**Differential effects of anesthetics and sex on supraventricular electrophysiology and atrial fibrillation substrate in rats**

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**Short title:** Influence of anesthetics on the cardiac electrophysiology of rats

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**Abstract**

**Introduction**: The utility of rodents for atrial electrophysiological (EP) research is rapidly growing. However, due to technical challenges, such studies are mostly conducted under anesthesia. Recently, we introduced an implantable device adapted for comprehensive atrial studies in ambulatory rats. We identified that rats implanted with an atrial electrode gradually developed arrhythmic substrate over a testing period of up to eight weeks. Here, we compared the effects of commonly used anesthetics on the supraventricular EP and arrhythmic substrate of male and female rats, relative to the unanesthetized state.

**Materials and methods:** Adult rats of both sexes were evaluated four weeks post-EP device implantation. Consecutive studies were conducted in the unanesthetized state (UAS), under 2% isoflurane (ISO) and under 40mg/kg pentobarbital (PEN). Stimulation protocols were performed to determine AVERP and AERP. Arrhythmic substrate was assessed following twenty conventional triggering bursts in each condition. Arrhythmic tendency was analyzed manually as well as by atrial fibrillation (AF) complexity ratio, an arrhythmia irregularity measure developed by our group recently.

**Results and discussion:** For both sexes, ISO and PEN significantly prolonged the AERP relative to the UAS. In contrast, PEN increased the AVERP of both sexes, but ISO affected males only. Only in the males, AF complexity ratio was significantly reduced under both anesthetic agents relative to the UAS. In the UAS, the AF complexity ratio was markedly lower in females. This sex difference was blunted under both anesthetic agents.

**Conclusion:** Our results demonstrate prominent impact of commonly used anesthetics on the supraventricular EP and the arrhythmic substrate of rats and a differential effect of these compounds according to sex. These findings should have important implications on AF research in rodents and stress the importance of methodologies enabling EP studies in unanesthetized rodents.

**Key Words:** Atrial remodeling; Atrial arrhythmia; Supraventricular arrhythmia; Rodent electrophysiology; Waveform complexity; Lempel-Ziv algorithm.

**Main**

Atrial fibrillation (AF), the most common sustained cardiac arrhythmia, presents a formidable medical challenge with substantial complications and an increased risk of mortality 1-3. The pathophysiology of AF is complex and progressive 4, influenced by mechanisms altering the electrical and structural properties of the atrial myocardium 5, 6. Aging as well as common conditions such as hypertension, diabetes mellitus, obesity, and obstructive sleep apnea converge together and increase the "AF substrate" i.e. the tissue susceptibility to recurrence and persistence of the arrhythmia 7, 8. Despite ongoing research, a comprehensive understanding of the mechanisms involved in AF substrate formation under various clinical settings remains elusive. Reliable biological models are crucial for a better understanding of underlying mechanisms and effective testing of new therapeutic strategies 9, 10. Although novel *in vitro* atrial models are evolving 11, 12, many challenges including proper differentiation, multi cellularity and realistic electrical and mechanical function still exist, leaving the need for animal models irreplaceable.

 Historically, AF research almost exclusively relied on large animals. However, recent years have witnessed a rapid increase in the use of rodents for AF research, largely due to the remarkable ability to increase the AF substrate of these small mammals using clinically relevant insults (e.g. 13-18). Nonetheless, technical challenges associated with the small and delicate atrial anatomy limit most electrophysiological (EP) studies and AF induction protocols to either *ex vivo* preparations or to invasive insertion of atrial pacing electrode under deep anesthesia 19, 20. Two of the most used anesthetics in rodent AF studies are isoflurane (ISO) and pentobarbital (PEN) (e.g. 21-24). While these agents enable the experimental procedures, they can alter the hemodynamic and cardiac EP parameters 19, 25-27. Thus, it is also likely that they can markedly modulate the arrhythmogenic substrate of the atrial tissue. However, the limited ability to compare the atrial EP and arrhythmogenic substrate of anesthetized rodents to that in the unanesthetized state (UAS), prevented direct evaluation of this issue up to now. In addition, there may be important sex-dependent variations in the effects of anesthetic agents on the cardiac EP of rodents 28-30. However, the sex-related effects of anesthetics on the supraventricular EP and AF inducibility of rodents are not defined to the best of our knowledge. Importantly, in humans, males have a 1.5–2 times higher risk of developing atrial fibrillation (AF) for reasons that are not yet understood 31. Recent *in vivo* data indicate that male mice exhibit increased AF substrate relative to females, which may be regulated by the presence of androgens 31. However, the situation in rats is not clear in this regard and the effects of anesthetics on the sex-dependent differences are unknown.

Our laboratory, recognizing the lack in proper tools allowing long-term cardiac EP studies in rodents, previously introduced an implanted system adapted for repeated pacing and EP recordings in freely moving rats and mice 32-36.More recently, we improved the rat system by introducing a miniature atrial quadripolar electrode composed of medical-grade silicon and fully biocompatible metal components. This electrode enables simultaneous atrial pacing and recordings exhibiting stable capture thresholds and high resolution atrial recordings over extended periods of at least eight weeks 37. Moreover, the high resolution of the recorded atrial signals enabled us to advance arrhythmia analysis and to develop an unbiased computerized approach to clean the atrial signal from ventricular mixing and thereafter evaluate the signal power spectrum as well as the irregularity of arrhythmic event in a potent manner 38. Importantly, while the atrial electrode was primarily designed for long-term pacing and recording purposes, we have found that when implanted for several weeks it gradually shortens that atrial effective refractory period (AERP) and concomitantly increase the AF substrate of adult male rats 36, 37. The mechanism/s leading to this phenomenon are not elucidated so far, but presumably reflect mechanical loading related to the electrode weight / resistance to the contraction, leading to localized atrial remodeling and AERP dispersion 37. Regardless of the exact mechanism, the arrhythmogenic substrate that develops in this model mimics some important aspects of AF-related remodeling and opens a window of opportunity to evaluate the effect of multiple manipulations on the obtained, atrial selective, AF substrate.

In the current study, taking advantage of our unique EP system capabilities, we explore the impact of different anesthetics on the rat supraventricular EP as well as the differential impact of these agents on males versus females. For that purpose, adult rats of both sexes were evaluated four weeks post device implantation by repeated EP studies conducted under a). UAS b). ISO 2% and c). PEN 40mg/kg (Figure 1). Our results indicate prominent impact of commonly used anesthetics on the supraventricular EP parameters and the arrhythmogenic substrate of rats, which is often sex dependent. Overall, our findings provide valuable data on the complex effects of anesthetics and emphasize the importance of methodologies enabling rodent EP studies in the UAS.

**Results**

**Supraventricular EP properties of unanesthetized rats markedly differ by sex**

Four weeks following EP device implantation, we first analyzed the EP properties of male and female rats in the unanesthetized state. While we performed the implantation procedure on rats having similar weight, the males were markedly heavier during the EP studies (Table 1), as expected. Basal ECG recordings revealed slower heart rate in the males as inferred from the longer RR interval of males compared to females (182.2 ms ± 4.21 vs. 168.6 ms ± 3.48, respectively, p = 0.019). The PR interval was also slightly but significantly longer in the males (Table 1).

In contrast with the RR interval, the CSNRT obtained during programmed stimulation was not different between males and females, suggesting similar SA nodal properties. The AERP measurements of both sexes revealed absence of typical rate-adaptation over the whole range of tested basic CLs (120-70 ms), as we already documented in the past for unanesthetized males 37. However, for all basic CLs the AERP of males tended to be shorter relative to females and this difference reached clear significance when 70ms basic CL was applied (26.2 ms ± 1.4 vs. 31.5 ms ± 1.4, respectively, p= 0.019). In regard to AV nodal function, AVERP measurements did not reveal significant differences between males and females over the whole range of tested basic CLs (130-100 ms). However, dynamic AV nodal properties (AV Wenckebach and AV 2:1 block) were significantly longer in the males (Table 1). Overall, in the unanesthetized state our data demonstrate marked differences in heart rate, atrial AERP and dynamic AV nodal conduction properties between males and female rats.

**Unanesthetized males demonstrate markedly increased AF substrate**

The application of burst pacing for arrhythmia induction did not reveal difference in the induction or the duration of regular arrhythmias (Table 1). However, we found markedly increased AF substrate in the males compared to the females. This result was noted both when AF substrate parameters (induction and duration) were measured manually (AF Induction: 30.77 % ± 6.72 vs. 9.69 % ± 3.04, respectively, p= 0.010. AF Duration: 48.57 s ± 28.83 vs 5.54 ± 1.89, respectively, p= 0.016) as well as using our recently developed objective tool 38 that performs irregular arrhythmia analysis based on CR measurements (Mean CR: 1.22 ± 0.03 vs 1.10 ± 0.02, respectively, p= 0.009, Arrhythmic CR seconds: 39.34 % ± 5.83 vs 16.81 % ± 4.30, respectively, p= 0.009). Overall, the AF substrate related to our implanted device was found to be markedly increased in the unanesthetized males relative to females (see discussion).

**Anesthetics markedly modulate the supraventricular EP properties of rats in a sex-dependent manner**

Initially, we evaluated the effects of ISO and PEN on the heart rate and SA nodal function of both males and females (Figure 2). In both sexes ISO did not affect the RR interval relative to UAS. In contrast, PEN prolonged the RR interval in both sexes relative to UAS and to ISO (Figure 2A). The similar prolonging effect of PEN on the RR interval of males and females is further emphasized when D changes from the UAS are compared for both sexes (Figure 2B). Interestingly, while ISO did not affect the RR interval, it significantly prolonged the CSNRT of males relative to both UAS and PEN. The CSNRT of females was insensitive to the effect of both anesthetics (Figure 2C). However, further analysis comparing the D changes of CSNRT relative to UAS in both sexes did not reveal statistical significance (Figure 2D). PR interval was also modified in a complex manner by anesthetics. While this parameter was insensitive to both anesthetics in the males, the inherently shorter PR interval of the females in UAS (Table 1) was significantly prolonged by both anesthetics (Figure 3A). Comparison of the D changes of PR relative to UAS between males and females did not reach significance however (Figure 3B). Overall, the above findings indicate complex and differential effects of ISO and PEN that are also somewhat variable between males and females in the case of CSNRT and PR interval.

As already noted, AERP was tested using three different basic CLs (70, 100, 120ms). Consistently with our previous publications 32, 37, we did not observe under any of the condition in both sexes, indications for typical rate-adaptation (i.e. shortening of AERP when basic CL was decreased). However, while 2-way ANOVA analysis indicated absence on any rate-adaptation in the males (Figure 3C), a small but significant reverse rate-adaptation (i.e. prolongation of AERP when basic CL is decreased) was noted in the females (Figure 3D). Notably, both ISO and PEN markedly prolonged the AERP in both sexes and over all the tested CLs (Figure 3C-D). However, while the AERP prolonging effect of ISO was increased relative PEN in the males, both anesthetics had a similar prolonging effect in the females. Detailed analyses of the AERP findings for each selected CL are also shown for 120ms CL and for the other CLs (Figure 3E and Supplemental Figure 1S, respectively), further supporting and stressing the noted above findings. Of note, comparison of the D changes of AERP relative to UAS indicated increased effect of ISO in males relative to the females (Figure 3F).

We next analyzed the effects on AV nodal properties. First, AVERP was tested using four different basic CLs (100, 110, 120, 130ms). As expected considering AV nodal physiology, we noted a gradual rate-dependent AVERP increase as the CL was decreased. This finding was noted in both sexes and was highly significant in 2-way ANOVA analysis (Figure 4A-B). Interestingly, both anesthetics markedly increased the AVERP in the males, although PEN had a greater effect compared to ISO. On the other hand, only PEN affected the AVERP in females. Detailed analyses of the AVERP findings for 120md CL as well as the other CLs are also shown (Figure 4C-D and Supplemental Figure 2S, respectively), further supporting the noted above findings. In contrast with the marked sex-dependent effect of ISO on the AVERP, the effect of both anesthetics on the dynamic properties of the AV node (AV Wenckebach block and AV 2:1 block) were note in for both agents and in both sexes, although for ISO they were more prominent and significant in the males (Figure 4E-F, Supplemental Figure 3S).

**ISO and to a lesser extent PEN inhibit the AF substrate in male rats**

Finally, we comprehensively assessed the effects of anesthetics on the atrial arrhythmias induced by burst pacing in our rat model. Manual analysis of irregular (AF) substrate parameters revealed that ISO significantly decrease AF Induction (%) in the males without a notable effect in females (Figure 5A). Comparison of the D changes of AF induction relative to UAS further stressed the inhibitory effect of ISO on the AF induction, which differentially affected males only (Figure 5B). A similar inhibitory tendency of ISO was also noted in regard to AF duration (Figure 5C). However, using conservative statistical analysis as required for non-gaussian distributions, this tendency did not reach significance. Comparison of the D changes in AF duration relative to UAS, reached significance and again supported an inhibitory effect of ISO in males only (Figure 5D). Interestingly, the inhibitory effect of ISO on the AF substrate parameters of males was associated with tendency of increase in the induction and duration of regular atrial arrhythmias. However, this tendency also did not reach statistical significance (Supplemental Figure 4S).

In contrast to ISO, the manual analysis of the AF substrate did not demonstrate a significant effect of PEN on the AF induction and duration, although a tendency for reduced induction was noted (Figure 5A-B), which was further supported by the objective analysis as described below. To further substantiate the above findings, we also analyzed the AF substrate of the rats using our recently developed objective tool aimed for cleaning of the atrial signal from ventricular mixing, followed by CR calculation (Murninkas et al. 2023 38, see also methods for detailed description). As we previously described, the CR parameter detects irregular atrial signals in a highly accurate manner. A clear inhibitory effect of both anesthetics on the mean CR post pacing was noted in the males, (Figure 6 A-B) without a notable effect in the females. A similar tendency was noted when we analyzed the percentage of seconds above the arrhythmic CR threshold (Figure 6 C-D). However, these findings reached statistical significance only for PEN in the males.

Finally, we performed power spectrum analysis of the pacing-induced AF signal under each condition. In both males and females, we found that both anesthetics significantly reduced the dominant frequency of the AF (Figure 7 A-B) a finding which may be consistent with the prolonged AERP under both anesthetics. In addition, a slower dominant frequency was noted for females under UAS and ISO (Figure 7C). This analysis may overall suggest that differences in the atrial EP properties of males vs. females as well as in response to ISO and PEN not only modulate the AF substrate, but also affect the characteristics of induced AF episodes. However, contribution of extrinsic factors such as autonomic activation on these properties cannot be excluded (see discussion).

**Discussion**

The current study is based on our recent advances in developing an implantable EP device adapted for atrial programmed stimulation protocols and AF substrate assessment in freely moving rats 35-38. Using this device, we aimed to comprehensively characterize how ISO and PEN, which are routinely used in conventional invasive EP studies, affect the EP results relative to the most physiological setting, i.e. the UAS. We characterized the results in both males and females to get a thorough overview of the effects of the selected anesthetics on the supraventricular EP. Our main findings indicate that although conventional doses of ISO and PEN do not prominently modulate heart rate, they affect multiple other aspects of the rat supraventricular EP in a complex manner that is often variable between the two agents and is also variable between males and females. In general terms, our findings clearly stress the importance of considering the effects of the applied anesthetic agent when EP results are reported in the literature. Indeed, while the use of anesthetic agents is inevitable in conventional rodent EP studies, there is great variability between studies in the used agents and the applied doses. Moreover, in many cases, the agent and/or the dose are not even mentioned in the relevant section of the methods (e.g. 21, 39). Our current findings stress the need to change this problematic practice and to further develop and utilize technologies enabling EP studies in the UAS. Below we will further discuss specific findings and their implications.

**Sex-dependent differences in the UAS supraventricular EP**

The initial comparison between the EP findings of males and females in the UAS (Table 1) indicates important differences. These include slower heart rate, longer PR interval, and prolonged AV Wenckebach and AV 2:1 blocks in the males, as well as longer AERP and substantially reduced AF substrate in the females. An important issue that should be considered is the increased weight of the males during the EP study. Since our recordings were performed four weeks after device implantation, this difference simply reflects the difference in growing curves between males and females. While we cannot exclude that the weight difference affected the EP findings to some extent, at least for heart rate, such differences were also previously reported in a study applying telemetric recordings in males and females of approximately similar weight 40. Interestingly, in this study, the heart rate difference was most prominent between males and females that were housed singly40, a condition that existed in our case as well (in order to prevent EP device extraction by cagemates). The prolonged AV Wenckebach block and AV 2:1 block, as well as the shorter AERP in males, might reflect either different intrinsic atrial properties or differences in extrinsic factors such as autonomic tone or sex hormones. Further experiments, which are out of the scope of the current study, will be needed to differentiate between such options. For the AERP sex-dependent difference, interpretation is somewhat more complex: We have previously shown that in male rats implanted with an atrial electrode, the AERP is progressively shortened over time 37, presumably reflecting a remodeling process as a result of mechanical loading of the electrode on the RA myocardium. Thus, a possible option is that the female atrium is less susceptible to the mechanical loading induced by the electrode and therefore the AERP sex difference that was detected four weeks post-device implantation mainly reflects less loading-dependent electrical remodeling in the females. Indeed, it is well documented that estrogens improve myocardial adaptation to ventricular pressure overload in hypertensive women 41 , and similar findings were found in rodent models of trans-aortic constriction42, 43. In addition, this possibility may also be supported by the reported absence of sex-dependent differences in action potential characteristics in mice atrial cardiomyocytes under basal conditions 31. In a future study, direct comparison between the AERP of males and females early post EP device implantation may be important based on our current findings. However, such experiment, although simple to perform technically, was not in the main scope of our current study. As mentioned above, sex-dependent differences in AF substrate were also remarkable. This issue will be discussed in detail later on in the discussion below.

**ISO and PEN markedly modulate the supraventricular EP**

Methodologically, for each parameter we compared the differences between UAS, ISO and PEN within each sex, as well as the D change from UAS in comparison between males and females. Initial RR interval analysis indicated that ISO does not affect the heart rate, while PEN has a modest bradycardic effect in both sexes. In an attempt to compare these results to the literature, we could not find additional studies that repeatedly measured the heart rate of the same rodents under UAS as well as under ISO or PEN as we performed. However, unmatched results in the literature also indicated relatively minor effects of these agents on the heart rate of rodents 32, 44, 45. While these findings may imply that the supraventricular EP properties retain their physiological values under these two agents, our further analyses indicate that this is clearly not the case. Indeed, CSNRT analysis in the males revealed marked prolongation under ISO compared to UAS and PEN. This finding may suggest that while the pacemaker properties within the SA node remain unaffected in the presence of ISO (resulting in unaltered heart rate), sinoatrial conduction pathways transferring the excitation from the SA node to the atria 46, 47 are affected by ISO, leading to sinoatrial exit block and prolonged CSNRT. Moreover, since the sinoatrial conduction pathways share properties with the AV nodal conduction system 47, the above possibility may also be supported by the marked effects of ISO on AV nodal function (Figure 4). Interestingly, in both cases, the effects of ISO in males were far more prominent than in females, a finding that may deserve further attention and an attempt for mechanistic understanding in future studies.

Our finding indicates that AERP is markedly prolonged by both ISO and PEN. Interestingly, at least for ISO, similar findings were also noted in humans 48, 49. However, it is hard to conclude whether similar mechanisms affect both rodents and humans in this regard. Since AERP is a surrogate of atrial APD, it is interesting to correlate our findings with reports describing the direct effects of ISO on cardiac APD. However, these reports are not fully consistent and also do not contain data on atrial tissue/myocytes. A study in isolated ventricular myocytes from guinea pigs demonstrated that the effects of ISO are complex and dose-dependent, leading to prolongation of the APD at low concentrations (<2%) followed by marked shortening at higher doses 50. The main mechanism leading to APD prolongation was attributed to the inhibitory effects of isoflurane on IKdr, a current which is not involved in the action potential repolarization in rodents. On the other hand, a report in isolated rat ventricular cardiomyocytes describes mainly APD shortening due to marked inhibition of L-type Ca2+ current with only modest inhibitory effect on Ito, the dominant repolarizing current in rodents 51. Interestingly, it was also found that desflurane, another volatile anesthetic with similarities to ISO, prolonged the APD in isolated rat ventricular myocytes by markedly suppressing Ito 52. It is possible that some of the above discrepancies may be related to differences in doses and experimental conditions. *In vivo*, indirect effects through autonomic modulation may also play a role. In any case, our data support the notion that atrial APD prolongation is the dominant effect of the conventional concentration of ISO (i.e. ~ 2%), at least in rats. Interestingly, while PEN prolonged the AERP in both sexes in a similar manner, ISO had a stronger effect in males, as also demonstrated above in regard to CSNRT and AV nodal properties. Regarding AERP rate-dependence, our group has previously shown that this property is practically absent in male rat and mice under ISO anesthesia 32 as well as in freely moving male rats 37. Our current data confirm rather flat AERP rate-dependence as a uniform finding under all conditions, although in females modest but significant reverse-adaptation was noted.

**Modulation of AF substrate and AF signal by sex and anesthetics**

Our EP studies were performed four weeks post EP device implantation. Thus, we had the opportunity to compare the effects of anesthetics on the AF substrate that progressively develops in our model over time 36-38. Our first finding was that females in UAS have markedly lower AF substrate compared to males (Table 1). In addition, their AF substrate is rather insensitive to the anesthetics (Figures 5, 6). There are several factors that can contribute to the reduced AF substrate in females including reduced atrial size, prolonged AERP and more. A recent study in mice noted similar sex-dependent AF substrate difference in CD-1 mice under 2% ISO, which was mainly attributed to testosterone-dependent changes in connexin lateralization 31. In this regard, it would be helpful to know the atrial conduction velocity in our rats. However, while our electrode could theoretically enable such recording 37, we found that such measurement was practically possible only in the minority of cases. In any case, the reduced AF substrate of females has important practical implications for the future design of AF studies. It will also be vital to study how orchiectomy and ovariectomy affect the obtained results as was performed in mice 31.

The inhibitory effect of ISO on the conventional AF substrate parameters of males is a finding of paramount importance considering the broad use of this agent in AF-related studies of rodents. This finding was confirmed both by conventional measurements (AF induction and duration) and by our recently developed objective analysis of CR that is dominantly detecting irregular arrhythmias 38. The latter also detected a reduction of AF substrate under PEN (Figure 6), which we did not detect manually. An important consideration in this regard is the signal resolution of the obtained arrhythmic recordings and the ability to differentiate between regular and irregular signals. Most conventional recordings, certainly those using peripheral ECG to identify the arrhythmias, cannot discriminate between totally regular arrhythmias and AF. Our findings stress the importance of this issue by showing how ISO, which markedly increases AERP, leads to markedly reduced AF substrate concomitant with clear tendency towards more regular arrhythmias (Supplemental Figure 4S). These findings, could not have been identifies using conventional low-resolution recordings and an analysis that pools together regular and irregular arrhythmias. Thus, our findings stress the importance of maintaining high resolution atrial signals, for proper AF substrate analysis. Of note, while there is no clear data in the literature on the EP mechanisms underlying regular and irregular supraventricular arrhythmias in rats, our observations are that the former are far more stable and are therefore consistent with a single reentry cycle. Importantly, regular arrhythmias might not even depend on properties of the atrial tissue per-see (e.g., AV nodal reentrant tachycardia).

Finally, the power spectrum analyses (Figure 7) are also directly related to our current abilities to record atrial signals with high resolution and digitally clean the signal from ventricular mixing 38. Our analyses indicated that in both males and females ISO and PEN reduced the dominant frequency of the AF signals. It is tempting to speculate that these findings may be related to the AERP prolonging effects of both anesthetics leading to increased AF wavelength. Similarly, the slower dominant frequency in females under UAS and ISO (Figure 7) may also correlate with the increased AERP of females. However, at this stage it is hard to know whether the differences in AERP are indeed the main determinant of these findings. Thus, further more mechanistic studies will have to be performed to address these findings in future studies.

**Methods**

**Animals**

The study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All animal studies reported in this study were approved by the institutional ethics committee of Ben-Gurion University of the Negev, Israel (Protocol No. IL42062021D). Adult male and female Sprague-Dawley rats were obtained from Harlan Laboratories (Jerusalem, Israel). Experiments were performed on male and female rats with a body weight of ~250 gr at the time of operation. The animals were kept under standardized conditions throughout the study, according to home office guidelines: 12:12 light:dark cycles at 20–24 °C and 30–70% relative humidity. Animals were free-fed autoclaved rodent chow and had free access to reverse osmosis filtered water. The animals were monitored on a daily basis for signs of stress or inappropriate weight loss, according to guidance from the Ben-Gurion University veterinary services [assured by the Office of Laboratory Animal Welfare, USA (OLAW) #A5060-01, and fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)]. At the end of all EP evaluation all rats were euthanized under deep anesthesia.

**EP device and Surgical implantation procedure**

The EP device that was used in this study as well as the details of the atrial quadripolar electrode were previously described in details 36, 37. In brief, the device includes an 8-pin connector that is attached by highly flexible insulated electrical wires (AS155-36, Cooner wires, Chatsworth, CA) to the atrial quadripolar electrode as well as to three peripheral ECG leads. The atrial quadripolar electrode contains four Platinum-Iridium electrical poles that are embedded in medical grade silicon (MED-6219P, Nusil, CA) and fixed to the tissue by miniature stainless steel hooking pins (26002-10, Fine Science Tools, Vancouver, Canada). For implantation, the animals were anesthetized by Ketamine/Xylazine (intramuscular 75/5 mg/kg). Rats were mechanically ventilated and placed on a heating pad (37oC). Under sterile conditions, right upper thoracotomy was performed, and the atrial pericardium was removed. The atrial quadripolar electrode was implanted on the epicardial surface of the right atrium and the ECG electrodes were positioned in the left forelimb, right forelimb and left leg subcutaneously. After the chest was closed, the 8-pin connector was exteriorized through the back skin, and a shielding ring with four plastic restraints was used to prevent the device from being extracted over time. The ring was inserted over the connector, sutured to the skin and glued to the connector over four plastic restraints 36. This procedure practically eliminated the risk for connector extraction by the animals. Following conventional post-operative recovery animals were maintained in normal cages for four weeks in order to allow for notable AF substrate to develop 36, 37. Thereafter, repeated EP measurements were conducted as described below.

**Experimental design of the repeated EP evaluations**

Thirty days following device implantation the animals went three consecutive EP measurements (Figure 1). For the initial UAS EP evaluation, each animal was placed in a dedicated EP cage where the back connector was attached by an elastic cable to the pacing and recording apparatus through a multi-channel commutator (PLA-SL12C/SB, PLASTICS One Inc., CA), allowing the rat to move freely in the cage without affecting the electrical connections. In each animal, a pair of atrial poles were selected for pacing and were electrically connected to an optically isolated pacing unit (STG4002-16 mA, Multichannels, Reutlingen, Germany). The remaining two atrial poles and the three peripheral ECG electrodes were connected to a voltage amplifier (Amplifier 1700, A-M systems, Carlsborg, WA). As previously described the electrode side that was used for pacing was empirically determined based on a relatively low capture threshold and an ability to differentiate the atrial signal from the stimulus artifact in the recordings from the other side. After a pacing and recording configuration was selected it remained without changes thorough the repeated EP studies under anesthesia. Signals were filtered (1–1000 Hz with a notch filter at 50 Hz) and sampled to the PC at a digital sample rate of 2 kHz. A self-made program developed using Labview 7.1 (National Instruments, Austin, TX) controlled data acquisition and electrical stimulation. UAS EP studies were performed in freely moving rats following overnight adaptation to the EP cages and during daylight hours of the circadian cycle when animals were inactive, as previously described 36, 37. After the UAS EP procedure each animal was moved to its regular cage for 24 hour. Subsequently, a second EP study was performed under ISO anesthesia (2% in O2 mixture) in a manner identical to the initial one. For the anesthetized procedures a temperature probe was rectally inserted, and the animals were placed on a heating pad and constantly kept at ~37.5oC throughout the EP study. Of note, while we could easily perform the ISO EP study under lower levels (as low as 1%), we intentionally selected 2% since this is the most common level of ISO required during acute EP studies that utilize conventional invasive catheters 20. Lastly, following the isoflurane EP procedure each animal was moved to its regular cage for a recovery period of 72 hours and then the final EP study was performed under PEN anesthesia (intraperitoneal, 40mg/kg). At the end of this study the experiment was terminated using high dose PEN euthanasia.

EP evaluation was performed as previously described in detail 37. RR, PR and QT interval were obtained averaged form 5 consecutive cycles on the non-paced ECG. Following baseline recordings, atrial pacing threshold was obtained using bipolar square current pulses (total duration 4 ms; 2 ms in each direction), and stimulus intensity was raised to double threshold (DT) for the rest of the EP study. A programmed S1S2 stimulation protocol (S1=10) was used to determine the atrioventricular node refractory period (AVERP) and the (AERP) in the millisecond range. To access rate dependence the S1-S1 cycle (basic cycle length - BCL) was varied between 130-100ms for the AVERP measurements and 120-70ms for the AERP measurements. All AVERP and AERP values in the study were confirmed three consecutive times. Sinus node recovery time (SNRT) was evaluated using burst pacing protocols (30s, 120 ms BCL) applied 3 times with 30s pause between the bursts. Spontaneous cycle length (SCL) was measured after each SNRT burst as the average of 3 consecutive spontaneous beats based on our previous detailed SNRT calibration pilot 37.

**Arrhythmic substrate analysis**

The arrhythmic substrate evaluation in each of the three EP studies (UAS, ISO, PEN) comprised twenty consecutive triggering bursts (1 s duration, 10 ms cycle length). Arrhythmic episodes lasting more than 4 minutes were aborted using short (1 s) pacing bursts of increasing intensity until sinus rhythm was restored. The minimal time between pacing bursts was 1 minute from the end of an event. If an episode of more than 60 s was detected, the delay from the end of this episode to the next pacing burst was equal to the duration of the AF episode. The cutoff for defining a positive arrhythmic event was defined as >1 s following the burst pacing protocol. To avoid any bias in the AF analysis by regular stable arrhythmic episodes we distinguished between regular and irregular events in our analysis. As we recently reported 53, we have previously noted that regular arrhythmic episodes are characterized by a stable cycle length > 60 ms. Thus, AF was defined in the current study as fast irregular atrial electrocardiograms or atrial waveforms in which the main repeating component had a duration < 55 ms. Regular arrhythmic waveforms were analyzed separately from the AF analysis.

In addition to the noted above manual analysis, we also applied our recently developed computerized algorithm to clean the atrial signal from ventricular contamination and thereafter quantity AF substrate and complexity in an objective manner as described in detail in Murninaks et al. 2023 38. Briefly, pre-burst ventricular complex sampling was initially performed followed by its automatic subtraction from the entire atrial signal based on QRS detection in ECG wave form. Next, the pure post-burst atrial signal was divided to 1S windows, and each window was analyzed for its complexity by applying the Lempel-Ziv complexity algorithm 38. The final Complexity-Ratio (CR) value was calculated for each window by normalizing its Lempel-Ziv values to the pre-burst value in the same trace. CR values close to one indicate low complexity that is similar to the pre-burst sinus rhythm values. In contrast, values higher than 1 indicate increased episode irregularity relative to the pre-burst signal. Our detail analysis previously revealed that a cutoff value of CR= 1.236 ideally separates between regular rhythms and irregular events (AF) in a highly accurate fashion 38. Finaly, for power spectrum analysis of the AF waveforms, conventional Fast-Fourier-Transform was performed as previously described 38, 53. Since recordings were all performed in the presence of a notch filter to reduce the signal levels proximal to 50 Hz, an artificial depression was detected around this frequency.

## **Statistical analysis**

A total of 29 rats (13 males and 16 females), which were successfully implanted with the EP device and demonstrated proper atrial pacing and recordings four weeks post-implantation, were included in our final analysis. Data analysis was performed using Prism 9.0 (GraphPad Software, Inc., San Diego, CA). All data were expressed as the mean ± SEM (standard error of the mean). We used Shapiro Wilk test to examine the normal distribution of various parameters. For parameters with a normal distribution in all groups, one-way ANOVA for repeated measurements, followed by post hoc Tukey's multiple-comparison test were used. For parameters without a normal distribution, Friedman test was used instead with post hoc Dunn’s multiple comparison correction. For direct comparison between males and females in the UAS (Table 1) student’s t-test and Mann-Whitney test were performed for parameters which passed or failed normality test, respectively. Comparisons of AERP and AVERP under different BCLs were performed using two-way ANOVA for repeated measurements. Since AF substrate parameters usually do not follow a normal distribution, we analyzed all of them using nonparametric testing. Comparison of delta values from the unanesthetized state for each anesthetic drug (ISO or PEN) between males and females were conducted using the Mann-Whitney test. The criterion for significance was set at p < 0.05. The specific tests that were used are mentioned in the legends of each figure / table.

**Data availability**

he data sets generated during and/or analyzed for the reported study are available from the corresponding author on reasonable request.

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**AUTHOR CONTRIBUTIONS**

M.M., O.L. and Y.E. conceived and designed research. M.M., O.L., S.E. and N.D. performed experiments; A.N., A.K. and N.M. fabricated the implanted EP devices and helped with the experiments. M.M., O.L. and G.G. analyzed data and prepared the figures. M.M. and O.L. drafted the manuscript; Y.E., G.G. edited and revised manuscript; All authors approved final version of manuscript.

**Ethics declarations**

Competing interests: The authors declare no competing interests.

**Figure Legends:**

**Figure 1. Schematic presentation of the experimental design.** On day 1All animals were implanted with an EP device including an atrial quadripolar device and three peripheral ECG leads all connected to a 8 pin connector in the back of the rat. For additional details see methods as well as detailed description including photos in previous publications 36, 37.

**Figure 2. Heart rate and SA node properties are differentially affected by ISO and PEN in both sexes. A**: Comparison of RR intervals under UAS, ISO and PEN, stratified by sex. Note significant prolongation of RR intervals by PEN relative to UAS and ISO in both males and females. **B**: Comparison between males and females. D change of RR interval relative to UAS under ISO and PEN conditions. **C-D**: Similar representation as in A-B, for CSNRT. Note in males only, marked prolongation of the CSNRT under ISO relative to both UAS and PEN. Statistical analysis: A, C: normality was confirmed. Thus, one-way ANOVA for repeated measurements was applied, followed by post hoc Tukey's multiple-comparison test. B, D: normality was confirmed. Thus, unpaired student’ t-test was applied to compare between males and females.

**Figure 3. Anesthetics prolong PR interval in females only and AERP in both sexes.**

**A**: Comparison of PR intervals under UAS, ISO and PEN, stratified by sex. Note small but significant prolongation of PR interval under ISO and PEN relative to UAS in females only. **B**: Comparison between males and females. D change of PR interval relative to UAS under ISO and PEN conditions. Results were non-significant in this analysis. **C-D**: AERP as a function of basic CL in males and females, respectively. Note absence of rate-adaptation in the males and small but significant reverse rate-adaptation the females (i.e. prolongation of AERP as the CL is decreased). **E-F**: Similar representation as in A-B, for AERP at 120 ms basic CL. Note prolongation of AERP by both ISO and PEN relative to UAS in both males and females. D change of AERP reached significance for ISO only. Statistical analysis: A, E: normality was confirmed. Thus, one-way ANOVA for repeated measurements was applied, followed by post hoc Tukey's multiple-comparison test. B, D: normality was confirmed. Thus, two-way ANOVA for repeated measurements was applied, followed by post hoc Tukey's multiple-comparison test. In C, normality was confirmed. Thus, unpaired student’ t-test was applied to compare between males and females. In F, normality was not confirmed. Thus, Mann-Whitney test was applied to compare between males and females.

**Figure 4. AV nodal properties are affected differentially by ISO and PEN in both Sexes. A-B**: AVERP as a function of basic CL in males and females, respectively. Note prolongation of AVERP as CL is decreased in both sexes under all experimental conditions. Also note marked prolongation of AVERP by both ISO and PEN in males and by PEN only in females. **C**: Comparison of AVERP at 120 ms basic CL under UAS, ISO and PEN, stratified by sex. Note prolongation of AVERP by both ISO and PEN relative to UAS males and by PEN only in females. **D**: Comparison between males and females. D change of AVERP relative to UAS under ISO and PEN conditions. Note the differential response of males and females to ISO. **E-F**: Similar representation as in Figure C-D, for AV Wenckebach. Note prolongation of this dynamic parameter in both males and females relative to UAS. Statistical analysis: A, B: normality was confirmed. Thus, two-way ANOVA for repeated measurements was applied, followed by post hoc Tukey's multiple-comparison test. In C, normality was confirmed. Thus, one-way ANOVA for repeated measurements was applied, followed by post hoc Tukey's multiple-comparison test. In E, normality was not confirmed. Thus, Friedman test was applied, followed by Dunn’s multiple comparison correction. D, F: normality was not confirmed. Thus, Mann-Whitney test was applied to compare between males and females.

**Figure 5 - AF substrate is inhibited by ISO in males only.** **A**: Comparison of AF induction (%) under UAS, ISO and PEN, stratified by sex. Note significant inhibition by ISO relative to UAS in males only. **B**: Comparison between males and females. D change of AF induction (%) relative to UAS under ISO and PEN conditions. Note marked difference between the effect of ISO in males vs. females. **C-D**: Similar representation as in A-B but for AF duration. Note differential effect of ISO in males vs. females in this parameter as well. Statistical analysis: A, C: Friedman and Dunn’s multiple comparisons. B, D: Mann-Whitney test. For clarity, 2 data points in B, 2 in C, and 5 in D are out of scale and are not represented. In Figures A and C, the comparison was performed by Friedman and Dunn’s multiple comparisons. In Figures B and D, the comparison was performed by the Mann-Whitney test. For clarity, 2 data points in B, 2 in C, and 5 in D are out of scale and are not represented.

**Figure 6. Objective AF substrate analysis indicate that both ISO and PEN inhibit the AF substrate of males. A**: Comparison of mean CR of the first 5 Sec post burst pacing under UAS, ISO and PEN, stratified by sex. Note significant inhibition by both ISO and PEN relative to UAS in males only. **B**: Comparison between males and females. D change of CR relative to UAS under ISO and PEN conditions. Results were non-significant in this analysis. **C-D**: Similar representation as in A-B but for the % of signals above the CR threshold for irregular arrhythmias (see methods for details). Significant inhibition of this parameter was noted only for PEN in the males. A non-significant inhibitory tendency was also noted for ISO in the males. Statistical analysis: A, C: Friedman and Dunn’s multiple comparisons. B, D: Mann-Whitney test.

**Figure 7. Slower AF dominant frequency is revealed under both anesthetics in both sexes. A:** *Upper graph*:Average power spectrum of AF signal inmales under UAS, ISO and PEN. Arrows mark the dominant frequency in each condition. *Lower graph*: detected dominant frequency of all AF signals in each condition. **B:** similar representation as in A but for females. Note in both sexes significantly reduced AF dominant frequency under ISO and PEN. **C**: AF dominant frequency. Comparison between males and females in each condition. Note decreased dominant frequency in females under UAS and ISO. Statistical analysis: A, B: one-way ANOVA was applied, followed by post hoc Tukey's multiple-comparison test. In C, Student’s t-test was applied to compare between males and females.