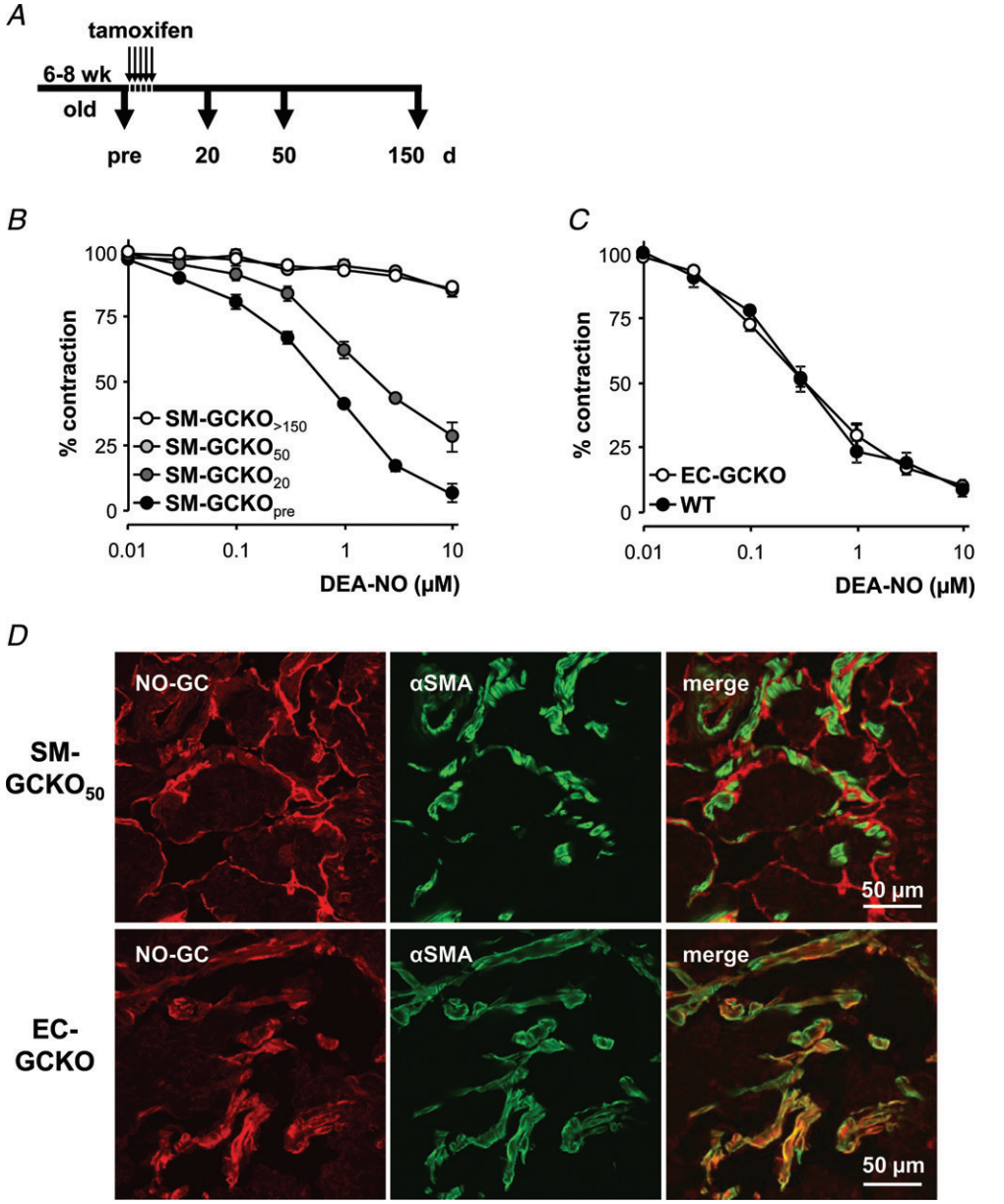

Figure 3. Lack of NO-induced relaxation in CC from SM-GCKO animals and preserved relaxation in EC-GCKO mice

A, time scheme for the generation of SM-GCKO mice. Male mice (6–8 weeks old) were injected with tamoxifen (1 mg I.P.) on five consecutive days. The last day of tamoxifen injection was defined as day 0; mice were analysed 20, 50 or 150 days after tamoxifen injection. Control animals used did not receive tamoxifen (SM-GCKO$_{pre}$). *B*, CCs were mounted into the organ bath. After pre-contraction with PE (3 μM), increasing concentrations of the NO donor DEA-NO were applied. In SM-GCKO animals, NO-induced relaxation decreased with increasing time distance from the last tamoxifen injection; unresponsiveness to NO was seen after approximately 50 days and did not recover even after 150 days. The differences between SM-GCKOpre and SM-GCKO20, SM-GCKO50 or SM-GCKO150 reached statistical significance ($P < 0.05$, Mann–Whitney *U* test) from 0.1 μmol l^{-1} to 10 μmol l^{-1} (SM-GCKOpre vs SM-GCKO20), from 0.03 μmol l^{-1} to 10 μmol l^{-1} (SM-GCKOpre vs SM-GCKO50) and from 0.03 μmol l^{-1} to 10 μmol l^{-1} (SM-GCKOpre vs SM-GCKO150). *C*, quantitative analysis of DEA-NO-induced relaxation of CC from EC-GCKO mice precontracted with PE (3 μM). Data shown are means ± SEM of $n = 4$–8 per genotype. No statistical significance was reached in either comparison ($P = 0.24$ to 1.00, Mann–Whitney *U* test). Data shown are means ± SEM of $n = 4$–8 per genotype. *D*, expression of NO-GC (β_1 subunit) in the CC of SM-GCKO$_{50}$ and EC-GCKO animals. Tissues were co-stained with an antibody specific for α-smooth muscle actin. Both sets indicate effective cell-specific deletion of NO-GC.

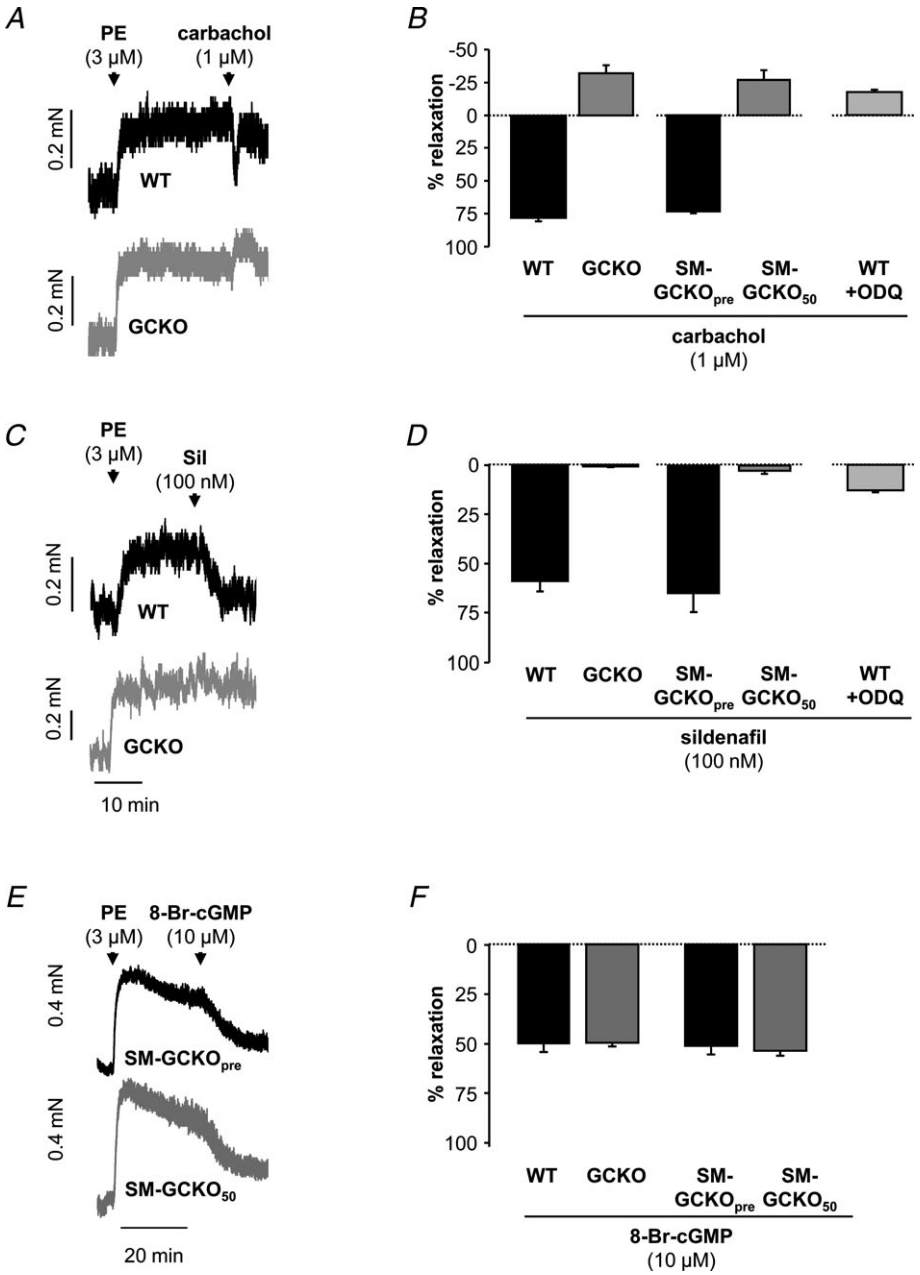

Figure 4. Effects of NOS and PKGI stimulation and PDE inhibition
CCs were mounted into the organ bath. After pre-contraction with PE (3 μM), relaxation was induced. *A* and *B*, representative original recordings (*A*) and quantitative analysis (*B*) of carbachol (1 μM)-induced relaxation of CC from WT and GCKO mice. Comparison of the data from WT animals to the data from GCKO animals or WT + ODQ reached statistical significance ($P = 0.0012$ and 0.0022, respectively, Mann–Whitney *U* test). Comparison of the data from SM-GCKOpre animals to the data from the SM-GCKO50 reached statistical significance ($P = 0.0025$, Mann–Whitney *U* test). *C* and *D*, representative original recordings (*C*) and quantitative analysis (*D*) of sildenafil (100 nM)-induced relaxation of CC from SM-GCKO$_{pre}$ and SM-GCKO$_{50}$ mice. Specific inhibition of NO-GC by ODQ (10 μM) blocked carbachol- and sildenafil-induced relaxation of WT CC as shown in *B* and *D*. Comparison of the data from WT animals to the data from GCKO animals or WT + ODQ reached statistical significance ($P = 0.0016$ and 0.0007, respectively, Mann–Whitney *U* test). Comparison of the data from SM-GCKOpre animals to the data from SM-GCKO50 reached statistical significance ($P = 0.0025$, Mann–Whitney *U* test). *E* and *F*, representative original recordings (*E*) and quantitative analysis (*F*) of 8-Br-cGMP (10 μM)-induced relaxation of CC from SM-GCKO$_{pre}$ and SM-GCKO$_{50}$ mice. No significant difference was detected between WT, GCKO, SM-GCKOpre and SM-GCKO50 ($P = 0.66$, Kruskal–Wallis test). Data shown are means ± SEM of $n = 3$–8 per genotype.

receptors, carbachol (1 μM) was applied to pre-contracted CC (PE; 3 μM). Original traces of this experiment are shown in Fig. 4*A* (statistical analyses in Fig. 4*B*). CC from controls (WT and SM-GCKO$_{pre}$ animals) showed an almost 75% relaxation upon administration of carbachol. Contraction rather than relaxation was observed in CC from GCKO and SM-GCKO$_{50}$ animals as well as in CC from WT treated with ODQ. Thus, relaxation by endogenous NO is conveyed exclusively by smooth muscle NO-GC.

Effects of sildenafil and 8-Br-cGMP on CC tension

Sildenafil is a specific inhibitor of phosphodiesterase 5 (PDE5) which is the major cGMP-degrading enzyme in SMCs (Rybalkin *et al.* 2003). Administration of sildenafil (100 nM) to PE-precontracted CC produced similar results to carbachol (Figs 4*C* and *D*): CC from WT and SM-GCKO$_{pre}$ animals relaxed upon sildenafil treatment whereas PDE5 inhibition did not induce relaxation in CC from GCKO, SM-GCKO$_{50}$ mice and WT in the presence of ODQ.

To exclude a possible change in signalling downstream of NO-GC we treated precontracted CC with the membrane-permeable cGMP analogue 8-Br-cGMP (10 μM). Relaxation by direct PKG activation was identical in CC from WT, GCKO, SM-GCKO$_{pre}$ and SM-GCKO$_{50}$ animals demonstrating PKG-mediated signal transduction to be undisturbed by the GC knockout (Figs 4*E* and *F*).

cAMP-mediated CC relaxation

Similar to cGMP, cAMP is known to relax CC smooth muscle. To investigate a possible cAMP-mediated

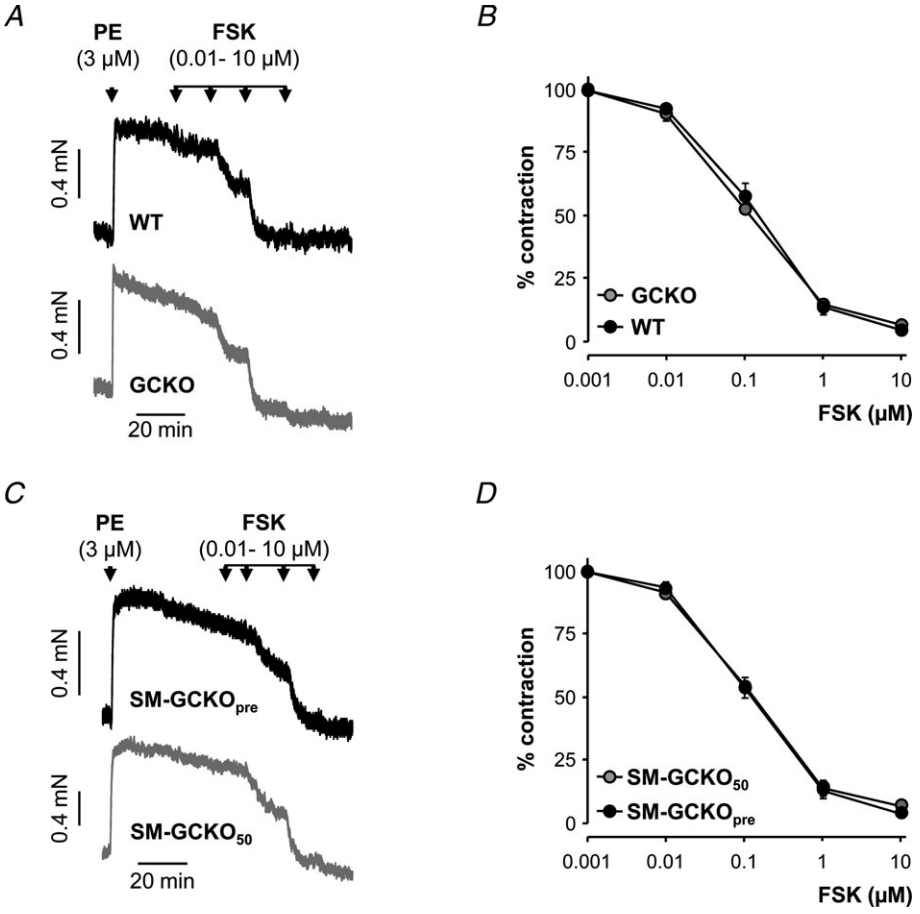

Figure 5. Preserved cAMP cascade in CC from GCKO and SM-GCKO mice
After pre-contraction (PE, 3 μM), CC relaxation was induced with increasing concentrations of forskolin (FSK). *A* and *B*, representative original recordings (*A*) and quantitative analysis (*B*) of CC from WT and GCKO mice. Comparison of the data from WT animals to the data from the GCKO animals did not reach statistical significance ($P = 0.2$ to 1.00, Mann–Whitney *U* test). *C* and *D*, representative original recordings (*C*) and quantitative analysis (*D*) of CC from SM-GCKO$_{pre}$ and SM-GCKO$_{50}$ mice. No statistical significance was reached in either comparison. Comparison of the data from SM-GCKOpre animals to the data from SM-GCKO50 animals did not reach statistical significance ($P = 0.7$ to 1.00, Mann–Whitney *U* test). Data shown are means ± SEM of $n = 3$ per genotype.

compensation in the NO-GC knockout models we studied the effect of forskolin, an activator of adenylyl cyclase. Forskolin-dependent relaxation of PE-contracted CC was identical in GCKO and SM-GCKO$_{50}$ animals and the respective controls (Figs 5*A* and *B*). These data clearly demonstrate that cAMP signalling is not affected by and does not compensate for the absence of NO-GC.

Lack of EFS-induced CC relaxation in GCKO and SM-GCKO mice

We then used EFS to stimulate release of neurotransmitters from nerve terminals onto CC smooth muscle. Relaxation of PE-contracted CC from WT and SM-GCKO$_{pre}$ was frequency dependent (0.125–8 Hz; Fig. 6). In contrast, EFS up to 1 Hz did not relax CC strips from GCKO mice; at higher stimulation frequencies (2–8 Hz), we saw EFS-induced contraction, as also noted by Hedlund *et al.* (2000). Similar results were observed in SM-GCKO$_{>50}$ animals.

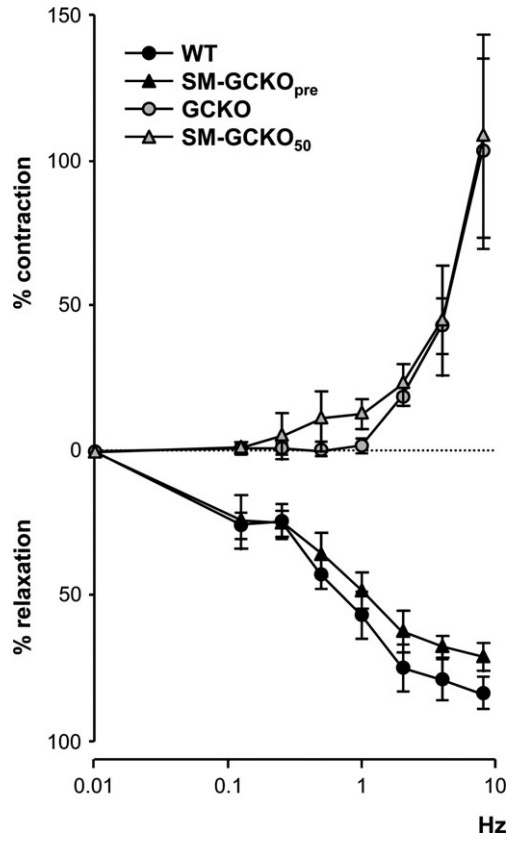

Figure 6. EFS-induced relaxation of CC from GCKO and SM-GCKO mice
After pre-contraction (PE, 3 μM), CC relaxation was induced by electric field stimulation. Quantitative analysis for EFS-induced relaxation of CC from WT, SM-GCKO$_{pre}$, GCKO and SM-GCKO$_{50}$ mice. The difference between WT and GCKO animals reached statistical significance ($P < 0.05$, Mann–Whitney *U* test) from 0.125 Hz to 8 Hz. The difference between SM-GCKOpre animals and SM-GCKO50 animals reached statistical significance ($P < 0.05$, Mann–Whitney *U* test) from 0.125 Hz to 8 Hz. Data shown are means ± SEM of $n = 4$–8 per genotype.

Table 1. Litter sizes and pup numbers of WT and GCKO mice

	WT	GCKO
Number of litters	54	14
Number of pups	273	69
Litters per week (mean ± SEM)	4.5 ± 1.2	1.2 ± 1.1
Pups per litter (mean ± SEM)	5.1 ± 2.7	4.9 ± 2.1

Four males of each genotype were mated to two females. Males were rotated to new females on a weekly basis for 12 weeks.

Fertility of KO models

To study the impact of the absence of NO-GC we crossed male mice of the different genotypes with WT females. We did not see any differences in fertility between EC-GCKO and WT animals. Although all GCKO males tested were capable of producing offspring, their fecundity was reduced. Table 1 shows the direct comparison of litter numbers and sizes between WT and GCKO. GCKO males produced only 25% of the number of litters compared to WT although the average size of the litters was similar. Crossing five SM-GCKO males with WT females over a period of 8 weeks resulted in only three litters with an mean size of 5.7 ± 2.3 pups. Taken together, deletion of NO-GC results in the loss of NO-mediated relaxation of CC smooth muscle but does not abolish fertility.

Discussion

NO released from nitrergic nerves as well as from ECs of the sinusoids is the major signalling molecule for inducing and sustaining penile erection (Burnett *et al.* 1992). A central nervous stimulus is thought to elicit a burst of neuronal NO release to relax cavernosal smooth muscle and increase blood flow. The resulting increase in shear stress then leads to activation of eNOS providing a sustained NO release which promotes maximal relaxation and thus erection (Hurt *et al.* 2002). Unexpectedly, deletion of either eNOS or nNOS did not result in erectile dysfunction (Huang *et al.* 1993, 1995; Burnett *et al.* 1996), and even double or triple deletion including iNOS did not impair the production of offspring of mutant mice (Son *et al.* 1996; Tranguch & Huet-Hudson, 2003; Morishita *et al.* 2005). These data are in stark contrast to those collected from mice lacking PKGI: Hedlund *et al.* (2000) clearly showed that lack of PKGI resulted in erectile dysfunction in combination with strongly reduced fertility.

To resolve this discrepancy we investigated CC relaxation and fertility in mice lacking NO-GC. In the current model NO-GC, as receptor for NO, integrates endothelial and neuronal NO-based signals to convey these via changes of the intracavernosal cGMP concentration on to PKGI. CC from NO-GC-deficient males failed to relax upon stimulation with exogenous or endogenous NO but relaxation was unperturbed when induced by cAMP. The lack of NO/cGMP signalling did, however, only partly impair the production of offspring. Over a period of 12 weeks (rotation to two females on a weekly basis) WT mice produced a large number of litters/pups (54/273, respectively). To our surprise, fertility of GCKO males was not totally abrogated but they still were able to produce approximately 25% of the litter/pup numbers seen for WT (14/69, respectively).

How can the difference in fertility between all these knockout lines be explained? Health conditions and survival rates in eNOS and nNOS knockouts appear to be WT-like, which is in contrast to the PKGI- and NO-GC-deficient mice. The general phenotype of animals lacking PKGI deteriorates between 5 and 6 weeks of age. Eighty per cent of them die before reaching 8 weeks (Pfeifer *et al.* 1998), probably because of gastrointestinal malfunction (Singh *et al.* 2012). GCKO mice are even worse off than mice lacking PKGI. Eighty per cent of the NO-GC-deficient mice die of unknown reason within 2 days after birth, the remaining mice dying after weaning between weeks 3 and 4. Using an omeprazole-supplemented fibre-free diet, we were able to greatly improve the survival and the general condition of the NO-GC-knockouts. It appears conceivable that a better overall fitness (on fibre-free diet) is responsible for the higher fertility rates in GCKO compared to PKGI-KO animals (without special diet). Thus, fertility measurements in animals with reduced health status should not be used for the estimation of erectile dysfunction. In our case, reduced fertility may arise from erectile dysfunction but also be due to a reduced fertilizing capability. Although the number of litters produced by GCKO males was only 25% of that by WT males, the number of pups per litter was unaltered. This indicates that the functional competence of sperm, e.g. maturation and motility, was probably not affected by the absence of the NO receptor. The normal fertility rates in eNOS and nNOS knockout mice may well be explained by the presence of the alternatively spliced β variant of nNOS which is present in all mentioned NOS-deficient strains (Hurt *et al.* 2006) or by a compensatory increase in eNOS expression in the case of nNOS deficiency (Burnett *et al.* 1996). Deletion of the α_1 subunit of NO-GC also led to reduced but not abolished CC relaxation to exogenous and endogenous NO (Nimmegeers *et al.* 2008). As NO-GC inhibition by ODQ abrogated the residual NO effect the α_2-containing isoforms (which are not deleted in the α_1-NO-GC knockout) obviously play a role in erectile function.

The absence of nitrergic relaxation of CC from GCKO mice did not impair effective mating. Thus, an alternative, NO/cGMP-independent mechanism to allow copulation has to exist. Vasoactive intestinal peptide (VIP) and VIP-related peptides have been shown to relax CC and cavernosal vessels *in vitro* (Andersson & Wagner, 1995; Mizusawa *et al.* 2001) but their role *in vivo* is questioned. It is worth noting that cAMP-induced CC relaxation appears to be different in PKGI- and NO-GC-deficient animals. Concentration–response curves for forskolin were identical between WT and GCKO animals, whereas a difference was documented for WT and PKGI-deficient animals, at least for elevated forskolin concentrations (Hedlund *et al.* 2000). So far, we have no explanation for this difference but it may indicate that besides PKGI, cAMP/cGMP may partially signal through some other effector, possibly PDEs in murine CC. Similar to Hedlund *et al*. we were unable to see EFS-induced relaxation in knockout CC. Other putative neurotransmitters involved in the initiation of penile erection thus appear to play a minor role.

A different explanation for the preserved fertility may be based on the presence of a penis bone present in many mammals including mice (Ramm, 2007). The baculum (os penis) is normally kept in the abdomen. During intercourse, it provides stiffness and thus allows entry and sperm deposition in the female reproductive tract. Therefore, it is conceivable that penile erection may be helpful but not absolutely necessary in mice for effective fertilization.

The discrepancy between *in vivo* and *in vitro* observations in our and in other labs may also be explained by different degrees of endothelial dysfunction in the respective animals, which was shown to be linked to reduction in fertility (Visioli & Hagen, 2011). Endothelial dysfunction regarding a reduced production of NO will probably not affect fertility in our mice as the effect of other signalling molecules (e.g. prostaglandins, sphingosine derivatives, reactive oxygen species) will probably be unaffected. NO signalling through a non-GC effector or a reduced effect of endothelium-derived non-NO mediators would possibly affect erectile function and thus fertility.

Immunohistochemical analysis revealed expression of NO-GC in cavernosal smooth muscle and in ECs, but not in ckit-positive interstitial cells. Absence of nitrergic relaxation in CC from smooth muscle-specific knockout mice clearly demonstrated that erectility is a function of NO/cGMP in SMCs. In endothelium-specific knockout animals, fertility (data not shown) as well as NO-induced relaxation was identical to WT. We have not yet detected a functional difference between the EC-GCKO and WT mice, and thus the function of NO-GC in the cavernosal endothelium is still enigmatic. A role of interstitial cells of

Cajal in penile function including nitrergic CC relaxation has been proposed in humans and guinea pigs (Uckert *et al.* 2010; Fu *et al.* 2011); the existence of interstitial cells of Cajal in murine CC, however, has not yet been reported. We only saw minor staining for ckit, a marker of interstitial cells of Cajal, in murine cavernosal tissue, resembling mast cells. As no co-staining for ckit and NO-GC was detected, we rule out a possible contribution of interstitial cells in nitrergic relaxation of the murine CC.

In summary, our data provide evidence that nitrergic relaxation leading to penile erection is dependent on NO-GC in SMCs and that mice lacking nitrergic signalling are capable of producing offspring by a secondary mechanism. Our mice provide useful models for investigation of cAMP/non-cGMP-induced regulation of erectile function.

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Study concept and design: D.G. and A.F.; acquisition of data: D.G., P.K., B.L., R.J. and A.F.; analysis and interpretation of data: D.G., B.L. and A.F.; critical revision of the manuscript for important intellectual content: D.G., B.L. and A.F.; obtained funding: P.K. and A.F.; study supervision: A.F.

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